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Enhancement of hydrophilic drug loading and release characteristics through micellization with new carboxymethyldextran-PEG block copolymers of tunable charge density

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Abstract

The micellization of a model cationic drug, diminazene diaceturate (DIM) and a series of new diblock copolymers, carboxymethyldextran-poly(ethylene glycols) (CMD-PEG), were evaluated as a function of the ionic charge density or degree of substitution (DS) of the carboxymethyldextran block and the molar ratio, [+]/[−], of positive charges provided by the drug to negative charges provided by CMD-PEG. Micelles ([+]/[−] = 2) incorporated up to 64% (w/w) DIM and ranged in hydrodynamic radius (R_H) from 36 to 50 nm, depending on the molecular weight and DS of CMD-PEG. The critical association concentration (CAC) was on the order of 15–50 mg/L for CMD-PEG of DS > 60%, and ca. 100 mg/L for CMD-PEG of DS ∼ 30%. The micelles were stable upon storage in solution for up to 2 months and after freeze-drying in the presence of trehalose. They remained intact within the 4 < pH < 11 range and for solutions of pH 5.3, they resisted increases in salinity up to ∼0.4 M NaCl in the case of CMD-PEG of high DS. However, micelles of DIM and a CMD-PEG of low DS (30%) disintegrated in solutions containing more than 0.1 M NaCl, setting a minimum value to the DS of copolymers useful in *in vivo* applications. Sustained *in vitro* DIM release was observed for micelles of CMD-PEG of high DS ([+]/[−] = 2).

Keywords: Polyion complex micelles; Dextran; Electrostatic interactions; Polyelectrolytes; Diminazene diaceturate; Hydrophilic drug

1. Introduction

Polysaccharides are ubiquitous components of traditional pharmaceutical formulations where they act as coatings or suspending agents, tablet binders and extended-release matrix formers (Skinner et al., 1999; Lee and Chen, 2000). They are also known to possess self-assembling qualities and to undergo stimuli-responsive transformations, such as heat- or salt-triggered gelation. More recently, polysaccharide-based nanostructures have emerged as promising materials for biological and medical applications (Payne, 2007). Micellar systems based on dextran (Francis et al., 2005), cellulose ethers, poly(ethylene glycol)(PEG)-grafted chitosans, hyaluronan-*block*-poly(2-ethyl-2-oxazoline) or pullulans, have been shown to be effective nanocarriers for various drugs (Francis et al., 2003a,b, 2005) and proteins (Nomura et al., 2003; Yang et al., 2005; Jeong et al., 2006). In most cases polysaccharide nanoparticles were designed for the delivery of hydrophobic drugs. Fewer studies have been devoted to polysaccharide-based nanoparticles for the delivery of highly water soluble drugs. To address this issue, we developed a straightforward synthesis of carboxymethyldextran-*block*-poly(ethylene glycol)s (CMD-PEG, Fig. 1) (Hernandez et al., 2007). The CMD-PEG copolymers were designed specifically as substrates *of tunable charge density*, able to form polyion complex (PIC) micelles upon interaction with an oppositely charged drug. The charge density of the ionic segment cannot be adjusted readily in the case of diblock copolymers used in most PIC-micelle-based drug delivery systems, in which the ionic fragment is usually a poly(aminoacid) bearing a charge on each repeat unit. Since the number of charged groups linked to the ionic segment determines the loading efficiency and drug release characteristics of

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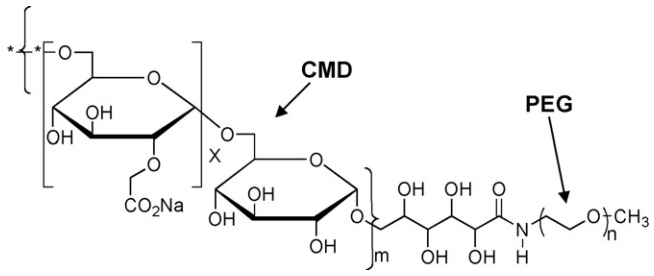

Fig. 1. Idealized chemical structure of carboxymethyldextran-*block*-poly(ethylene glycol) (CMD-PEG); *n* represents the number of ethylene glycol units, *m* is the number of glucopyranose rings of the polysaccharide block, and *x* represents the fraction of glucose units of the dextran chain that bear a carboxymethyl group. The polysaccharide segment consists of a random distribution of glucopyranose units and carboxymethylglucopyranose units.

PIC micelles, the control of charge density adds a new dimension in the design of drug-loaded PIC micelles which we set about to explore.

We compare the properties of PIC micelles formed by four CDM-PEG copolymers: (i) two copolymers identical in terms of the length of each block, but different in terms of the charge density of the CMD block and (ii) two copolymers in which the charged block is the same, but the neutral block is of different molecular weight. This strategy enabled us to determine the optimal charge density required to form stable micelles with high drug loading efficiency, small size, and suitable drug release profiles of diminazene diaceturate (DIM), a dicationic molecule used as model drug. DIM is effective in the treatment of trypanosomiasis in animals (Peregrine and Mamman, 1993). It has been used previously to demonstrate the formation of PIC micelles with poly(aspartic acid)-*block*-poly(ethylene glycol) (Govender et al., 2001) and poly(ethylene oxide)-*block*-poly(L-glutamate) (Thünemann et al., 2006).

We characterize the micelles formed between diminazene and the four CMD-PEG copolymers and assess the effect of charge density on the physico-chemical properties of the micelles, on their stability as a function of salinity, pH, and storage time, and on the drug release kinetics. In order to determine the level of drug loading as a function of charge density, we used static and dynamic light scattering which, together with ^{1}H NMR spectroscopy, allow one to characterize drug-loaded micelles and to detect drug molecules dissolved in the aqueous medium (free drug).

2. Material and methods

2.1. Materials

Trizma® hydrochloride (Tris–HCl), diminazene diaceturate (≥90% pure, as stated by the supplier), D(+) trehalose dihydrate, Amberlite® IR-120 and all other chemicals were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). The drug (m.p. = 215–217 °C) was used without further purification. The purity of DIM was estimated to be ≥96% on the basis of the ^{1}H NMR spectrum of DIM in D_2O. Dextran-PEG (DEX-PEG) samples were synthesized as described previously (Hernandez et al., 2007). Dialysis tubing (SpectraPore, MWCO: 1000 or 3500 g/mol) was purchased from Fisher Scientific (Rancho Dominguez, CA, USA). All solvents were reagent grade and used as received.

2.2. Synthesis of carboxymethyldextran-block-poly(ethylene glycols) (CMD-PEG)

The CMD-PEG samples of high charge density were obtained according to the protocol previously reported (Hernandez et al., 2007). The method is described briefly below and the amounts of reagents and solvents employed in each synthesis are given in Table 1. Sodium hydroxide was added to a solution of DEX-PEG in an isopropanol–water mixture (85:15 v:v) kept at room temperature. The reaction mixture was heated to 60 °C and kept at this temperature for 30 min. Monochloroacetic acid was added portionwise to the mixture while stirring. The reaction mixture was kept at 60 °C for 90 min. It was cooled to room temperature, transferred in a dialysis bag and dialyzed against water for 24 h. The purified copolymers were isolated by lyophilization and characterized by ^{1}H NMR (D_2O, 400 MHz) δ/ppm: 5.07 (anomeric proton on glucopyranose bearing a carboxymethyl group at C_2), 4.89 (anomeric proton on glucopyranose unsubstituted at C_2), 4.15–4.08 (–CH_2COONa), 3.97–3.36 (CMD C-2 to C-6 glucopyranosyl protons), 3.61 (PEG, –CH_2CH_2O–), 3.29 (–OCH_3).

