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In-vitro and in-vivo evaluation of pH-responsive polymeric micelles in a photodynamic cancer therapy model

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Abstract

pH-sensitive polymeric micelles of randomly and terminally alkylated *N*-isopropylacrylamide copolymers were prepared and characterized. Aluminium chloride phthalocyanine (AlClPc), a second generation sensitizer for the photodynamic therapy of cancer, was incorporated in the micelles by dialysis. Their photodynamic activities were evaluated in-vitro against EMT-6 mouse mammary tumour cells and in-vivo against EMT-6 tumours implanted intradermally on each hind thigh of Balb/c mice. pH-sensitive polymeric micelles were found to exhibit greater cytotoxicity in-vitro than control Cremophor EL formulations. In the presence of chloroquine, a weak base that raises the internal pH of acidic organelles, in-vitro experiments demonstrated the importance of endosomal/lysosomal acidity for the pH-sensitive polymeric micelles to be fully effective. Biodistribution was assessed by fluorescence of tissue extracts after intravenous injection of 2 μmol kg^{-1} AlClPc. The results revealed accumulation of AlClPc polymeric micelles in the liver, spleen and lungs, with a lower tumour uptake than AlClPc Cremophor EL formulations. However, polymeric micelles exhibited similar activity in-vivo to the control Cremophor EL formulations, demonstrating the higher potency of AlClPc polymeric micelles when localized in tumour tissue. It was concluded that polymeric micelles represent a good alternative to Cremophor EL preparations for the vectorization of hydrophobic drugs.

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Introduction

In recent years, there has been growing interest in photodynamic therapy for the treatment of a variety of solid tumours (Fisher et al 1995; Kessel 1996; Dougherty et al 1998; Bonnett 1999). Therapy is based on the administration of a tumour-localizing photosensitizer and illumination of the lesions with visible light. Photodynamic therapy efficacy depends on formation of the cytotoxic species $^1O_2$, $^{\bullet}O_2^-$ or $^{\bullet}OH$ by the photosensitizers (Ochsner 1997). The porphyrin photosensitizer, Photofrin, presently approved for clinical use, has several disadvantages such as skin photosensitivity and weak absorption of tissue-penetrating red light, which has led to the search for second-generation photosensitizers.

Phthalocyanines are second-generation photosensitizers that show reduced cutaneous photosensitivity and a higher absorption coefficient in the red part of the spectrum where light transmission through tissue is optimal (Roberts et al 1989; Tralau et al 1989). The depth of light penetration in tissues at 675 nm (extinction

coefficient 2.5×10^5 M^{-1} cm^{-1}) is twice that obtained at 630 nm, which is the optimal wavelength for Photofrin (Svaasand 1984). There are two classes of phthalocyanines, the hydrophilic type, which are freely soluble in physiological media, and hydrophobic phthalocyanines, which require appropriate drug formulation, such as liposomes (Isele et al 1995), nanoparticles (Allémann et al 1995) or micelles (Taillefer et al 2000). Some studies have demonstrated that water-insoluble aluminium chloride phthalocyanine (AlClPc) is a better photosensitizer of tumour cells than its water-soluble sulphonated derivative in-vitro (Ben-Hur & Rosenthal 1986) and in-vivo (Chan et al 1997). Cremophor EL-formulated AlClPc is currently one of the most potent therapeutic photosensitizer preparations for in-vivo administration (Chan et al 1997). However, Cremophor EL surfactant is known to induce anaphylactic reactions in patients (Dye & Watkins 1980), and no data are available on the stability of Cremophor EL micelles in-vivo.

Polymeric micelles are interesting candidates for the delivery of water-insoluble components by virtue of increasing their efficiency while at the same time reducing unwanted side-effects (Jones & Leroux 1999). Polymeric micelles offer several advantages over conventional colloidal drug carriers. Their generally small size (< 100 nm) allows them to minimize scavenging by the mononuclear phagocyte system, and water-insoluble drugs can be loaded in their hydrophobic-hydrophilic core-shell structure (Jones & Leroux 1999). The presence of a highly-hydrated outer shell further prevents non-specific interactions and uptake by the mononuclear phagocyte system (Kwon & Okano 1996). Polymeric micelles generally exhibit good stability because of their low critical association concentration (CAC). Moreover, the introduction of a polymer responsive to physical stimuli such as temperature (Cammas et al 1997) or pH (Meyer et al 1998) could theoretically enhance the targeting of tumours by polymeric micelles. We were interested in the preparation of pH-responsive polymeric micelles because the pH in the tumour interstitium can be lower than normal (Tannock & Rotin 1989) and also because, after cellular uptake, the carrier may end up in cellular compartments such as endosomes/lysosomes that exhibit an acidic pH (Collins et al 1989). Such micelles could either accumulate in acidic regions of the body (e.g. tumours) or destabilize the endosomal/lysosomal membrane after internalization. Therefore, we anticipated that polymeric micelles with pH-sensitization could improve the efficiency of AlClPc.

In this study, we examined the in-vitro photodynamic activity of pH-sensitive polymeric micelles of randomly and terminally alkylated *N*-isopropylacrylamide copolymers loaded with AlClPc against EMT-6 mouse mammary tumour cells, and compared it with AlClPc Cremophor EL formulation. We further investigated the biodistribution and pharmacokinetics of the micelles as well as their in-vivo photodynamic activity against EMT-6 tumours implanted intradermally in Balb/c mice.

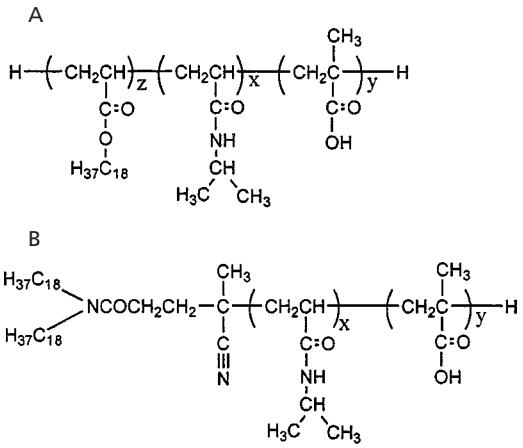

Figure 1 Chemical structure of poly(*N*-isopropylacrylamide-*co*-methacrylic acid-*co*-octadecyl acrylate) (A) and DODA-poly(*N*-isopropylacrylamide-*co*-methacrylic acid) (B).

Materials and Methods

Materials

N-Isopropylacrylamide, methacrylic acid, octadecyl acrylate, 1,1′-azobis(cyclohexanecarbonitrile) (ACCN) and AlClPc were purchased from Aldrich Chemical Co. (Milwaukee, WI). *N*-Isopropylacrylamide was purified by re-crystallization from heptane–acetone (3:2, v/v). Methacrylic acid was purified using an inhibitor-remover disposable column for hydroquinone and monomethylether hydroquinone (Aldrich). ACCN was dissolved in ethanol, filtered, re-crystallized in water and dried under vacuum. 1,2-Propanediol, chloroquine and Cremophor EL were from Sigma (St Louis, MO). The lipophilic initiator DODA-501, 4,4′-azobis(4cyano-*N*,*N*-dioctadecylvaleramide), was prepared by reaction of dioctadecylamine with disuccinimidyl 4,4′-azobis(4-cyanovalerate), as described previously (Kitano et al 1991). All other chemicals were of analytical grade and used as received.

