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Research Paper

Polymeric Micelles Based on Amphiphilic Scorpion-like Macromolecules: Novel Carriers for Water-Insoluble Drugs

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Purpose. The objective was to evaluate amphiphilic scorpion-like macromolecules (AScMs) as drug carriers for hydrophobic drugs.
Methods. Indomethacin (IMC) was incorporated into two AScM micelles ($M_{12}P_5$ and $M_{12}P_2$) by the O/W emulsion technique. The influences of IMC:polymer feed ratio and molecular weight of the hydrophilic block of AScMs on the micelle size, IMC entrapment efficiency and release behavior were investigated. Furthermore, cytotoxicity of the AScMs was evaluated with human umbilical vein endothelial cells (HUVEC).
Results. The maximal IMC entrapment efficiency in $M_{12}P_5$ and $M_{12}P_2$ micelles (72.3 and 20.2%, respectively) was obtained at ratios of 0.1 to 1 for indomethacin:polymer. The sizes of IMC-loaded $M_{12}P_5$ and $M_{12}P_2$ polymeric micelles were <20 nm with a narrow size distribution. *In vitro* release studies revealed that IMC released from $M_{12}P_5$ and $M_{12}P_2$ polymeric micelles showed sustained release behavior during the 24 h of experiment. Additionally, $M_{12}P_5$ and $M_{12}P_2$ polymeric micelles did not induce remarkable cytotoxicity against HUVEC cells at concentrations up to 1 and 0.5 mM, respectively.
Conclusion. The amphiphilic scorpion-like macromolecules may be useful as novel drug carriers because of their small size, ability to encapsulate hydrophobic drugs and release them in a sustained manner as well as low cytotoxicity.

KEY WORDS: amphiphilic block copolymers; polymeric micelles; drug carriers; drug delivery system; poly(ethylene glycol); water-insoluble drugs; AScMs.

INTRODUCTION

Polymeric micelles have attracted much attention in drug delivery partly because of their ability to solubilize hydrophobic molecules, small particle size, good thermodynamic solution stability, extended release of various drugs, and prevention of rapid clearance by the reticuloendothelial system (RES) (1–5). Generally, the amphiphilic core/shell structure of polymeric micelles is formed from block copolymers, which are hydrophobic polymer chains linked to hydrophilic polymer chains (6). The inner micelle core is created by association of the hydrophobic portions of the block copolymers due to their cohesive interactions with each other in aqueous media (i.e., hydrophobic interactions), while the outer hydrophilic portions surround the inner hydrophobic core as a hydrated shell (7,8). Various types of drug can be loaded into the hydrophobic core of polymeric micelles (e.g., hydrophobic low molecular weight drugs, cisplatin and DNA) by chemical conjugation or physical entrapment utilizing various interactions such as hydrophobic interactions, ionic interactions and hydrogen bonding (8–10). Furthermore, the hydrophobic core serves as a reservoir from which drug is released slowly over extended periods of time (5,10,11). The hydrophobic inner core is solubilized by the hydrophilic shell, which prevents the inactivation of core-encapsulated drug molecules by decreasing the contact with the inactivating species in the aqueous (blood) phase. Because the outer hydrophilic shell of the polymeric micelles interacts with biocomponents such as cells and proteins, it affects their pharmacokinetics, disposition as well as their surface properties (12–15).

Similar to low-molecular-weight surfactants, the critical micelle concentration (CMC) is a key characterization parameter; CMC is the concentration at which amphiphilic polymers in aqueous solution begin to form micelles (i.e., self-aggregate) while co-existing in the equilibrium with individual polymer chains, or unimers. At CMC and slightly above it, the micelles form loose aggregates and contain some water in the

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ABBREVIATIONS: AScMs, amphiphilic scorpion-like macromolecules; DLS, dynamic light scattering; DMF, *N,N*-dimethylformamide; ECGS, endothelial cell growth supplement; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells; IMC, indomethacin; IMC-$M_{12}P_5$, indomethacin-loaded $M_{12}P_5$ micelles; IMC-$M_{12}P_2$, indomethacin-loaded $M_{12}P_2$ micelles; $M_{12}P_5$, poly(ethylene glycol-5000)–mucic acid based amphiphilic scorpion-like macromolecules; $M_{12}P_2$, poly(ethylene glycol-2000)–mucic acid based amphiphilic scorpion-like macromolecules; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PEG, poly(ethylene glycol); PTFE, polytetrafluoroethylene; TEM, transmission electron microscopy.