To prepare samples of low degree of carboxymethylation, such as 30-CMD_{68}-PEG_{64}, the carboxymethylation was achieved by adding monochloroacetic acid to a stirred solution of DEX_{68}-PEG_{64} in aqueous NaOH kept in an ice/water bath, followed by treatment at 60 °C for 1 h. The resulting

Table 1
Experimental conditions for the carboxymethylation of DEX-PEG copolymers

Polymer	DEX_m-PEG_n		NaOH (mmol)	MCA[a] (mmol)	Isopropanol/water (mL)
	g	mmol Glu[b]			
85-CMD_{40}-PEG_{140}[c]	2.20[d]	–	16.51	8.80	9.95/1.75
80-CMD_{40}-PEG_{64}	0.50	2.19	10.68	5.69	6.43/1.17
60-CMD_{68}-PEG_{64}	0.27	1.6	7.50	4.00	4.53/0.80
30-CMD_{68}-PEG_{64}	0.50	3.0	24.00	14.0	0.00/4.00

[a] MCA: monochloroacetic acid.
[b] Glu: glucopyranosyl.
[c] The prefix denotes the degree of carboxymethylation of the dextran block.
[d] Mixture of DEX_{40}-PEG_{140} and unreacted PEG-NH_2.

polymer was purified as described above. ^{1}H NMR (D_2O, 400 MHz) δ/ppm: 5.07 (anomeric proton on glucopyranose bearing a carboxymethyl group at C_2), 4.88 (anomeric proton on glucopyranose unsubstituted at C_2), 4.15–4.08 (–CH_2COONa), 3.95–3.36 (CMD C-2 to C-6 glucopyranosyl protons), 3.61 (PEG, –CH_2CH_2O–), 3.29 (–OCH_3).

2.3. Methods

2.3.1. General methods

^{1}H NMR spectra were recorded for solutions in D_2O (25 °C) using a Bruker AV-400 MHz spectrometer operating at 400 MHz. Chemical shifts are given relative to external tetramethylsilane (TMS = 0 ppm). Gel permeation chromatography (GPC) measurements were carried out using a GPC system with an Agilent 1100 isocratic pump, a Dawn EOS multiangle laser light scattering detector (Wyatt Technology Corp., Santa Barbara, USA) and an Optilab DSP interferometric refractometer (Wyatt Technology Corp.) using PL-aquagel-OH 40 (8 μm) and PL-aquagel-OH 30 (8 μm) columns (Polymer Laboratories, Amherst, MA, USA) eluted with a pH 7.02 buffer composed of 0.2 M $NaNO_3$, 0.01 M NaH_2PO_4, 0.08 mM NaN_3 at a flow rate of 0.5 mL/min. Solutions for analysis had a polymer concentration of 10.0 mg/mL and the injection volume was set at 100 μL. For dn/dc measurements, solutions of each polymer of concentration ranging from 0.2 to 1.0 mg/mL were prepared in the same buffer. UV–vis absorption spectra were recorded with an Agilent 8452A photodiode array spectrometer. Zeta-potential measurements were carried out with a Malvern ZetaSizer Nanoseries ZS (Malvern Instruments, Worcestershire, UK). Lyophilizations were performed with a Virtis (Gardiner, NY, USA) Sentry Benchtop (3L) freeze-dryer. Melting points were measured with a Büchi 535 capillary melting point apparatus (Büchi, Switzerland).

2.3.2. Light scattering

Static (SLS) and dynamic (DLS) light scattering experiments were performed on a CGS-3 goniometer (ALV GmbH) equipped with an ALV/LSE-5003 multiple-τ digital correlator (ALV GmbH), a He-Ne laser (λ = 632.8 nm), and a C25P circulating water bath (Thermo Haake). The SLS data were analyzed according to the Zimm method (Harada and Kataoka, 1999). The refractive index increment (dn/dc) values of the CMD-PEG samples (Table 2) and of diminazene diaceturate (0.2543 mL/g) in 25 mM Tris–HCl buffer, pH 5.3 were measured using an Optilab DSP interferometric refractometer (Wyatt Technology Corp.). The dn/dc value of the micelles was calculated from Eq. (1) (Tanodekaew et al., 1997; Harada and Kataoka, 1998):

$$\left(\frac{dn}{dc}\right)_{\text{micelle}} = W_{\text{CMD-PEG}}\left(\frac{dn}{dc}\right)_{\text{CMD-PEG}} + W_{\text{drug}}\left(\frac{dn}{dc}\right)_{\text{drug}} \quad (1)$$

where $(dn/dc)_{\text{CMD-PEG}}$ and $(dn/dc)_{\text{drug}}$ are the refractive index increments of CMD-PEG and diminazene diaceturate, respectively, and $W_{\text{CMD-PEG}}$ and W_{drug} are the weight fractions of CMD-PEG and diminazene diaceturate, respectively. A cumulant analysis was applied to obtain the diffusion coefficient (D) of the micelles in solution. The hydrodynamic radius (R_H) of the micelles was obtained using the Stokes–Einstein Eq. (2),

$$D = \frac{k_B T}{6\pi\eta_s R_H} \quad (2)$$

where η_s is the viscosity of the solvent, k_B is the Boltzmann constant, and T is the absolute temperature. The constrained regularized CONTIN method was used to obtain the particle size distribution (Vamvakaki et al., 2006). The data presented are the mean of six measurements ± S.D. Solutions for analysis were filtered through a 0.45 μm Millex Millipore PVDF membrane prior to measurements.

2.4. Preparation and characterization of the micelles

2.4.1. General method

Stock solutions of the diblock copolymers (1.0 g/L) and diminazene diaceturate (4.0 g/L) were prepared in Tris–HCl buffer (25 mM, pH 5.3). Specified volumes of the diminazene diaceturate solution were added dropwise to a magnetically stirred polymer solution over a 10-min period to obtain solutions with a [+]/[−] ratio ranging from 0.2 to 5.0. For simplicity reasons the [+]/[−] ratio was calculated assuming a drug purity of 100%. The uncertainty of the ratio is estimated to be ∼0.08 knowing that the purity of the drug is ≥96%. The volume of each sample was adjusted to 5.0 mL by addition of the same buffer. The final CMD-PEG concentration was 0.2 g/L in all samples.