Synthesis

The randomly alkylated copolymers (Figure 1A) poly(*N*-isopropylacrylamide$_{93}$-*co*-methacrylic acid$_5$-*co*-

octadecyl acrylate$_2$) and poly(*N*-isopropylacrylamide$_{95}$-*co*-methacrylic acid$_3$-*co*-octadecyl acrylate$_2$) were prepared by free radical polymerization. *N*-Isopropylacrylamide, methacrylic acid, octadecyl acrylate (at different molar ratios as indicated by the monomers' subscripts) and ACCN (1.3 mol%) were dissolved in distilled 1,4-dioxane. The solution was degassed by bubbling with N_2 for 15 min. Polymerization occurred as the solution was heated while stirring at 65°C for 5 h. Polymers were recovered by precipitation in diethyl ether, re-solubilized in tetrahydrofuran (THF), re-precipitated and washed extensively with diethyl ether. They were then dissolved in water, filtered and freeze-dried (Taillefer et al 2000). The terminally alkylated polymer (Figure 1B) DODA-poly(*N*-isopropylacrylamide$_{96}$-*co*-methacrylic acid$_3$) in which the hydrophobic group is attached to one end of the polymeric chain was prepared using DODA-501 (Kitano et al 1991) as radical initiator (0.9 mol%). *N*-Isopropylacrylamide, methacrylic acid and DODA-501 were dissolved in dioxane. The solution was degassed by bubbling with N_2 for 15 min and heated to 70°C for 17 h. The polymer was recovered by precipitation in diethyl ether. It was dissolved in THF and re-precipitated in diethyl ether. The polymer was dried in-vacuo for 24 h, dissolved in water, filtered and freeze-dried.

Characterization

The copolymers were characterized by ^{1}H NMR spectrometry and titration for methacrylic acid content (Han & Bae 1998). ^{1}H NMR spectra were recorded on a Bruker AMX600 spectrometer in deuterated chloroform solutions at 25°C with a relaxation time of 10 s. The weight- (M_w) and number-average (M_n) molecular weights of the polymers were established by gel-permeation chromatography (Waters Model 600, Milford, MA) employing the Millennium software program. HR1, HR3 and HR4 Styragel columns (Waters, 4.6 × 300 mm) and a differential refractometer detector (Waters 2410) were used. The mobile phase was THF (30°C and 1 mL min^{-1}). The calibration curve was produced with polystyrene standards (Aldrich). The CAC was determined by a steady-state pyrene fluorescence method described elsewhere (Yamazaki et al 1998). It has been shown previously that increasing the concentration of amphiphilic polymers in aqueous pyrene solution causes a shift of the (0,0) band from 333 to 338.5 nm in the excitation spectra of pyrene. This change, as measured by the I_{338}/I_{333} intensity ratio, accompanies the transfer of pyrene molecules from a water environment to the hydrophobic micellar cores, and can be used to estimate the apparent CAC. Several polymeric solutions in water containing 10^{-7} M pyrene were prepared and stirred overnight in the dark at room temperature. Steady-state fluorescent spectra were measured (λ_{em} = 390 nm) after 5 min stirring at 20°C using a Series 2 Aminco Bowman fluorimeter (Spectronic Instruments Inc., Rochester, NY). The pH at which the polymer precipitates (25 μg mL^{-1}) was determined by 90° static light scattering ($\lambda_{ex} = \lambda_{em}$ = 480 nm) after incubation at 37°C for 5 min in phosphate-buffered saline (PBS) pH 4.8–7.4 using a 650 Perkin-Elmer fluorescence detector (Norwalk, CT). Micelle sizes were measured at room temperature by dynamic laser light scattering (DLS) employing differential size distribution processor (SDP) analysis (Coulter N4 Plus, Hialeah, FL).

Incorporation of AlClPc

The drug was incorporated into polymeric micelles according to a dialysis procedure described elsewhere (Taillefer et al 2000). Briefly, the drug and the polymer were solubilized in *N*,*N*-dimethylformamide (DMF) at an initial drug/copolymer mass ratio of 0.04 and dialysed at room temperature for 24 h, in the dark, against water using a Spectra/Por membrane with a molecular weight cut-off of 6000–8000 g mol^{-1} (Spectrum Laboratories, Inc., Rancho Dominguez, CA). The solutions were filtered through a 0.22-μm pore-size filter and freeze-dried. Drug content was assayed by spectrophotometry in DMF at 670 nm, in a Hewlett Packard 8452A diode array spectrophotometer (Boise, ID).

In-vitro cytotoxicity

Cells

Mouse mammary tumour EMT-6 cells were maintained in Waymouth's medium (Gibco, Burlington, Canada) supplemented with 15% fetal bovine serum (FBS) (ICN, Aurora, OH), 1% L-glutamine and 1% Penicillin-Streptomycin (Gibco).

Cellular photo-inactivation

Stock solutions of AlClPc-loaded polymeric micelles in dextrose 5% (w/v) and AlClPc in PBS containing 10% (v/v) Cremophor EL and 3% (v/v) 1,2-propanediol (AlClPc-Cremophor EL) (Chan et al 1997) were prepared and filtered with a 0.22-μm filter. The solutions were then diluted to the desired concentration with Waymouth's medium (Gibco) containing 1% FBS (ICN). Cell survival was estimated by means of the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Tada et al 1986).

Table 1 Characterization of the polymer and polymeric micelles.

	Poly(*N*-isopropylacrylamide$_{93}$-*co*-methacrylic acid$_5$-*co*-octadecyl acrylate$_2$)-	Poly(*N*-isopropylacrylamide$_{95}$-*co*-methacrylic acid$_3$-*co*-octadecyl acrylate$_2$)	DODA-poly (*N*-isopropylacrylamide$_{96}$-*co*-methyl acrylic acid$_3$)
M_n	27900	35500	9700
M_w	48500	126000	31150
CAC (mg L^{-1})	10	8	20
Phase transition pH	5.7	6.0	6.1
Micelle size (nm)	Water: 13 ± 6 PBS: 35 ± 17	Water: 17 ± 8 PBS: 146 ± 64	Water: 53 ± 25 PBS: 58 ± 25
Drug loading (% w/w)	3.9	3.3	1.9