core (9). With further increases in amphiphilic polymer concentration, the unimer-to-micelle equilibrium shifts toward micelle formation; the micellar structure becomes more compressed and stable, residual solvent is excluded from the core, and the micelle sized decreases. In summary, lower CMC values correlate to more stable micelles. This concept is especially important from the pharmacological point of view; upon dilution with a large volume of the blood, micelles with high CMC values may dissociate into unimers and their content may precipitate (4,9), whereas micelles with low CMC value are more likely to remain. Thus, to develop improved drug delivery systems, amphiphilic molecules that are able to form more stable micelles with lower CMC values are appropriate candidates (9) that include amphiphilic scorpion-like macromolecules (AScMs).

The synthesis of amphiphilic scorpion-like macromolecules (AScMs) as potential drug carriers was recently described. (16) The design rationale of AScMs was to develop nontoxic, nonimmunogenic, biodegradable block copolymers with a tunable hydrophilic-lipophilic balance (HLB) that were more thermodynamically stable than other block copolymer micelles (16). The individual chains of AScMs are referred to as MxPy, in which M denotes mucic acid; x denotes the total carbon number of each acyl chain; P denotes poly(ethylene glycol) (PEG); and y refers to molecular weight of the PEG in thousands (Fig. 1a). Choice of PEG as a micellar outer shell was based on its hydrophilicity and biocompatibility (12,16,17). In addition, the hydrophilic PEG on the surface of AScMs was expected to sterically stabilize the polymeric micelles against opsonization and phagocytosis, as documented with other PEG-based block copolymers (18,19). As previously determined (16), AScMs have critical micellar concentrations (CMC) in the range of 10^{-5} to 10^{-7} M; in aqueous media, they self-associate to form micelles in which hydrophilic PEG forms the outer shell whereas acylated mucic acid portion forms the hydrophobic inner core (Fig. 1b) (16). Typically, block copolymers evaluated as drug delivery systems display CMC values ranging from 1×10^{-3} M to 1×10^{-6} M (e.g., commercially available Pluronic block copolymers) (4–6). Compared to other block copolymers, AScMs have lower CMC values. Because of the slower rate of micellar dissociation, loaded drugs should be retained for longer periods of time, and eventually, achieve higher accumulation of a drug at the target site (6). From the eight amphiphilic scorpion-like macromolecules (AScMs) synthesized (16), $M_{12}P_5$ and $M_{12}P_2$ were shown to be good candidates for drug delivery due to their low CMC (1.25×10^{-7} M and 1.27×10^{-6} M, respectively) and satisfactory aqueous solubility (higher than 1 mg/ml) (Table I).

The aim of this work was to evaluate the AScMs ($M_{12}P_5$ and $M_{12}P_2$) as potential drug carriers using indomethacin as an example. Indomethacin (IMC) is an acidic nonsteroidal anti-inflammatory drug belonging to the class of acetic acid and is used to reduce pain, fever and inflammation. The anti-inflammatory activity of IMC is due to inhibition of cyclooxyganase-2 (COX-2), an enzyme crucial to the production of inflammatory mediators (20). Possible side effects of IMC are renal dysfunction and gastrointestinal alteration of hemorrhage (20,21). Thus, to overcome the side effects of IMC, several experimental approaches such as encapsulation, film coating and matrix forms have been studied (20). In this paper, IMC-loaded amphiphilic scorpion-like macromolecule (AScM) micelles were prepared as an alternative approach to decrease the side effects of IMC. More specifically, incorporation of the IMC into the micelles by organic solvent/water emulsion technique (o/w) was analyzed as well as the influ-