2.4.2. pH studies

A micellar solution (CMD-PEG: 0.2 g/L; [+]/[−] = 2.0) was prepared in 25 mM Tris-HCL buffer, pH 5.3. Aliquots of this

Table 2
Molecular properties of the CMD-PEG samples prepared

Polymer	dn/dc[a] (mL/mg)	M_w[b] (g mol^{-1})	M_n[b] (g mol^{-1})	DS[c]
85-CMD_{40}-PEG_{140}[d]	0.1416	14,800	10,800	0.86 ± 0.09
80-CMD_{40}-PEG_{64}	0.1434	12,200	10,200	0.76 ± 0.08
60-CMD_{68}-PEG_{64}	0.1376	16,800	13,400	0.62 ± 0.06
30-CMD_{68}-PEG_{64}	0.1392	15,900	12,000	0.31 ± 0.03

[a] Values recorded for polymer solutions in 25 mM Tris–HCl pH 5.3, 25 °C.
[b] From GPC measurements in aqueous $NaNO_3$ (0.2 M)/NaH_2PO_4 (0.01 M)/NaN_3 (0.8 mM); pH 7.02.
[c] Degree of substitution: mol fraction of glucopyranose units carrying a –CH_2–COONa group; determined by potentiometric titration.
[d] In this nomenclature, the prefix denotes the degree of carboxymethylation of the dextran block; the subscripts designate the average number of glucopyranosyl and –CH_2–CH_2–O– repeat units of the CMD and PEG segments, respectively.

solution were treated either with 1N NaOH or with 1N HCl to obtain solutions ranging in pH from 11 to 2. After each pH adjustment, the sample was stirred for 5 min prior to measurement. The hydrodynamic radius, polydispersity index and scattered light intensity of an aliquot of the sample were determined by DLS. A control experiment was carried out with CMD-PEG solutions (0.2 g/L) treated in the same pH range. The mean ± S.D. of six measurements was determined.

2.4.3. Ionic strength studies

Micellar solutions (CMD-PEG: 0.2 g/L; [+]/[−] = 2.0) were prepared in a 25 mM Tris–HCl buffer of pH 5.3. Aliquots of a NaCl stock solution (2.5 M) in the same buffer were added to the micellar solutions in volumes such that [NaCl] in the sample ranged from 50 to 600 mM. The mixture was stirred for 5 min and the volume of each sample was adjusted to 5.0 mL with Tris–HCl buffer, pH 5.3. The hydrodynamic radius, polydispersity index and scattered light intensity of an aliquot of each sample were determined by DLS measurements. The mean ± S.D. of six measurements was determined.

2.4.4. Critical association concentration

Micellar solutions were prepared using the general procedure described above, with a polymer concentration of 0.2 g/L and [+]/[−] = 2. The micellar solutions were serially diluted with Tris–HCl (25 mM, pH 5.3) and the intensity of light scattered by the solutions was determined by DLS at a scattering angle of 90°. Six consecutive scattered light intensity measurements were performed. Their average value is reported. Normalized intensities, $I_c/I_{0.2}$ where I_c is the intensity of the light scattered by a solution of concentration c and $I_{0.2}$ is the intensity of the light scattered by a solution of polymer concentration 0.2 g/L were plotted against polymer concentration. The CAC was determined from the plot, following methods reported previously (Li and Kwon, 2000).

2.4.5. Zeta-potential

The ζ-potential of polymer micelles (CMD-PEG concentration: 0.2 g/L) of various [+]/[−] molar ratios, prepared in Tris–HCl buffer (25 mM, pH 5.3) following the general procedure described above, was measured for solutions kept at 25 °C. Each sample was measured four times and the mean ± S.D. is presented. The ζ-potential of the particles was calculated from the electrophoretic mobility values using Smoluchowski equation.

2.4.6. Stability of micellar solutions upon storage

The R_H and size distribution of polymer micelles (CMD-PEG concentration: 0.2 g/L), [+]/[−] = 2), prepared in Tris–HCl buffer (25 mM, pH 5.3) following the general procedure described above, were measured by DLS as described above at various time intervals up to 60 days. Solutions were kept at 25 °C between measurements.

2.4.7. ^{1}H NMR spectra of DIM/CMD-PEG mixtures

Specified volumes of a DIM stock solution of in D_2O (10 g/L) were added dropwise to a magnetically stirred solution of CMD-PEG in D_2O over a period of 10 min in amounts necessary to prepare mixed solutions of CMD-PEG (3.0 g/L) with [+]/[−] ranging from 0.2 to 10.0. ^{1}H NMR spectra of the mixed solutions were recorded as described above.

2.5. Lyophilization/redissolution of DIM/CMD-PEG micelles

Micellar solutions of DIM/85-CMD_{40}-PEG_{140} (10 mL, polymer concentration: 0.2 g/L; [+]/[−] = 2.0) in a Tris–HCl buffer (25 mM, pH 5.3) or in aqueous trehalose (5%, w/v) were frozen by placing the glass vials containing the samples in a dry ice/acetone mixture (temperature: −78 °C). After 30 min the vials were placed in the freeze-dryer and lyophilized for 48 h. The resulting powder was rehydrated with deionized water (10 mL) to reach a polymer concentration of 0.2 g/L. The resulting mixture was stirred at room temperature for 10 min and analyzed by DLS.

2.6. Diminazene release studies

The release of diminazene diaceturate from micelles (3.0 mL, [DIM] = 1.2 g/L, [+]/[−] = 2) in a Tris–HCl buffered saline (25 mM, pH 5.3, 0 mM NaCl or 25 mM, pH 7.4, 150 mM NaCl) was evaluated by the dialysis bag method at 37 °C (Prompruk et al., 2005; Nishiyama et al., 2003) against the buffer (200 mL) used to prepare the micelles and using a dialysis membrane of MWCO = 3500 g/mol). The concentration of diminazene in the dialyzate was determined from the absorbance at 370 nm using a calibration curve. A control experiment to determine diminazene diffusion through the membrane in the absence of the polymer was carried out using a solution of diminazene (1.2 g/L, 3 mL) in the same Tris–HCl buffer. The concentration of diminazene released from the micelles is expressed as the cumulative percentage of the total drug available and plotted as a function of dialysis time.

3. Results and discussion

3.1. Synthesis of carboxymethyldextran-block-poly(ethylene glycols)

The ionic diblock copolymers CMD-PEG were obtained by reaction of monochloroacetic acid (MCA) with DEX-PEG in the presence of sodium hydroxide (Hernandez et al., 2007). Reaction conditions were adjusted in order to obtain copolymers of desired degree of substitution (DS), defined as the molar fraction of glucopyranose rings bearing a $–CH_2COO^-$ group. To obtain a high substitution level (DS > 0.50), solutions of DEX-PEG in a 85/15 (v/v) isopropanol/water mixture were treated with aqueous NaOH (9.0 M) at 60 °C (Huynh et al., 2001). To achieve moderate carboxymethylation yields (DS ≤ 0.30), MCA was added to a solution of DEX-PEG in aqueous NaOH cooled to ∼0 °C, with subsequent treatment at 60 °C for 1 h (Rebizak et al., 1997). All CMD-PEG samples were isolated as their sodium salts. The successful incorporation of carboxylate groups onto the dextran block was ascertained by analysis of the ^{1}H NMR spectrum of the CMD-PEG samples, which exhibits

two doublets (δ 4.89 and 5.07 ppm) ascribed to the resonance of the anomeric protons, a series of signals between δ 4.08 and 4.15 ppm, due to the methylene protons α to the carboxylate group, and two signals characteristic of the PEG block: a singlet at δ 3.28 ppm due to the methoxy end group of the PEG block and a broad signal at δ 3.60 ppm due to the $–CH_2–CH_2–O–$ groups (Hernandez et al., 2007). The average molar mass of the CMD-PEG diblock copolymers measured by gel permeation chromatography are listed in Table 2, together with the degree of substitution (DS) of the polymers determined by potentiometric titration carried out following the procedure reported previously (Hernandez et al., 2007).