Briefly, 15×10^3 EMT-6 cells per well were inoculated in 100 μL Waymouth's growth medium in 96-well plates and incubated overnight at 37°C and 5% CO_2. The cells were rinsed twice with PBS and incubated for 1 or 24 h with 100 μL of the drug (AlClPc-Cremophor EL or AlClPc polymeric micelles) at various concentrations in Waymouth's medium containing 1% FBS at 37°C and 5% CO_2. After incubation, the cells were rinsed twice with PBS, fed with 100 μL Waymouth's medium and exposed to red light. The light source consisted of two 500 W tungsten/halogen lamps (GTE Sylvania, Montreal, Canada) fitted with a circulating, refrigerated, aqueous Rhodamine filter. The incident light intensity calculated over the absorbance peak of AlClPc (660–700 nm) was 10 mW cm^{-2}, and the plates were illuminated for 10 min at a total light dose of 6 J cm^{-2}. The cells were incubated overnight at 37°C and 5% CO_2 before cell viability was assessed. Fifty microlitres of a 5-fold diluted MTT stock solution (Aldrich; 5 mg mL^{-1} PBS) in Waymouth's medium were added to each well. After 3 h, 100 μL of sodium dodecyl sulfate 10% (Gibco) in 0.01 N HCl were added to the wells. The plates were incubated overnight at 37°C, after which absorbance was read at 570 nm by means of a microplate reader (Molecular Devices, Thermo Max, Sunnyvale, CA). Average absorbance of the blank wells in which cells were omitted was subtracted from readings of the other wells. Average absorbance of the control cells, which were incubated with drug-free Waymouth's 1% FBS, represents 100% cell survival. The extracellular drug dose required to inactivate 90% of the cells (LD90) was extrapolated from the survival curves. In another series of experiments, cells were pre-incubated with chloroquine 50 μM in Waymouth's medium containing 1% FBS for 1 h and then incubated for 1 or 24 h with AlClPc polymeric micelles or AlClPc-Cremophor EL in the presence of chloroquine 50 μM as described above. Chloroquine is a weak base which accumulates in endosomes/lysosomes and raises the internal pH of these organelles (de Duve et al 1974). These experiments were carried out to determine whether the activity of AlClPc polymeric micelles was dependent on their pH-responsiveness. Eight-fold replicates were run per drug and light dose, and each experiment was repeated at least 3 times.

In-vivo studies

Tumour models

Experiments were performed on male BALB/c mice (18–22 g) (Charles River Breeding Laboratories, Montreal, Canada) bearing the EMT-6 tumour following a protocol approved by the Canadian Council on Animal Care and in-house ethics committee. The animals were allowed free access to water and food throughout the course of the experiments. Before tumour implantation, hair on the hind legs and back of the mice was removed by shaving and chemical depilation (Nair, Whitehall, Mississauga, Canada). One EMT-6 tumour (2–3 for biodistribution) was implanted on each hind thigh by intradermal injection of 2×10^5 cells suspended in 0.05 mL growth medium.

Biodistribution

Mice were used 10 or 11 days after cell inoculation when tumour diameter and thickness reached 4–8 mm and 2–4 mm, respectively. Tumour-bearing mice were injected via the caudal vein with 2 μmol kg^{-1} of drug (0.2 mL/20 g body weight). At different time intervals after drug administration (30 min–1 week), blood was collected by intracardiac puncture via a heparinized syringe, whereafter the animals ($n = 4$ per time interval) were killed. The blood was centrifuged at 4°C in Eppendorf tubes for 5 min at 2000 *g*, and the plasma collected. Plasma or blood (100 μL) was mixed with 1.9 mL DMF. Organs and tissues of interest were re-

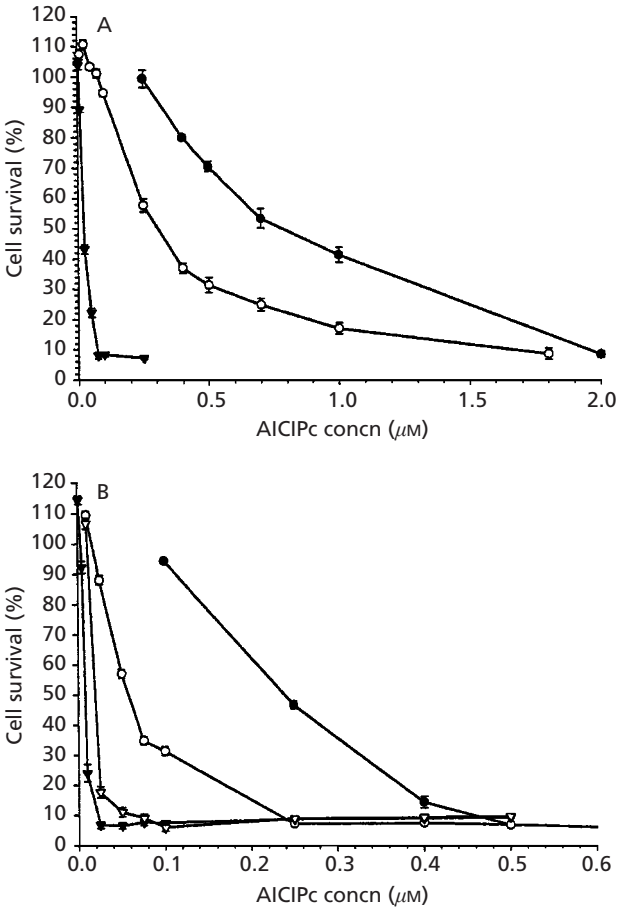

Figure 2 Cell survival after 1 h (A) or 24 h (B) of incubation with AlClPc loaded into poly(*N*-isopropylacrylamide$_{93}$-*co*-methacrylic acid$_5$-*co*-octadecyl acrylate$_2$) (▽), poly(*N*-isopropylacrylamide$_{95}$-*co*-methylacrylic acid$_3$-*co*-octadecyl acrylate$_2$) (▼), DODA-poly(*N*-isopropylacrylamide$_{96}$-*co*-methacrylic acid$_3$) (○) or Cremophor EL (●) micelles.

moved, washed with saline (0.9%) and blotted dry. Whole tumours (2 or 3), samples of other organs and minced skin (80–150 mg) were homogenized with 20 volumes of DMF in a Polytron fitted with a PT 10/35 rotor (Brinkmann, Mississauga, Canada). The homogenates were centrifuged at 4°C (2790 *g* for 10 min). The drug concentration in the clear supernatant was assayed by fluorescence (Fluorescence spectrophotometer F-2000, Hitachi, Tokyo, Japan) (λ_{ex} 606 nm, λ_{em} ~ 680 nm; band pass 5 nm and 10 nm, respectively). Calibration curves were established by adding known amounts of drug to 100 μL of plasma or blood or 80–150 mg of tissue samples from control mice, after which the tissues were treated as described above. No fluorescence was found in control tissue samples to which no drug had been added.

Photodynamic therapy

For photodynamic therapy studies, mice (4–12 per group) were used 6–8 days after tumour inoculation (tumour size: 3–5 mm diameter, 2–3 mm thickness). They were given an intravenous injection of drugs at a dose of 0.1–0.5 μmol kg^{-1} (0.2 mL/20 g body weight), and the right tumour was treated with red light 24 h later while the left one served as control. Animals were discarded from the analysis if the control tumour underwent spontaneous regression or showed abnormally slow growth. Tumours were illuminated with an 8-mm diameter beam of 650–700 nm light (200 mW cm^{-2} for a total light dose of 400 J cm^{-2}) generated by a 1000 W Xenon lamp, equipped with a 10-cm circulating water filter and 2 glass filters (Corion LL650 and LS700, Holliston, MA). A positive tumour response was assigned to tumours that appeared macroscopically as flat and necrotic tissues within a few days after photodynamic therapy. Complete tumour regression was defined as the absence of a palpable tumour at 3 weeks after photodynamic therapy.