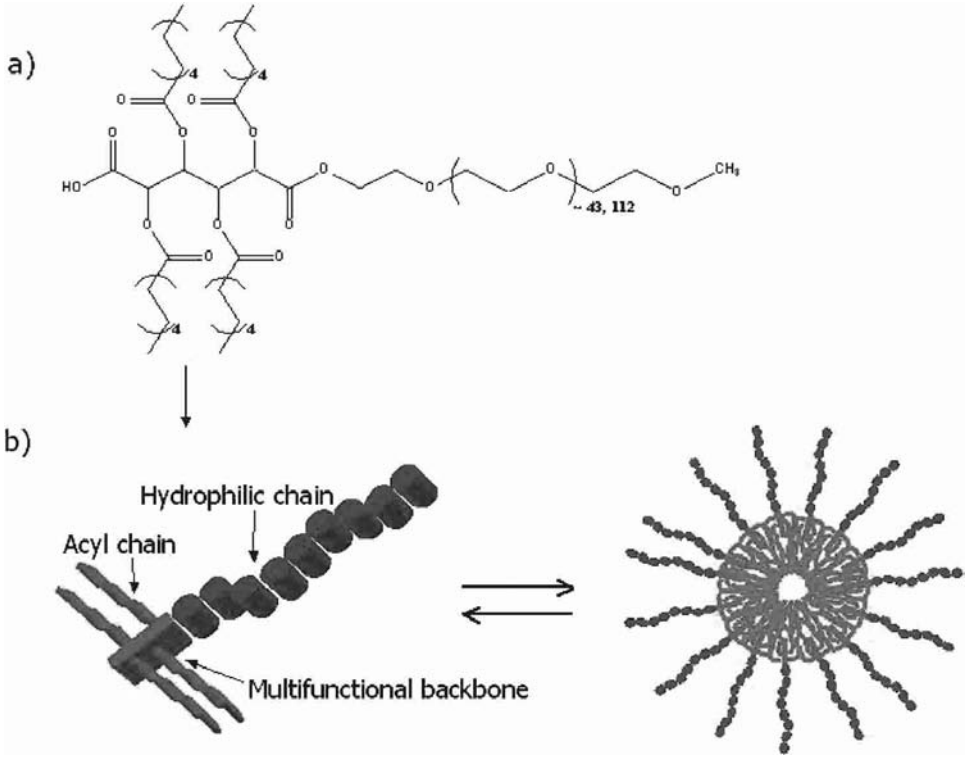

Fig. 1. (a) Chemical structure of AScMs and (b) self-assembly of AScMs in aqueous media.

Table I. Characteristics of the Investigated AScMs

Amphiphilic scorpion-like macromolecules (AScMs)	Polymer molecular weight (Da)	Molecular weight of PEG (Da)	CMC (M)
$M_{12}P_5$	5900	5000	1.25×10^{-7}
$M_{12}P_2$	2800	2000	1.27×10^{-6}

Modified from Tian *et al.,* (16).

ences of drug:polymer feed ratio and molecular weight of hydrophilic block of AScMs (PEG 2000 and PEG 5000) on the micelle size, surface morphology, and drug loading. Furthermore, drug release profiles and the cytotoxicity toward HUVEC cells are also discussed.

MATERIALS AND METHODS

Materials

Indomethacin (IMC), phosphate buffer tablets, heparin sodium salt (grade I-A from porcine intestinal mucosa), triton-X 100, endothelial cell growth supplement (ECGS), cellulose acetate membranes (Spectra/Por MWCO 3500), and *in vitro* toxicology assay kit - MTT based, were purchased from Sigma-Aldrich (St. Louis, MO, USA). The AScMs polymers, $M_{12}P_5$ and $M_{12}P_2$, were synthesized according to previously described methods (16). Pluronic P-85 was a gift from BASF Corporation (Mount Olive, NJ, USA). Fetal bovine serum (FBS, non heat-inactivated), Ham's F-12 media, penicillin-streptomycin 100× solution, and human umbilical vein endothelial (HUVEC) cells were obtained from American Tissue Culture Collection (ATCC, Manassa, VA, USA). Tissue culture plates, flasks, and all solvents (HPLC grade) were purchased from Fisher Scientific (Atlanta, GA, USA).

Preparation of IMC-Loaded Micelles

AScM polymeric micelles containing IMC were prepared according to organic solvent/water (o/w) emulsion technique (5,20,21). In the o/w emulsion technique, hydrophobic drugs are dissolved into a volatile, water-immiscible solvent (e.g., chloroform or methylene chloride) and the resulting solution is added dropwise into water under vigorous stirring in an open air system to evaporate the organic solvent; the micellar system is formed as the solvent evaporates (5,9). IMC-loaded $M_{12}P_5$ and $M_{12}P_2$ micelles were prepared in the following way. Briefly, $M_{12}P_5$ or $M_{12}P_2$ block copolymer (50 mg) was dissolved in 100 ml of water and the IMC was dissolved in 3 ml of methylene chloride (Table II). The IMC:polymer feed ratio by weight was varied from 0.1:1 to 4:1 (Table II). The IMC solution was added drop-wise to the aqueous polymer solution, and the resulting mixture stirred overnight to allow evaporation of methylene chloride. To remove free IMC, the solution was purified by filtration using an Amicon YM-30 membrane (Millipore Corporation, Bedford, MA, USA) (20,21).