3.2. Preparation and size of diminazene/CMD-PEG micelles

Simple mixing of diminazene diaceturate ($pK_a = 11$) (Atsriku et al., 2002) and CMD-PEG in a Tris–HCl buffer (25 mM) of pH 5.3 should trigger the formation of micellar complexes via electrostatic interactions, since both DIM and CMD-PEG are fully ionized at this solution pH. These conditions were used throughout, unless specified otherwise. Evidence for the formation of nanoparticles was provided by dynamic light scattering (DLS) measurements, exemplified in Fig. 2 (top) which presents the size distribution recorded for a solution of diminazene/60-CMD_{68}-PEG_{64} of charge ratio [+]/[−] = 2.0, where [+]/[−] is the ratio of the molar concentration of positive charges provided by the drug to that of the negative charges provided by the polymer. The changes in the particles hydrodynamic radius (R_H) and polydispersity index (PDI) as a function of the ratio [+]/[−] are shown in Fig. 2 (bottom) for the same drug/CMD-PEG system. Particles of $R_H \sim 50$ nm with a PDI of ∼0.5 were detected in mixed solutions containing a large excess of polymer ([+]/[−] < 0.2) signaling the formation of loose polymer/drug aggregates as a result of the competition between drug/polymer attractive forces and repulsive forces between the negative charges on the CMD segments. The hydrodynamic radius and polydispersity index of the scattering objects reached minimum values, ∼20 nm and 0.05, respectively, in mixed solutions of [+]/[−] ∼ 1, i.e. when charge neutralization is achieved. Further increase in drug concentration, with respect to polymer concentration, resulted in a gradual increase in the size of the nanoparticles until [+]/[−] ∼ 2, implying further incorporation of diminazene within the micellar core, as observed also by ^{1}H NMR spectroscopy (see below). No changes in R_H or PDI took place upon further addition of drug, signifying that micelles with [+]/[−] ∼ 2 are unable to incorporate additional drug molecules. The R_H and PDI values recorded for all DIM/CMD-PEG systems are listed in Table 3 for solutions containing 0.2 g/L of polymer and drug in amounts such that [+]/[−] = 2.0. The hydrodynamic size of DIM/85-CMD_{40}-PEG_{140} micelles is slightly larger than that of DIM/80-CMD_{40}-PEG_{64}. This difference in size can be attributed to the difference in the length of the PEG segment of the two copolymers (140 EG units or M_n(PEG) ∼ 6200 g/mol vs. 40 EG units or M_n(PEG) ∼ 2800 g/mol).

Diminazene/CMD-PEG micelles of low polydispersity index, such as those represented in Fig. 2 for systems of

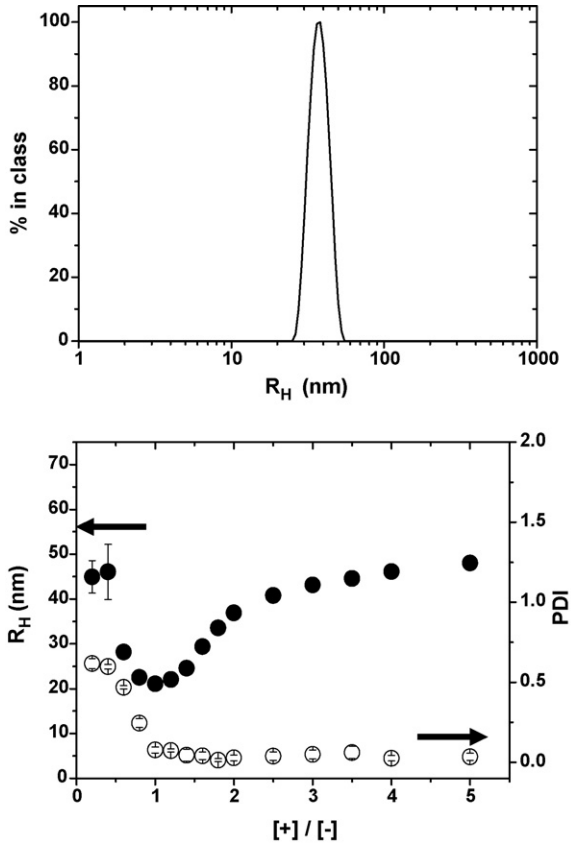

Fig. 2. (Top) distribution of the hydrodynamic radius (R_H) of micelles in a solution of DIM/60-CMD_{68}-PEG_{64} ([+]/[−] = 2; polymer concentration: 0.2 g/L; solvent: Tris–HCl buffer, 25 mM, pH 5.3; temperature: 25 °C; θ: 90°); (bottom) plots of the changes of R_H (●) and the polydispersity index (PDI, ◯) as a function of [+]/[−] in mixtures of diminazene diaceturate and 60-CMD_{68}-PEG_{64}; polymer concentration: 0.2 g/L; temperature: 25 °C; θ: 90°.

[+]/[−] > 1 were prepared by dropwise addition of a drug solution to a magnetically stirred polymer solution. This method consistently led to micelles of identical size for a given [+]/[−] ratio. However, when the drug solution was added in one shot to the polymer solution, the resulting micelles were significantly more polydisperse in size (PDI > 0.1). These PDI values are similar to those reported by Govender et al. in the case of DIM/poly(aspartic acid)-*block*-PEG systems which were prepared by a "one-shot" mixing (Govender et al., 2001). In order to ascertain reproducibility of the micellar properties, throughout this study diminazene/CMD-PEG nanoparticles were prepared via the dropwise addition of the drug solution to the polymer solution. In the case of DIM/30-CMD_{68}-PEG_{64}, micelles of uniform size distribution were obtained only for [+]/[−] > 1.6. The micelles were larger than DIM/60-CMD_{68}-PEG_{64} micelles of identical [+]/[−] ratio (Table 3). The backbone of the two copolymers (30-CMD_{68}-PEG_{64} and 60-CMD_{68}-PEG_{64}) is the same, but 30-CMD_{68}-PEG_{64} contains about half as many charges as 60-CMD_{68}-PEG_{64}. Consequently, the level of drug incorporation in 30-CMD_{68}-PEG_{64} micelles is lower, for identical [+]/[−], compared to the situation in DIM/60-CMD_{68}-PEG_{64}. With fewer drug molecules bound to the CMD segments,

Table 3
Characteristics of DIM/CMD-PEG micelles ([+]/[−] = 2)[a] in a Tris–HCl buffer (25 mM, pH 5.3) for four different diblock copolymers

Polymer	R_H (nm)[b]	PDI[b]	CAC (g/L)	$M_{w,app}$ ($\times 10^{-6}$ g/mol)	N_{DIM}	N_{agg}	% DIM[c]
85-CMD_{40}-PEG_{140}	48.7 ± 0.6	0.05 ± 0.03	0.048	8.25	12,300	363	64.3
80-CMD_{40}-PEG_{64}	43.5 ± 0.7	0.01 ± 0.01	0.032	7.21	10,400	348	62.0
60-CMD_{68}-PEG_{64}	36.9 ± 0.5	0.02 ± 0.01	0.014	4.99	7,300	174	60.1
30-CMD_{68}-PEG_{64}	49.7 ± 0.6	0.10 ± 0.02	0.095	3.89	3,700	178	41.4

[a] [+]/[−]: ratio of the molar concentration of positive charges provided by the drug to that of negative charges provided by the polymer.
[b] Mean of six measurements ± S.D.
[c] % DIM loading = weight of drug/(weight of micelles) × 100.

the micellar core remains more hydrated leading to the formation of larger micelles.