Statistical methods

The statistical significance of the difference between the LD90 values with and without chloroquine was calculated by Student's *t*-test. In other cases (in-vitro experiments and biodistribution studies), statistical significance of differences was calculated using a one-way analysis of variance followed by a post-hoc Scheffé test for multiple comparisons. Only probability values of $P < 0.05$ were considered statistically significant.

Results

Characterization of polymers and polymeric micelles

Table 1 shows the characteristics of the polymers and polymeric micelles. All copolymers self-associated into supramolecular aggregates as evidenced by DLS which showed particle sizes in the range 10–150 nm. The CACs, as determined by spectrofluorimetry, were below 25 mg L^{-1} for all polymeric micelle formulations. The terminally alkylated copolymers showed the highest CAC probably because each polymeric chain carries only two alkyl segments. These micelles should, in theory, be less stable upon dilution. The phase-transition pH was dependent on the methacrylic acid content, since the copolymer bearing 5 mol% methacrylic acid precipitated at a pH value lower than the copolymers con-

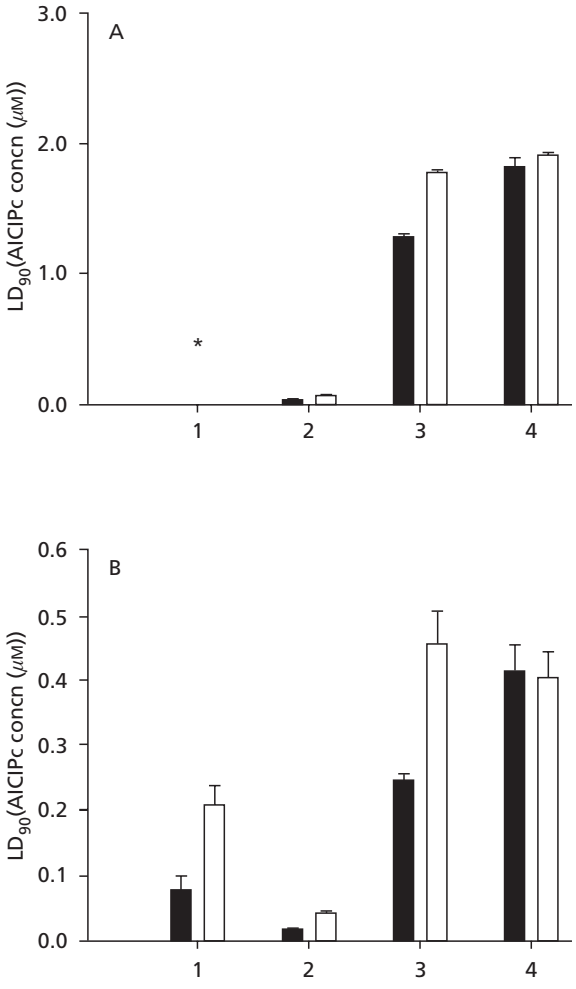

Figure 3 LD90 of AlClPc after 1 h (A) or 24 h (B) of incubation, with (□) or without (■) 50 μM chloroquine. Poly(*N*-isopropylacrylamide$_{93}$-*co*-methacrylic acid$_5$-*co*-octadecyl acrylate$_2$) (1), poly(*N*-isopropylacrylamide$_{95}$-*co*-methacrylic acid$_3$-*co*-octadecyl acrylate$_2$) (2), DODA-poly(*N*-isopropylacrylamide$_{96}$-*co*-methacrylic acid$_3$) (3) and Cremophor EL (4) micelles. *Not determined. Mean ± s.e.m. (n = 3).

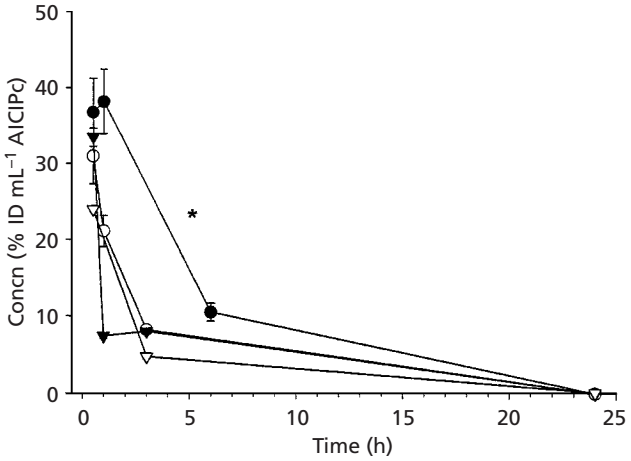

Figure 4 Drug blood concentration of AlClPc after intravenous injection of 2 μmol kg^{-1} loaded into poly(*N*-isopropylacrylamide$_{93}$-*co*-methacrylic acid$_5$-*co*-octadecyl acrylate$_2$) (▽), poly(*N*-isopropylacrylamide$_{95}$-*co*-methacrylic acid$_3$-*co*-octadecyl acrylate$_2$) (▼), DODA-poly(*N*-isopropylacrylamide$_{96}$-*co*-methacrylic acid$_3$) (○) or Cremophor EL (●) micelles, in EMT-6 tumour-bearing mice. ID, injected dose. *Taken from Brasseur et al (1999). Mean ± s.e.m. (n = 4).

taining 3 mol% methacrylic acid. Finally, polymeric micelles prepared with the terminally alkylated copolymer exhibited a lower drug loading than their randomly alkylated counterparts, reflecting the lower proportion of alkyl chains.

Cell photo-inactivation

The effect of AlClPc incorporated in different carriers on cell survival is presented in Figure 2. No dark toxicity was observed against EMT-6 cells with any of the micelles at the concentrations and incubation times studied (data not shown). Upon light treatment, all AlClPc polymeric micelle formulations exhibited greater photoactivity than AlClPc-Cremophor EL. The terminally alkylated copolymer appeared to be significantly less efficient than its random counterpart with an LD90 value of 1.3 and 0.25 vs 0.05 and 0.02 μM after 1-h (A) and 24-h (B) incubation, respectively. Randomly alkylated copolymer micelles of poly(*N*-isopropylacrylamide$_{95}$-*co*-methylacrylic acid$_3$-*co*-octadecyl acrylate$_2$) prepared with 3 mol% methacrylic acid were found to be slightly more effective ($LD90_{24h} = 0.02\ \mu$M) than micelles of poly(*N*-isopropylacrylamide$_{93}$-*co*-methacrylic acid$_5$-*co*-octadecyl acrylate$_2$) bearing 5 mol% methacrylic acid ($LD90_{24h} = 0.08\ \mu$M), although the LD90 values were not statistically different. To determine whether endosome/lysosome acidification was required for pH-sensitive polymeric-micelle-mediated cytotoxicity to EMT-6, the experiments were repeated in the presence of chloroquine, a weak base that raises the internal pH of acidic organelles (de Duve et al 1974). As shown in Figure 3, in the presence of chloroquine, the activity of the drug loaded in the pH-sensitive micelles decreased ($P < 0.05$) whereas it remained unchanged in the case of the control formulation (AlClPc-Cremophor EL, $P > 0.05$). These results point to the importance of endosomal/lysosomal acidity for the pH-sensitive polymeric micelles to be fully effective. The influence of chloroquine was more pronounced after 24 h (Figure 3B) than after 1 h (Figure 3A) incubation,

Table 2 Tissue concentration of AlClPc in EMT-6 tumour-bearing mice (n = 4) after intravenous injection of dye (2 μmol kg^{-1} of AlClPc) formulated in different vehicles.