The amount of IMC entrapped, or loaded, into the hydrophobic portion of the polymeric micelles was determined by measuring UV absorbance at 320 nm (DU 520, Beckman, Fullerton, CA) after disruption of the micelles by DMF (10-fold volume) addition. The drug loading content and drug entrapment efficiency of IMC were calculated as follows (22,23) and are displayed in Table II:

$$\text{Drug loading content (wt\%)} = \frac{\text{the total amount of IMC in micelles}}{\text{the amount of polymer added initially}} \times 100 \quad (1)$$

$$\text{Entrapment efficiency (\%)} = \frac{\text{the total amount of IMC in micelles}}{\text{the amount of IMC added initially}} \times 100 \quad (2)$$

Micelle Size and Size Distribution

The average size and the size distribution of the polymeric micelles (i.e., AScMs) were estimated by dynamic light scattering (DLS) using a Nicomp 380 Submicron Particle Sizer (Santa Barbara, CA, USA) equipped with helium-neon laser and a temperature-controlled cell holder (21,24). The intensity of scattered light was detected at room temperature and at 90° to the incident beam. All measurements were made after the supernatant solution was filtered with a PTFE filter (Whatman, USA) having an average pore size of 0.45 μm (25,26). The data were analyzed by volume- and number-weighted Nicomp distribution (26).

In Vitro Indomethacin Release Studies

For *in vitro* release studies, IMC-loaded polymeric micelle solutions (defined as IMC-$M_{12}P_5$ and IMC-$M_{12}P_2$) were prepared according to the procedure described above. As the highest drug loading was achieved with a IMC:polymer ratio of 0.1 to 1, the samples for *in vitro* release studies were prepared using the same ratio.

The *in vitro* IMC release profiles from IMC-$M_{12}P_5$ and IMC-$M_{12}P_2$ micelles were evaluated using modified Franz diffusion cells (PermeGear, Riegelsville, PA, USA) each having a receptor volume of 5.1 ml and diffusion area of 0.64 cm^2 (27,28). Before the experiment, cellulose acetate membranes (MWCO 3500) were soaked in receptor medium for 12 h. The membranes were then dried with two filter papers (Fisher Scientific, Pittsburgh, PA, USA), mounted between donor and receptor compartment of Franz diffusion cells, and equili-

Table II. Effect of IMC:Polymer Ratios on Micelle Size, IMC Loading Content, and Entrapment Efficiency (n = 4)

Polymers	IMC:polymer ratio (by weight)	Mean particle size ± SD (nm)	IMC loading (wt%)	Entrapment efficiency (%)
$M_{12}P_5$	0:1	12.2 ± 1.5	0.0	0.0 ± 0.0
	0.1:1	15.8 ± 2.8	7.1 ± 1.5	72.3 ± 2.5
	0.2:1	14.1 ± 2.1	3.7 ± 0.8	18.4 ± 1.5
	0.6:1	12.3 ± 1.2	1.4 ± 0.6	2.3 ± 0.7
	1:1	12.4 ± 1.2	0.9 ± 0.2	0.9 ± 0.2
	2:1	14.6 ± 1.5	0.8 ± 0.1	0.4 ± 0.1
	4:1	16.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0
$M_{12}P_2$	0:1	8.3 ± 1.2	0.0	0.0 ± 0.0
	0.1:1	12.6 ± 2.7	1.9 ± 0.1	20.2 ± 1.5
	0.2:1	11.2 ± 1.2	1.7 ± 0.1	8.6 ± 1.2
	0.6:1	9.4 ± 1.7	0.5 ± 0.1	0.9 ± 0.2
	1:1	11.3 ± 2.1	0.3 ± 0.1	0.3 ± 0.1
	2:1	8.2 ± 0.1	0.4 ± 0.1	0.2 ± 0.0
	4:1	8.7 ± 1.3	0.5 ± 0.1	0.1 ± 0.0

brated for 1 h. The receptor compartment was filled with phosphate buffer solution (pH 7.4), and continuously stirred with magnetic stirrer at 600 rpm to ensure uniform distribution and maintain sink conditions. The temperature of the entire diffusion cell assembly was maintained at 37 ± 0.5°C, using a recirculating water jacket (21,27).