The apparent molecular weight ($M_{w,app}$) of DIM/CMD-PEG nanoparticles ([+]/[−] = 2) was obtained by a Zimm plot analysis of static light scattering measurements. From this value, and knowing the weight average molecular weight of individual chains determined by GPC (Table 2), it is possible to estimate (1) the aggregation number (N_{agg}) of the micelles, defined here as the number of CMD-PEG chains associated in each micelle and (2) the number (N_{DIM}) of drug molecules incorporated in a micelle. In this calculation, it is assumed that there is no free drug in the mixed solutions and that each carboxylate substituent of the CMD block is bound to one diminazene molecule. Values of $M_{w,app}$, N_{DIM} and N_{agg} calculated for micelles formed by CMD-PEG samples of different block lengths and degrees of substitution are listed in Table 3. The N_{agg} value depends primarily on the length of the CMD block: it is the same for the two copolymers, 30-CMD_{68}-PEG_{64} and 60-CMD_{68}-PEG_{64}, which differ greatly in DS but are of identical length. The N_{agg} and N_{DIM} of micelles formed by two polymers with similar DS and CMD block length, but different PEG segments (85-CMD_{40}-PEG_{140} and 80-CMD_{40}-PEG_{64}) are similar, implying that the PEG segments play a passive role in directing the micellar composition, which is driven primarily by the CMD block. Control experiments using isothermal titration calorimetry confirmed the absence of interactions between PEG and DIM.

3.3. Determination of the [+]/[−] ratios corresponding to the onset of micellization and to the maximum drug loading capacity by ^{1}H NMR spectroscopy

Incorporation of drug molecules in the core of polymeric micelles restricts the motion of the protons linked to the drug as well as that of the polymer fragments directly bound to the drug. This loss of mobility is reflected by a significant line broadening and/or disappearance of the ^{1}H NMR signals due to the corresponding protons. We used this inherent property of solution NMR spectroscopy to detect the [+]/[−] ratio for which the drug is effectively entrapped into micelles (onset of micellization) as well as the [+]/[−] value for which the maximum loading capacity of a PIC micelle is attained. The method also allows one to ascertain the absence of free drug in a PIC-micelle formulation. It is described in detail, since it is applicable readily to other drug/diblock copolymer systems.

The ^{1}H NMR spectrum of diminazene diaceturate in D_2O at room temperature (Fig. 3, bottom) presents signals at δ 7.5 and 7.7 ppm, attributed to the aromatic protons, H_c and H_d, respectively (Lee et al., 2006), as well as singlets at δ 1.92 and 3.63 ppm assigned, respectively, to the methyl (H_a) and methylene (H_b) protons of the aceturate counterions. Also shown in Fig. 3 are the ^{1}H NMR spectra of drug/60-CMD_{68}-PEG_{64} solutions of different [+]/[−] ratios. Turning our attention first to the signals of these spectra corresponding to the drug, we note that (1) the sig-

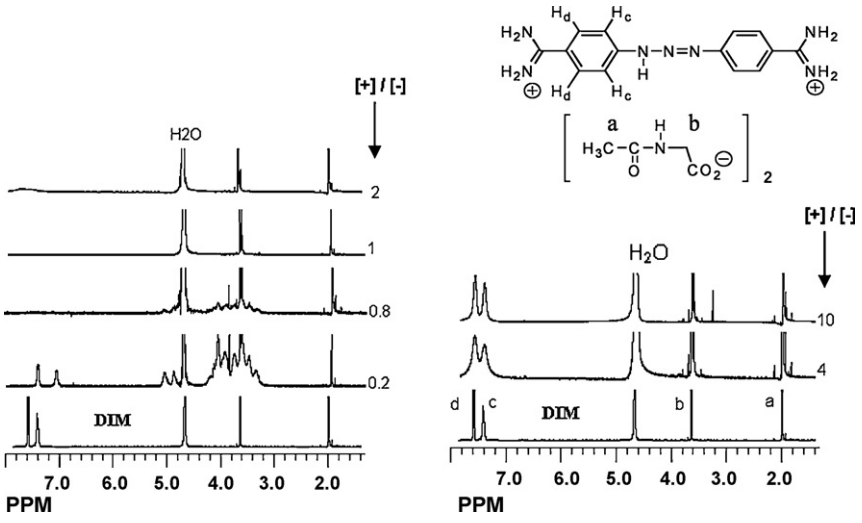

Fig. 3. ^{1}H NMR spectra recorded for diminazene diaceturate (DIM, lower spectra) and solutions of DIM and 60-CMD_{68}-PEG_{64} of 0 < [+]/[−] < 2 (left) and [+]/[−] = 4, 10 (right); polymer concentration: 3.0 g/L, solvent: D_2O; temperature: 25 °C.

nals at δ 1.92 and 3.63 ppm due to the drug counterion (aceturate) are sharp and well resolved in all spectra, indicating that the aceturates remain in solution, preserving their freedom of motion; (2) the signals in the aromatic region (δ 7.5 and 7.7 ppm) due to the protons of the drug are strongly affected by the presence of the polymer. They appear weak and broadened in the spectrum of the mixture with [+]/[−] = 0.4. Moreover, in the spectrum of this system, the signal attributed to the resonance of the protons H_d undergoes a significant upfield shift, implying a change in the local environment of these protons upon binding to the polymer-linked carboxylates. Both signals in the aromatic region vanish in spectra of mixed solutions of [+]/[−] = 1.0–2. They reappear in spectra of mixtures with [+]/[−] > 2.0, signaling the presence of free drug in the micellar solution, as seen in Fig. 3 (right) where we present spectra of mixed systems with [+]/[−] = 4 and 10.