Tissue	Concentration (% injected dose/g)[a]				
	0.5 h	1 h	3 h	24 h	168 h
Poly(*N*-isopropylacrylamide$_{93}$-*co*-methacrylic acid$_5$-*co*-octadecyl acrylate$_2$)					
Blood	24.00 (0.37)	ND	4.65 (0.33)	0.13 (0.02)	< 1
Plasma	50.29 (0.65)	25.31 (1.96)	8.03 (0.75)	< 1	< 1
Tumour	0.96 (0.07)	2.02 (0.36)	1.70 (0.44)	3.27 (0.57)	2.16 (0.24)
Muscle	1.13 (0.19)	0.82 (0.05)	1.73 (0.28)	1.49 (0.19)	1.53 (0.08)
Skin	0.95 (0.03)	1.04 (0.02)	1.74 (0.05)	1.13 (0.13)	1.63 (0.15)
Skin tumour	0.70 (0.14)	1.04 (0.02)	1.23 (0.07)	2.18 (0.21)	2.51 (0.28)
Liver	24.36 (2.22)	35.27 (3.09)	34.46 (6.27)	46.25 (2.89)	17.87 (1.30)
Spleen	22.65 (0.64)	26.48 (0.34)	37.49 (4.22)	63.13 (1.50)	48.66 (1.62)
Lung	16.65 (0.23)	15.49 (0.39)	19.26 (0.37)	65.98 (5.29)	26.88 (1.62)
Kidney	6.96 (0.22)	7.42 (0.24)	7.12 (0.10)	11.02 (0.32)	12.41 (0.60)
DODA-poly(*N*-isopropylacrylamide$_{96}$-*co*-methacrylic acid$_3$)					
Blood	30.99 (3.63)	21.15 (2.06)	8.18 (0.53)	< 1	< 1
Plasma	78.80 (8.74)	51.21 (8.29)	17.99 (1.08)	< 1	< 1
Tumour	1.19 (0.32)	0.55 (0.14)	0.91 (0.03)	1.94 (0.63)	1.90 (0.29)
Muscle	0.69 (0.08)	0.67 (0.05)	0.61 (0.05)	0.65 (0.05)	0.70 (0.06)
Skin	0.75 (0.07)	0.64 (0.07)	0.73 (0.11)	0.83 (0.19)	0.71 (0.06)
Skin tumour	0.67 (0.06)	0.46 (0.03)	0.57 (0.03)	0.57 (0.07)	0.48 (0.03)
Liver	13.66 (4.09)	20.09 (2.42)	22.65 (1.87)	30.54 (5.76)	20.52 (1.92)
Spleen	146.2 (33.17)	607.00 (19.04)	613.5 (92.47)	540.5 (55.73)	550.9 (80.9)
Lung	21.03 (2.52)	37.86 (9.56)	13.30 (1.27)	6.66 (1.44)	7.14 (0.56)
Kidney	8.02 (0.89)	7.15 (0.68)	5.87 (0.81)	6.74 (2.17)	3.47 (1.32)
Poly(*N*-isopropylacrylamide$_{95}$-*co*-methacrylic acid$_3$-*co*-octadecyl acrylate$_2$)					
Blood	33.51 (0.22)	7.35 (0.34)	7.96 (0.38)	< 1	< 1
Plasma	88.31 (4.30)	12.11 (0.38)	2.34 (0.29)	< 1	< 1
Tumour	1.93 (0.46)	0.75 (0.10)	1.36 (0.06)	1.19 (0.14)	2.34 (0.33)
Muscle	0.69 (0.07)	1.20 (0.14)	0.87 (0.12)	1.55 (0.17)	1.56 (0.04)
Skin	0.88 (0.10)	0.93 (0.07)	0.86 (0.15)	0.97 (0.05)	1.33 (0.03)
Skin tumour	1.10 (0.16)	0.73 (0.06)	1.22 (0.31)	1.40 (0.12)	1.78 (0.03)
Liver	13.43 (3.25)	18.27 (1.20)	27.39 (0.59)	57.67 (10.00)	36.21 (1.72)
Spleen	83.05 (4.14)	73.5 (4.00)	106.6 (19.45)	100.3 (5.66)	113.3 (11.25)
Lung	23.82 (5.49)	79.03 (6.82)	187.8 (26.41)	109.1 (29.21)	27.52 (3.14)
Kidney	3.31 (0.21)	5.66 (0.23)	15.92 (1.63)	8.94 (0.88)	8.23 (2.82)

[a]1 % injected dose = 0.23 μg AlClPc. Values in brackets are s.e.m.

indicating the progressive accumulation of chloroquine in acidic organelles (Poole & Ohkuma 1981). However, even in the presence of chloroquine, the pH-responsive randomly alkylated micelles remained more potent than the Cremophor EL formulation (Figure 3).

Biodistribution

The data on AlClPc pharmacokinetics and biodistribution after intravenous administration of 2 μmol kg^{-1} pH-sensitive AlClPc polymeric micelles to EMT-6 tumour-bearing mice are given in Figure 4 and Table 2, respectively. The drug loaded into pH-sensitive polymeric micelles was cleared from blood more rapidly than AlClPc-Cremophor EL ($t_{\frac{1}{2}} = 23$ min) (Brasseur et al 1999) (Figure 4) and accumulated mainly in the liver and spleen, probably reflecting uptake by the mononuclear phagocyte system (Table 2). The highest spleen drug levels were obtained with the terminally alkylated polymers at all time points ($P < 0.05$) except 0.5 h. AlClPc levels in the tumour 24 h post injection were substantially lower than those previously obtained with the Cremophor EL formulation (Brasseur et al 1999) and were stable for 1 week. High drug levels were found in the lung with poly(*N*-isopropylacrylamide$_{95}$-*co*-methacrylic acid$_3$-*co*-octadecyl acrylate$_2$) ($P < 0.05$ for 1- and 3-h time points), which is the most hydrophobic polymer. This probably reflects aggregation of the micelles

Table 3 Photodynamic therapy results.