Sample (IMC-$M_{12}P_5$, IMC-$M_{12}P_2$ and control) was applied on the membrane surface, and the donor side occluded with Parafilm (American National, Menasha, WI, USA). At predetermined time intervals, 300 μl samples were withdrawn from receptor compartments and replenished with the same volume of fresh phosphate buffer after each sampling. Each experiment was repeated four times and receptor samples were frozen at –30°C prior to HPLC analysis (21).

HPLC Analysis

HPLC analysis was performed using a Hewlett-Packard 1100 instrument with an auto- sampler equipped with a quaternary pump and variable-wavelength UV detector. All samples were analyzed using a reverse phase C18 column (Microsorb-MV C18 15 cm, 5 μm). Indomethacin was detected at 320 nm using a mobile phase acetonitrile:water (0.1% TFA) 80:20, and flow rate of 1 ml/min (27,29).

Transmission Electron Microscopy

The morphology of polymeric micelles was observed using a JEM-100 CX II (Jeol Ltd., Tokyo, Japan) at 60 kV. A drop of the sample solution (0.5 mM) was placed onto a mesh copper grid coated with carbon. About 1 min after deposition, the grid was tapped with a filter paper (Fisher Scientific) to remove surface water and negatively stained using a 1% phosphotungstic acid solution (10,30). After 1 min, excess fluid was removed, the surface air-dried for 5 min and the grid loaded in the transmission electron microscope (30).

Cytotoxicity Studies

The cytotoxicity properties of AScMs polymeric micelles were evaluated *in vitro* with human umbilical endothelial cells (HUVECs) and compared with the cytotoxicity of two commercially available polymers, PEG and Pluronic P-85. The choice of this cell line was governed by our interest in endothelium, an important target for drug and gene therapy. The cells were cultured in Ham's F-12K media supplemented with 10% FBS, heparin (100 μg/ml), ECGS (40 μg/ml), and 1% penicillin-streptomycin solution, for a minimum of two passages prior to experimental use (31). They were subsequently harvested by trypsinization, suspended in fresh medium, plated at 5000 cells/well in 96-well plates (Costar, Cambridge, MA, USA) and cultured over 2 days to allow reattachment. The growth media was then replaced with fresh media containing various $M_{12}P_5$ (0.0001 mM to 2 mM), $M_{12}P_2$ (0.0001 mM to 0.5 mM), PEG (0.0001 mM to 2 mM), or Pluronic P-85 (0.0001 mM to 2 mM) polymer concentrations. Following 24 and 48 h incubation at 37°C and 5% CO_2, cell survival was measured using the standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test, in which MTT reagent is converted to a purple formazan product by mitochondrial enzymes in viable cells (25,33). Briefly, MTT was dissolved in phosphate buffer at 5 mg/ml. Ten microliters of the MTT solution was added to the medium for a final concentration of 500 μg/ml, then the cells incubated for 3 h at 37°C. The resulting purple formazan product was dissolved by adding 100 ml/well of solubilization solution containing 10% triton X-100 plus 0.1 N hydrochloric acid in anhydrous isopropanol (33,34). The absorbance of the colored product was measured at 570 nm with a background subtraction at 630 nm in a universal microplate reader (Bio-Tek Instrument, Highland Park, VT, USA). All experiments were performed in triplicate. Values are shown as the percentage of cell viability, and calculated as follows (1,35):

$$\text{Viability}\,(\%) = \frac{Nt}{Nc} \times 100 \tag{3}$$

where *Nt* and *Nc* are the number of surviving cells in the group treated with different concentration of polymers (*Nt*) and in control (no polymer added) group (*Nc*), respectively.