In the ^{1}H NMR spectra of mixed systems, one notices also changes in the signals due to the resonance of protons linked to the polymer. Thus, signals at δ 4.08–4.15, 4.89 and 5.07 ppm ascribed to protons of the CMD block decrease in intensity with increasing [+]/[−]. They are still detectable in mixed solutions of [+]/[−] = 0.8, but disappear for mixed systems of [+]/[−] > 1, signaling severe loss of mobility of the CMD block under these conditions (Fig. 3). In contrast, the signals due to the PEG protons (–CH_2–CH_2–O–, δ 3.61 ppm) remain unaffected by changes in [+]/[−], an indication that the PEG chains preserve their mobility within the corona of the PIC micelles. As noted earlier, signals due to the DIM protons are visible in spectra of mixed systems with [+]/[−] > 2, yet the signals due to the CMD protons remain undetectable up to [+]/[−] = 10, the highest ratio tested. Thus the PIC micelles preserve their integrity even in the presence of a large excess of free drug.

Taken together, the results of ^{1}H NMR experiments suggest the formation of micelles with some ordered structure, presumably a core-corona system, where PEG segments form a highly hydrated corona surrounding a core composed of diminazene electrostatically bound to CMD segments. Remembering that each drug molecule possesses two cationic centres, the ^{1}H NMR data may be taken as an indication that micelles formed upon charge neutralization ([+]/[−] ∼ 1), in which each drug molecule interacts with two polymer-bound carboxylates, are able to incorporate additional drug molecules, until only one of the two binding sites of the drug is involved in the complexation. This conclusion can be drawn from the combined facts that (i) signals ascribed to protons of the CMD block gradually decrease in intensity in spectra of mixed solutions of 0 < [+]/[−] < 1 and (ii) in the same mixed systems, signals of protons linked to the drug cannot be detected, whereas signals due to the drug protons reappear in mixtures of [+]/[−] > 2, a ratio corresponding to maximum drug loading in micelles of this copolymer. For this ratio, the weight percent loading of drug in the micelle ranges from ∼40 to ∼65 wt% depending on the type of CMD-PEG (Table 3). An identical spectroscopic analysis was performed also to monitor the interactions between DIM and the copolymer 30-CMD_{68}-PEG_{64}, which has a lower DS than 60-CMD_{68}-PEG_{64}, but has the same molar mass. The DIM/30-CMD_{68}-PEG_{64} mixed system followed the same trends as those depicted in Fig. 3, except that the signals due the drug aromatic protons and the CMD protons remained detectable as long as [+]/[−] < 1.6, confirming the observation from DLS experiments (see above) that micelles of this copolymer only form in solutions of [+]/[−] > 1.6. In the case of the samples 85-CMD_{40}-PEG_{140} and 80-CMD_{40}-PEG_{64}, the ^{1}H NMR experiments revealed tends similar to those exhibited by the DIM/60-CMD_{68}-PEG_{64} system. The results of the ^{1}H NMR study go beyond mere structural information. They indicate that for *in vivo* applications it is crucial to use drug-loaded micelles of $1 \leq$ [+]/[−] ≤ 2 in order to ascertain the absence of free drug, which is easily accessible to the external harsh conditions, such as those found in the GIT.

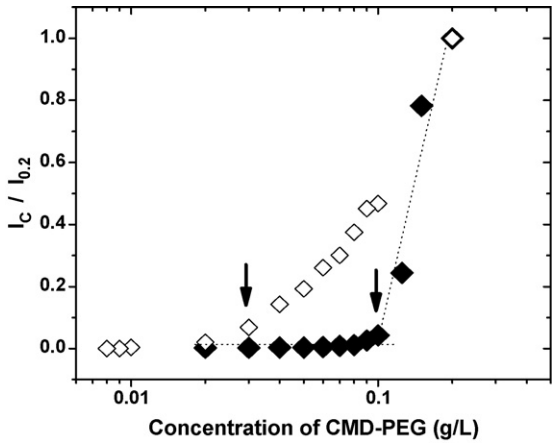

Fig. 4. Plots of the changes as a function of polymer concentration of the ratio ($I_c/I_{0.2}$) for a solution of DIM and 60-CMD_{68}-PEG_{64} (◊) or 30-CMD_{68}-PEG_{64} (♦); polymer of concentration 0.2 g/L; solvent: Tris–HCl buffer, 25 mM, pH 5.3; the arrows indicate the critical association concentration.

3.4. Critical association concentration of diminazene/CMD-PEG micelles

The minimal polymer concentration for which PIC micelles can be detected for a given [+]/[−] ratio, or critical association concentration (CAC), is an important parameter controlling the *in vivo* stability of a drug delivery system subjected to extensive dilution upon administration (Allen et al., 1999). The CAC value of diminazene/CMD-PEG micelles depends on the chemical composition of the ionic diblock copolymer and on the level of drug loading within the micelle. It was determined for micelles formed in Tris–HCl buffer, pH 5.3 by each of the four diblock copolymers in the presence of diminazene in amounts such that [+]/[−] = 2.0. Micellar solutions ranging from 5×10^{-3} g/L to 0.2 g/L were prepared by dilution of a stock micellar solution (CMD-PEG: 0.2 g/L). The intensity of the light scattered by each solution was measured. The CAC values (Table 3) were taken as the concentration corresponding to the onset of the increase in scattered light intensity, determined graphically from plots of $I_c/I_{0.2}$ vs. CMD-PEG concentration, where I_c is the intensity of light scattered by a solution of CMD-PEG of concentration c and $I_{0.2}$ is the intensity of light scattered by a solution of CMD-PEG concentration = 0.2 g/L, as shown in Fig. 4. The CAC value of all micelles is very low, (<0.1 g/L of polymer) vouching for

the stability of the micelles against dilution. The lowest value was recorded for micelles formed by the copolymer of longest CMD block and highest DS (60-CMD_{68}-PEG_{64}), presumably as a consequence of their high drug loading capacity. The length of the PEG block has only a minor influence on the CAC value of the micelles, as seen by comparing the values determined for 85-CMD_{40}-PEG_{140} and 80-CMD_{40}-PEG_{64} (Table 3). Similar trends have been reported in previous studies of other micelles (Alexandridis et al., 1994).

3.5. Effect of salt (NaCl) on micelle formation and stability

Low molecular weight salts screen the charges of the ionic diblock copolymer, such that above a given salt concentration the micellar assemblies fall apart (Harada and Kataoka, 1997; Mao et al., 2006). For *in vivo* applications it is crucial to ascertain that a specific drug/diblock copolymer system can resist the salinity of the biological milieu. Therefore we evaluated by light scattering measurements the salt concentration beyond which diminazene/CMD-PEG micelles do not form, using aqueous DIM/CMD-PEG solutions containing from 0 to 0.6 M [NaCl]. Fig. 5, bottom, illustrates the dependence on salt concentration of the micellar R_H and the scattered light intensity in the case of the DIM/85-CMD_{40}-PEG_{140} system (polymer concentration: 0.2 g/L; [+]/[−] = 2.0). The profile can be divided into three domains: (i) for $0 < [NaCl] \leq 0.2$ M, both R_H and the scattered light intensity (I_{Sc}) increase; (ii) for $0.2 < [NaCl] \leq 0.4$ M, R_H increases whereas I_{Sc} sharply decreases; and (iii) for [NaCl] > 0.4 M, R_H decreases while the scattering intensity remains weak and constant. The micelles of diminazene and 80-CMD_{40}-PEG_{64} as well as the copolymer of lower DS (60-CMD_{68}-PEG_{64}) respond to changes in salinity according to the same three-zone pattern.