AlClPc formulation	Drug dose (μmol kg^{-1})	n	Volume[a] (mm^3)	Tumour response (% of mice)	
				Regression[b]	Cure[c]
Cremophor EL[d]	0.25	8	8.7 (0.7)	100	100
Poly(*N*-isopropylacrylamide$_{93}$-*co*-methylacrylic acid$_5$-*co*-octadecyl acrylate$_2$)	0.1	12	16.4 (1.2)	83	66
	0.25	8	13.9 (1.1)	100	75
	0.5	4	22.4 (3.0)	100	100
Poly(*N*-isopropylacrylamide$_{95}$-*co*-methylacrylic acid$_3$-*co*-octadecyl acrylate$_2$)	0.1	4	16.9 (3.5)	75	0
	0.25	7	17.9 (1.8)	100	100
DODA-poly-(*N*-isopropylacrylamide$_{96}$-*co*-methylacrylic acid$_3$)	0.1	4	16.3 (2.7)	63	0
	0.25	6	17.5 (2.1)	100	83

[a]Tumour volume at the time of photodynamic therapy. [b]Flat and necrotic within 3–5 days after therapy. [c]Complete tumour regression at 21 days post-inoculation. [d]Taken from Brasseur et al (1999). Values in brackets are s.e.m.

and subsequent trapping in lung capillaries. From the data presented in Table 2, the tumour-to-tissue (skin, tumour skin and muscle) ratio at 24 h was calculated. It varied between 1.5 and 3.4 for poly(*N*-isopropylacrylamide$_{93}$-*co*-methacrylic acid$_5$-*co*-octadecyl acrylate$_2$) and DODA-poly(*N*-isopropylacrylamide$_{95}$-*co*-methacrylic acid$_3$) and was lower than 1.22 for poly(*N*-isopropylacrylamide$_{95}$-*co*-methacrylic acid$_3$-*co*-octadecyl acrylate$_2$).

Photodynamic therapy

Photodynamic therapy of the EMT-6 tumour showed that pH-sensitive AlClPc polymeric micelles were able to induce substantial tumour control at the same drug dose as AlClPc-Cremophor EL (Table 3). Complete EMT-6 tumour regression was achieved in 85% of animals, or more, with 0.25 μmol kg^{-1} of all three drug preparations compared with control tumours.

Discussion

The efficiency of a photosensitizer is dependent upon its ability to be taken up by cells and upon its photochemical properties (Berg et al 1989). The increased in-vitro activity of pH-sensitive AlClPc polymeric micelles vs AlClPc-Cremophor EL may be related to differences in micelle stability or cellular uptake. Polymeric micelles may be partly taken up by cells via an endocytotic process. Indeed, it has been demonstrated recently that polymeric micelles composed of polycaprolactone-*b*-poly(ethylene oxide) were endocytosed by PC12 cells (Allen et al 1999). Furthermore, the fact that micelles produced with the randomly alkylated copolymer were more efficient than terminally alkylated polymeric micelles could be explained by differences in interactions with cell membranes. The terminally alkylated copolymer associates in such a manner that the linear hydrophilic segments that create a steric barrier remain freely mobile and are excluded from the micellar core (Figure 5) (Chung et al 1998). Such a conformation is less likely to interact with macromolecules and cell membranes (Blume & Cevc 1993; Torchilin et al 1994). In contrast, the chain mobility of the randomly alkylated copolymer is restrained, and the hydrophobic core can be partially exposed to water (Figure 5). This may result in increased binding to the plasma membrane and thus enhanced uptake. Alternatively, terminally alkylated copolymer micelles are less stable than their random counterparts because of their higher CAC (Table 1), and thus dissociate more easily upon dilution in the culture medium. Experiments are currently underway to clarify this issue. It was further shown that acidification of

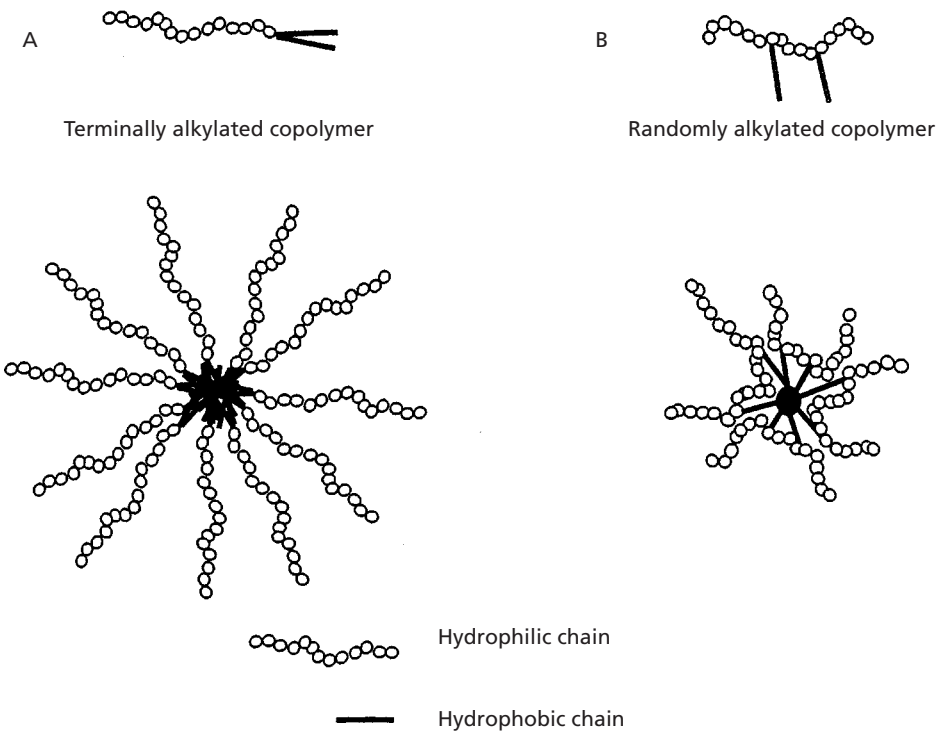

Figure 5 Schematic representation of terminally and randomly alkylated copolymer micelles.

endosomes/lysosomes was required for pH-responsive micelles to be fully effective (Figure 3). In a previous study, we demonstrated that copolymers of *N*-isopropylacrylamide could destabilize phospholipid membranes at acidic pH (Meyer et al 1998). Thus, following endocytosis, pH-responsive micelles may undergo phase transition which may, in turn, result in drug release (Chung et al 1999) or destabilization of the endosomal/lysosomal membrane. This may alter the intracellular localization of the drug, providing an explanation for the increased potency of AlClPc. Indeed, it has been reported that some photosensitizers are less photocytotoxic when located in plasma membranes than in other cellular compartments (Moan et al 1984). There is also some evidence for a lower quantum yield of cell inactivation for phthalocyanine located within lysosomes than that located outside lysosomes (Moan et al 1992). For instance, drugs located within mitochondria have been found to be highly efficient in sensitizing cells to photo-inactivation (Woodburn et al 1992).

The hypothesis of pH-induced membrane destabilization or drug release is further supported by the fact that the randomly alkylated copolymer micelles prepared with 3 mol% methacrylic acid seemed to be more efficient in-vitro than those prepared with 5 mol% methacrylic acid, although the difference could not be statistically demonstrated. The former micelles underwent phase transition at pH 6.0, compared with pH 5.7 for the latter (Table 1). Under weakly acidic conditions, micelles exhibiting the higher phase transition pH are expected to more readily destabilize cell membranes.