The increase of R_H and I_{Sc} in region I may be attributed to an overall increase in micellar size as a result of partial salt-induced dehydration of the PEG corona, which facilitates merging of micelles upon collision and promotes the formation of large micelles. In this region, the salinity is too low to disrupt the drug/CMD-PEG ionic interactions within the core of the micelle. Micelles begin to show signs of disintegration for [NaCl] ∼ 0.3 M as detected by a decrease in scattered light intensity. This salt concentration corresponds to the beginning of region II. The disintegration of the micelles occurs gradually by progressive loosening of the core interactions and expansion of the micelle size. The breadth of region II is narrow, however, and in solutions of [NaCl] > 0.4 M the solution contains primarily isolated drug molecules and polymer chains, with possibly loose drug/polymer associates. Thus, all CMD-PEG micelles in which the DS of the CMD block was 60% or higher are able to resist salt-induced disintegration up to 0.4 M, a value significantly higher than the physiological salt concentration (0.15 M). In contrast, micelles formed between diminazene and the copolymer 30-CMD_{68}-PEG_{64}, proved to be unable to withstand [NaCl] > 0.1 M, even under conditions of charge neutralization ([+]/[−] = 2). For this system, a plot of I_{Sc} vs. [NaCl] (Fig. 5, top) reveals that region I is limited to $0 < [NaCl] < 0.05$ M and region II spans from 0.05 to 0.15 M. Solutions of higher [NaCl] exhibit low scattered light intensity ascribed to the presence of loosely bound objects with $R_H \sim 60$ nm. This observation leads us to conclude that the drug loading must be above a threshold value for charge-neutralized PIC micelles to remain stable under physiological conditions. In the micelles studied here, this value is reached for diminazene/60-CMD_{68}-PEG_{64} ([+]/[−] = 2.0) micelles, but not for diminazene/30-CMD_{68}-PEG_{64} ([+]/[−] = 2.0). This result sets the lowest limit for the charge density of diblock copolymers useful in PIC-type drug delivery systems.

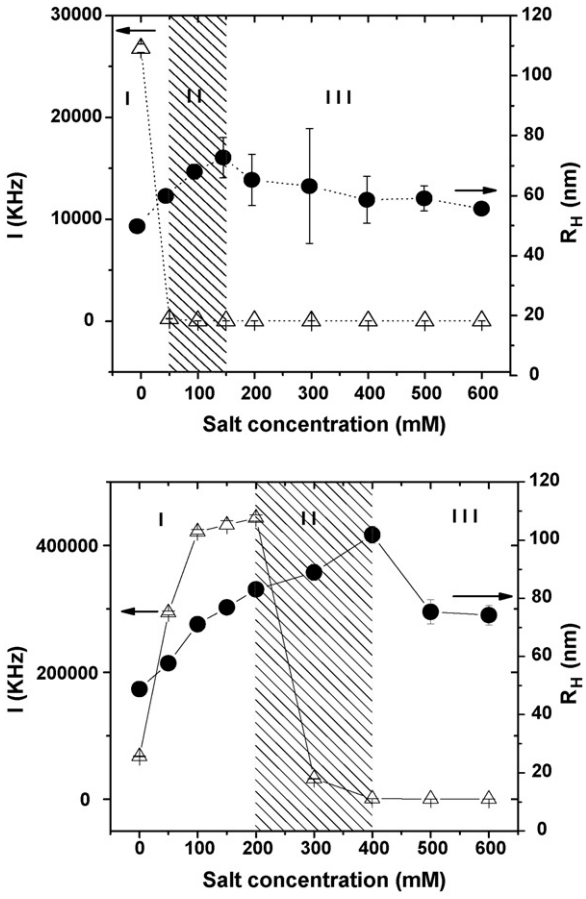

Fig. 5. Plots of the changes of R_H of micelles (●) and the intensity of scattered light (I, △) as a function of NaCl concentration in mixtures of DIM and 30-CMD_{68}-PEG_{64} (top) or 85-CMD_{40}-PEG_{140} (bottom) in Tris–HCl buffer, 25 mM, pH 5.3; polymer concentration: 0.2 g/L; [+]/[−] = 2; temperature: 25 °C; θ: 90°; the hatched area corresponds to region II (see text).

3.6. Zeta-potential studies

The interactions of nanoparticles with cells and cellular components are governed, at least in part, by their surface charge. The zeta (ζ) potential of DIM/85-CMD_{40}-PEG_{140} micelles in Tris–HCl (25 mM, pH 5.3) ranged from ∼−7.6 mV for [+]/[−] = 0.6 to ∼−3.4 mV for [+]/[−] = 2, as expected since their charge is determined by that of the corona (PEG).

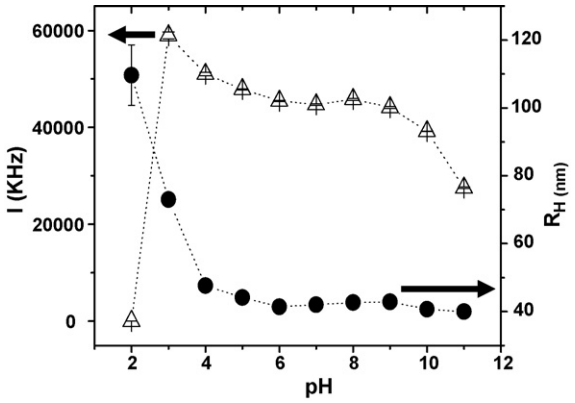

Fig. 6. Plots of the changes of R_H of micelles (●) and of the intensity of scattered light (I, △) as a function of solution pH in mixtures of DIM and 85-CMD_{40}-PEG_{140} in 25 mM Tris–HCl; polymer concentration: 0.2 g/L; [+]/[−] = 2; temperature: 25 °C; θ: 90°.

3.7. Effect of solution pH on the stability of diminazene/CMD-PEG micelles

Since the formation of polyion micelles relies on electrostatic interactions between oppositely charged drug and copolymer, there may exist pH conditions for which one of the interacting components will be neutral, triggering the disruption of the micellar core. In the case of the micelles described here, these conditions are attained when pH < 4 (neutralization of CMD-PEG) or pH > 11 (neutralization of diminazene). The pH-dependence of the R_H of micelles and of the intensity of the light scattered by the solutions was monitored by DLS measurements which indicated that diminazene/85-CMD_{40}-PEG_{140} micelles (polymer concentration: 0.2 g/L; [+]/[−] = 2, Fig. 6) were of constant size ($R_H \sim 50$ nm) and scattering intensity for 4 < pH < 11. Solutions brought to pH < 4.0 rapidly lost their ability to scatter light, presumably as a consequence of the near complete destruction of the micellar assemblies. In the high pH region (pH > 11), similar changes in the scattering characteristics of the samples took place, although the decrease in scattering intensity was not as severe. Similar DLS measurements carried out with polymer solutions (0.2 g/L) in the absence of drug gave no evidence of polymer self-assembly.