The enhanced activity of polymeric micelles might not be solely attributed to pH sensitivity since, in the presence of chloroquine, AlClPc was still more active than AlClPc-Cremophor EL. It had been previously shown that the addition of 50–100 μM chloroquine brings the pH of lysosomes above 6 (Reijngoud & Tager 1976; Poole & Ohkuma 1981). Although we cannot exclude residual pH sensitivity even when the pH gradient is reduced, the observed enhanced activity is more likely attributed to more significant uptake or a different uptake pattern, as discussed above.

The pharmacokinetic parameters of colloidal carriers are complex, depending on size, surface charge and steric stabilization. Several studies have shown that polymeric micelles could enhance the plasma half-life of drugs (Kwon et al 1994), while others have failed to demonstrate such an effect (Zhang et al 1997). In this study, it was noted that AlClPc polymeric micelles were cleared more rapidly and accumulated less in the tumour

than AlClPc-Cremophor EL (Brasseur et al 1999). Such a rapid uptake was unexpected, especially because *N*-isopropylacrylamide copolymers have been reported to reduce the absorption of plasma proteins on liposomal membranes when they were in their extended conformation (i.e. below the temperature at which they phase-separate) (Yamazaki et al 1999). Thus, it was expected that these micelles, owing to their small size (Table 1) and potential stealth effect, might exhibit prolonged circulation times in-vivo. Rapid uptake by the mononuclear phagocyte system and, in the case of poly (*N*-isopropylacrylamide$_{95}$-*co*-methacrylic acid$_3$-*co*-octadecyl acrylate$_2$), significant accumulation in the lungs could result from some micelle aggregation in plasma. Indeed, it is known that the phase transition of *N*-isopropylacrylamide copolymers is influenced, for instance, by the presence of inorganic ions (Schild & Tirrell 1990), and partial dehydration with aggregation of these polymers in blood at 37°C resulting from accelerated phase transition cannot be excluded. An increase in micelle hydrophobicity could lead to their aggregation, heightened opsonization and thus rapid removal by the mononuclear phagocyte system. If the size of the aggregates is sufficiently important, the micelles may end up in lung capillaries (Davis et al 1993). More importantly, the polymers used in this work were negatively charged at pH 7.4 (ionization of the methacrylic acid moiety), and this may accelerate micelle removal by the mononuclear phagocyte system. For instance, it was demonstrated in a number of studies that the presence of negatively charged lipids in liposomes can lead to rapid uptake by mononuclear phagocytes (Senior et al 1985). However, despite lower tumoral drug concentrations, polymeric micelles exhibited activity similar in-vivo to that of the control Cremophor EL formulation, revealing the higher potency of AlClPc polymeric micelles when localized in tumour tissue.

Shielding of liposomal charges by bulky hydrophilic moieties has been shown to improve the circulation times of liposomes (Woodle et al 1992), and experiments are currently ongoing in our laboratory to determine whether this could apply to polymeric micelles. It would also be interesting to investigate the influence of the polymer molecular weight on AlClPc biodistribution. Finally, it is important to note that the molecular weight of these copolymers was relatively high (Table 1). Since *N*-isopropylacrylamide polymers are a-priori not biodegradable in-vivo, it would be worthwhile to study the pharmacokinetics and biodistribution of lower-molecular-weight polymers which may interact differently with blood components and which could be eliminated by renal excretion.

Conclusion

In the experimental model used, pH-responsive polymeric micelle formulations were more potent than control formulations. In-vitro, efficacy was influenced by polymeric structure and endosomal/lysosomal pH. However, the polymeric micelle formulations still had to be optimized to decrease their uptake by the mononuclear phagocyte system and increase their localization in tumours. They represent a good alternative to Cremophor EL preparations for the vectorization of hydrophobic drugs.

References

Allémann, E., Brasseur, N., Benrezzak, O., Rousseau, J., Kudrevich, S. V., Boyle, R. W., Leroux, J.-C., Gurny, R., Van Lier, J. E. (1995) PEG-coated poly(lactic acid) nanoparticles for the delivery of hexadecafluoro zinc phthalocyanine to EMT-6 mouse mammary tumours. *J. Pharm. Pharmacol.* **47**: 382–387

Allen, C., Yu, Y., Eisenberg, A., Maysinger, D. (1999) Cellular internalization of PCL_{20}-b-PEO_{44} block copolymer micelles. *Biochim. Biophys. Acta* **1421**: 32–38

Ben-Hur, E., Rosenthal, I. (1986) Photosensitization of chinese hamster cells by water-soluble phthalocyanines. *Photochem. Photobiol.* **43**: 615–619

Berg, K., Bommer, J. C., Moan, J. (1989) Evaluation of sulfonated aluminum phthalocyanines for use in photochemotherapy. Cellular uptake studies. *Cancer Lett.* **44**: 7–15

Blume, G., Cevc, G. (1993) Molecular mechanism of lipid vesicle longevity in vivo. *Biochim. Biophys. Acta* **1146**: 157–168

Bonnett, R. (1999) Photodynamic therapy in historical perspective. *Rev. Contemp. Pharmacother.* **10**: 1–17

Brasseur, N., Ouellet, R., La Madeleine, C., van Lier, J. E. (1999) Water-soluble aluminium phthalocyanine-polymer conjugates for PDT: photodynamic activities and pharmacokinetics in tumour bearing mice. *Br. J. Cancer* **80**: 1533–1541

Cammas, S., Suzuki, K., Sone, C., Sakurai, Y., Kataoka, K., Okano, T. (1997) Thermo-responsive polymer nanoparticles with a core-shell micelle structure as site-specific drug carriers. *J. Control. Release* **48**: 157–164

Chan, W.-S., Brasseur, N., La Madeleine, C., Ouellet, R., van Lier, J. E. (1997) Efficacy and mechanism of aluminum phthalocyanine and its sulphonated derivatives mediated photodynamic therapy on murine tumours. *Eur. J. Cancer* **33**: 1855–1859

Chung, J. E., Yokoyama, M., Aoyagi, T., Sakurai, Y., Okano, T. (1998) Effect of molecular architecture of hydrophobically modified poly(N-isopropylacrylamide) on the

formation of thermoresponsive core-shell micellar drug carriers. *J. Control. Release* **53**: 119–130

Chung, J. E., Yokoyama, M., Yamato, M., Aoyagi, T., Sakurai, Y., Okano, T. (1999) Thermo-responsive drug delivery from polymeric micelles constructed using block copolymers of poly(N-isopropylacrylamide) and poly-(butylmethacrylate). *J. Control. Release* **62**: 115–127

Collins, D., Maxfield, F., Huang, L. (1989) Immunoliposomes with different acid sensitivities as probes for the cellular endocytic pathway. *Biochim. Biophys. Acta* **987**: 47–55