Interestingly, the pH-window of micellar stability reported in the case of DIM/poly(aspartic acid)-PEG micelles does not extend beyond 7.2 (Govender et al., 2001) while in our study we ascertained that micellar systems formed by all CMD-PEG copolymers exhibit the same behavior as diminazene/85-CMD_{40}-PEG_{140}, independently on the charge density of the copolymer and of drug loading. The pH sensitivity of these PIC micelles, however, can be taken into advantage in the case of drug delivery systems targeted to cancerous tumors for which the drug must be kept protected under physiological conditions (pH 7.4) and must be released in the mild acidic environment of the extracellular spaces of tumors or in the acidic environment of endosomes (pH ∼ 5–6) or lysosomes (pH ∼ 4–5) following cellular uptake of the PIC micelles (Ulbrich and Šubr, 2004). Nonetheless, the pH window of micellar stability (4–11) prohibits the use of DIM/CMD-PEG micelles in oral formulations, unless care is taken to avoid premature drug release in the stomach, such as application of an appropriate enteric coating (Sinha and Kumria, 2001).

3.8. Storage stability of diminazene/CMD-PEG micelles

We assessed the stability of diminazene/CMD-PEG micelles ([+]/[−] = 2.0) in Tris–HCl buffer, pH 5.3 at room temperature by following the evolution of their R_H over a period of 2 months. In the case of diminazene/80-CMD_{40}-PEG_{140}, for instance, the micelle R_H increased slightly (from 48.5 to 60.1 nm) over the course of the first week and remained constant upon further storage. Tests carried out with micelles of diminazene/CMD-PEG of different composition yielded similar trends, confirming the stability of the micelles. A slight increase in size over the first few days after micelle preparation was noticed in all cases. Initial experiments carried out on micellar formulations in Tris–HCl buffer (pH 5.3, [+]/[−] = 2.0) indicated that redissolution of the lyophilized micelles was incomplete, even after treatment in a sonicator bath. Moreover, the size and size distribution of the micelles were significantly larger, compared to those of the micelles prior to freeze-drying, with an R_H approximately twice that of the original value and a PDI > 0.10. However, micellar solutions complemented with 5% (w/v) of the cryoprotectant trehalose, readily dissolved in water after freeze-drying, yielding diminazene/CMD-PEG micelles of size slightly larger than the original micelles. Thus, diminazene/85-CMD_{40}-PEG_{140} micelles had R_H values of 50 and 75 nm, respectively, before and after freeze-drying/redissolution. The tendency of nanoparticulate formulations to agglomerate upon freeze-drying has been observed previously. Addition of cryoprotectants (Huh et al., 2005; Abdelwahed et al., 2006) or crosslinking of the micellar core (Miyata et al., 2005) are effective means to prevent agglomeration.

3.9. Drug release studies

The release of diminazene diaceturate from DIM/CMD-PEG micelles ([+]/[−] = 2.0) was monitored *in vitro* by the dialysis bag method using micelles formed between the drug and 85-CMD_{40}-PEG_{140}, which were shown to be stable under physiological conditions, as well as micelles formed with 30-CMD_{68}-PEG_{64} known to disintegrate under these conditions. The profile recorded under physiological conditions of pH and ionic strength ([NaCl] = 0.15 M, Tris–HCl buffer 25 mM, pH 7.4) (Fig. 7) reveals complete drug release after ∼8 h. Nonetheless this profile differs significantly from that recorded for a drug solution used as control, especially in the initial part of the release experiment, implying that micelles sustain the drug release over 8 h. In salt-free conditions diminazene/85-CMD_{40}-PEG_{140} micelles retained ∼40% of the drug after 24 h (50% after 8 h, Fig. 7), while diminazene/30-CMD_{68}-PEG_{64} nanoparticles released ∼72% drug after 8 h. These release profiles differ from observations of Prompruk et al. (2005) who noted that DIM/poly(aspartic acid)-PEG micelles undergo immediate DIM

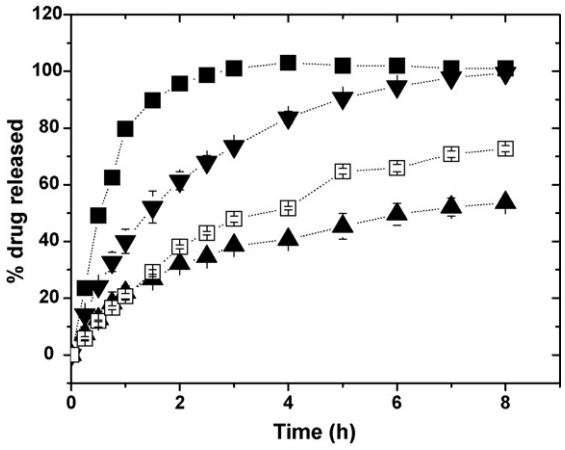

Fig. 7. Release of DIM evaluated by the dialysis bag method from (■) DIM alone in Tris–HCl, 25 mM [NaCl] = 150 mM, pH 7.4; (▼) DIM/85-CMD_{40}-PEG_{140} micelles, [+]/[−] = 2, in 25 mM Tris–HCl, [NaCl] = 150 mM, pH 7.4; (▲) DIM/85-CMD_{40}-PEG_{140} micelles, [+]/[−] = 2, in 25 mM Tris–HCl [NaCl] = 0 mM, pH 5.3, and (□) DIM/30-CMD_{68}-PEG_{64} at [+]/[−] = 2, in Tris–HCl, 25 mM [NaCl] = 0 mM, pH 5.3.

release upon dialysis. We suggest that the enhanced stability of DIM/CMD-PEG micelles, compared to DIM/poly(aspartic acid)-PEG micelles, may be due to the formation of hydrogen bonds between the drug and the CMD block which possesses a large number of hydroxyl groups able to interact with the drug. This synergistic effect of weak bonds is akin to the stabilizing effect of drug/polymer hydrophobic interactions taking place in DIM/poly(aspartic acid-*stat*-phenylalanine which exhibits sustained drug release (Prompruk et al., 2005) or between poly(L-aspartic acid)-PEG in its free acid form and [Arg^8]-vasopressin (Aoyagi et al., 1999). In our case, however, the enhanced stability is an inherent property of the charged copolymers.

4. Conclusion

Four different CMD-PEG block copolymers have been tested for their ability to form PIC micelles with a cationic water soluble drug. The micelles formed were of small size (36–50 nm) and unimodal size distribution (PDI < 0.1). Properties of the micelles such as their stability under different salt concentrations and drug release patterns depend primarily on the degree of substitution of the CMD block, which was readily adjusted by the synthesis protocol. Stable micelles with sustained drug release are formed if the DS of the CMD block exceeds a threshold value (∼40%). ^{1}H NMR spectroscopy was used to determine the [+]/[−] molar ratio for which complete drug incorporation in the micelle core is achieved and the maximum drug loading attained, Further studies are aimed at widening the scope of drug/CMD-PEG micelles by assessing the characteristics of micelles formed by CMD-PEG and other cationic therapeutic agents, proteins and peptides. The *in vivo* properties of drug/CMD-PEG micelles will be monitored next, since preliminary studies indicate that CMD-PEG samples present no toxicity towards several cell lines (Maysinger et al., unpublished data).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2007.12.029.

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