Davis, S. S., Illum, L., Moghimi, S. M., Davies, M. C., Porter, C. J. H., Muir, I. S., Brindley, A., Christy, N. M., Norman, M. E., Williams, P., Dunn, S. E. (1993) Microspheres for targeting drugs to specific body sites. *J. Control. Release* **24**: 157–163

de Duve, C., Barsy, T., Poole, B., Trouet, A., Tulkens, P., Van Hoof, F. (1974) Lysosomotropic agents. *Biochem. Pharmacol.* **23**: 2495–2531

Dougherty, T. J., Gomer, C. J., Henderson, B. W., Jori, G., Kessel, D., Korbelik, M., Moan, J., Peng, Q. (1998) Photodynamic therapy. *J. Natl Cancer Inst.* **90**: 889–905

Dye, D., Watkins, J. (1980) Suspected anaphylactic reaction to Cremophor EL. *Br. Med. J.* **280**: 1353

Fisher, A. M. R., Murphree, A. L., Gomer, C. J. (1995) Clinical and preclinical photodynamic therapy. *Lasers Surg. Med.* **17**: 2–31

Han, C. K., Bae, Y. H. (1998) Inverse thermally-reversible gelation of aqueous N-isopropylacrylamide copolymer solutions. *Polymer* **39**: 2809–2814

Isele, U., Schieweck, K., Ressler, R., van Hoogevest, P., Capraro, H.-G. (1995) Pharmacokinetics and body distribution of liposomal zinc phthalocyanine in tumor-bearing mice: influence of aggregation state, particle size, and composition. *J. Pharm. Sci.* **84**: 166–173

Jones, M.-C., Leroux, J.-C. (1999) Polymeric micelles – a new generation of colloidal drug carriers. *Eur. J. Pharm. Biopharm.* **48**: 101–111

Kessel, D. (1996) Photodynamic therapy of neoplastic disease. *Drugs Today* **32**: 385–396

Kitano, H., Akatsuka, Y., Ise, N. (1991) pH-responsive liposomes which contain amphiphiles prepared by using lipophilic radical initiator. *Macromolecules* **24**: 42–46

Kwon, G. S., Okano, T. (1996) Polymeric micelles as new drug carriers. *Adv. Drug Del. Rev.* **21**: 107–116

Kwon, G., Suwa, S., Yokoyama, M., Okano, T., Sakurai, Y., Kataoka, K. (1994) Enhanced tumor accumulation and prolonged circulation times of micelle-forming poly(ethylene oxide-aspartate) block copolymer-adriamycin conjugates. *J. Control. Release* **29**: 17–23

Meyer, O., Papahadjopoulos, D., Leroux, J.-C. (1998) Copolymers of N-isopropylacrylamide can trigger pH sensitivity to stable liposomes. *FEBS Lett.* **42**: 61–64

Moan, J., Christensen, T., Jacobsen, P. B. (1984) Photodynamic effects on cells in vitro labelled with hematoporphyrin derivative. *Photobiochem. Photobiophys.* **7**: 349–358

Moan, J., Berg, K., Steen, H. B., Warloe, T., Madslien, K. (1992) Fluorescence and photodynamic effects of phthalocyanines and porphyrins in cells. In: Henderdon, B. W., Dougherty, T. J. (eds) *Photodynamic Therapy: Basic Principles and Clinical Applications*. Marcel Dekker, New York, pp 19–36

Ochsner, M. (1997) Photophysical and photobiological processes in the photodynamic therapy of tumours. *J. Photochem. Photobiol. B: Biology* **39**: 1–18

Poole, B., Ohkuma, S. (1981) Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages. *J. Cell Biol.* **90**: 665–669

Reijngoud, D.-J., Tager, J. M. (1976) Chloroquine accumulation in isolated rat liver lysosomes. *FEBS Lett.* **64**: 231–235

Roberts, W. G., Smith, K. M., McCullough, J. L., Berns, M. W. (1989) Skin photosensitivity and photodestruction of several potential photodynamic sensitizers. *Photochem. Photobiol.* **49**: 431–438

Schild, H. G., Tirrell, D. A. (1990) Microcalorimetric detection of lower critical solution temperatures in aqueous polymer solutions. *J. Phys. Chem.* **94**: 4352–4356

Senior, J., Crawley, J. C. W., Gregoriadis, G. (1985) Tissue distribution of liposomes exhibiting long half-lives in the circulation after intravenous injection. *Biochim. Biophys. Acta* **839**: 1–8

Svaasand, O. (1984) Optical dosimetry for direct and interstitial radiation therapy of malignant tumors. In: Doiron, D. R., Gomer, C. J. (eds) *Porphyrin Localisation and Treatment of Tumours*. Alan R. Liss Inc., New York, pp 91–114

Tada, H. R., Shibo, O., Kuroshima, K., Koyama, M., Tsukamoto, K. (1986) An improved colorimetric assay for interleukin 2. *J. Immunol. Methods* **93**: 157–165

Taillefer, J., Jones, M.-C., Brasseur, N., van Lier, J. E., Leroux, J.-C. (2000) Preparation and characterization of pH-responsive polymeric micelles for the delivery of photosensitizing anticancer drugs. *J. Pharm. Sci.* **89**: 52–62

Tannock, I. F., Rotin, D. (1989) Acid pH in tumors and its potential for therapeutic exploitation. *Cancer Res.* **49**: 4373–4384

Torchilin, V. P., Omelyanenko, V. G., Papisov, M. I., Bogdanov, A. A., Trubetskoy, V. S., Herron, J. N., Gentry, C. A. (1994) Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. *Biochim. Biophys. Acta* **1195**: 11–20

Tralau, C. J., Young, A. R., Walker, N. P., Vernon, D. I., Macrobert, A. J., Brown, S. B., Brown, S. G. (1989) Mouse skin photosensitivity with dihaematoporphyrin ether (DHE) and aluminium sulphonated phthalocyanine (AlSPc): a comparative study. *Photochem. Photobiol.* **49**: 305–312

Woodburn, K. W., Vardaxis, N. J., Hill, J. S., Kaye, A. H., Reiss, J. A., Phillips, D. R. (1992) Evaluation of porphyrin characteristics required for photodynamic therapy. *Photochem. Photobiol.* **55**: 697–704

Woodle, M. C., Matthay, K. K., Newman, M. S., Hidayat,

J. E., Collins, L. R., Redemann, C., Martin, F. J., Papahadjopoulos, D. (1992) Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes. *Biochim. Biophys. Acta* **1105**: 193–200

Yamazaki, A., Song, J. M., Winnik, F. M., Brash, J. L. (1998) Synthesis and solution properties of fluorescently labeled amphiphilic (N-alkylacrylamide) oligomers. *Macromolecules* **31**: 109–115

Yamazaki, A., Winnik, F. M., Cornelius, R. M., Brash, J. L. (1999) Modification of liposomes with N-substituted polyacrylamides: identification of proteins adsorbed from plasma. *Biochim. Biophys. Acta* **1421**: 103–115

Zhang, X., Burt, H. M., Mangold, G., Dexter, D., Von Hoff, D., Mayer, L., Hunter, W. L. (1997) Anti-tumor efficacy and biodistribution of intravenous polymeric micellar paclitaxel. *Anti-Cancer Drugs* **8**: 696–701