Data and Statistical Analyses

All data are expressed as mean ± SD of at least three measurements. Statistical analysis of all data was performed using one-way analyses of variance (one-way ANOVA), followed by Holm-Sidak test if the ANOVA indicated that a difference existed. A p value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

IMC Loading and Entrapment Efficiency

An important aspect in designing polymeric micelles is controlling the amount of drug incorporated in the core, because this quantity is likely to influence micelle stability (13,14). Therefore, it is imperative to analyze the encapsulation process to obtain stable drug carriers for efficient passive and/or active targeting (15).

The loading and entrapment efficiency of indomethacin (IMC) in $M_{12}P_5$ and $M_{12}P_2$ polymeric micelles was evaluated by varying the IMC:polymer feed ratio by weight; the results are summarized in Table II. As shown in Table II, IMC loading decreases with increasing feed ratio of IMC: polymer for both block copolymers. The maximum IMC loadings and entrapment efficiencies in $M_{12}P_5$ and $M_{12}P_2$ micelles were obtained with IMC:polymer ratios ($p < 0.05$) of 0.1 to 1. These results agree well with other research groups who report that the highest drug loading and entrapment efficiency is obtained when drug:polymer ratio is 0.1 to 1 (8,29,36). However, maximal IMC encapsulation efficiency in $M_{12}P_5$ micelles (72.3%) was much higher then in other PEG-based amphiphilic polymers (e.g., 42.2% in methoxy poly(ethylene glycol)/ε-caprolactone micelles) (29). Thus, compared to the literature values of IMC encapsulation efficiency in PEG-based amphiphilic molecules, $M_{12}P_5$ more efficiently encapsulates hydrophobic molecules.

Our results clearly indicate that the lower IMC:polymer ratios (e.g., 0.1:1 and 0.2:1) lead to a statistically significant increase ($p < 0.05$) in IMC loading as well as entrapment efficiency (Table II) for both polymers. However, a large amount of precipitate was observed with IMC: polymer ratios exceeding 1:1. As reported by Yokoyama *et al.,* hydrophobic interactions among a hydrophobic polymer chain, indomethacin and solvent may be an important key to controlling the

drug incorporation process (8). Our results reveal that the excess IMC precipitated; indomethacin is a hydrophobic drug with aqueous solubility of 10 μg/ml at 25°C (37,38). The incorporation of IMC into micelles competes with IMC precipitation in the aqueous phase as the IMC ratio increased, resulting in lowered drug loading content and loading efficiency (Table II). In summary, IMC molecules interact with each other more strongly than with the hydrophobic polymer chains of the AScMs, thus IMC precipitates rather then being incorporated into polymeric micelles at higher IMC:polymer ratios. Therefore, the IMC:polymer ratio significantly influences IMC loading and loading efficiency, which is closely correlated to the IMC:polymer interactions during the micelle formation and drug incorporation processes.

Higher drug loading and entrapment efficiency was achieved with the $M_{12}P_5$ polymer ($p < 0.05$). As stated in the Introduction, $M_{12}P_5$ contains higher molecular weight PEG (MW 5000), whereas $M_{12}P_2$ contains lower molecular weight PEG (MW 2000) as the hydrophilic segment. Therefore, IMC loading content and entrapment efficiency is highly dependent on the molecular weight of hydrophilic segment of the AScM studied.

Morphology and Size Distribution of AScM Polymeric Micelles

The formation of AScM polymeric micelles is based on the concept of multimolecular micellization of block copolymers in a selective solvent: an amphiphilic block copolymer in a solvent that solubilizes one block as the other block remains insoluble creates a micelle-like structure through the association of the insoluble blocks (39–41). As previously stated, micelle formation of AScMs ($M_{12}P_5$ and $M_{12}P_2$) and indomethacin-loaded AScMs were initially investigated by dynamic light scattering measurements (21). The mean diameters of the polymeric micelles are listed in Table II as a function of IMC:polymer ratio. In general, $M_{12}P_5$ forms micelles larger than the $M_{12}P_2$ micelles ($p < 0.05$), which is explained by the higher molecular weight of $M_{12}P_5$ (42,43). However, both $M_{12}P_5$ and $M_{12}P_2$ micelles have a diameter less than 20 nm and maintain narrow size distributions (data not shown).

Generally, the size of AScMs (~20 nm) was much smaller compared to amphiphilic block copolymeric micelles (~100 nm); for example, methoxy poly(ethylene glycol)/ε-caprolactone forms micelles with sizes in the range 114–160 nm (29,38). Similarly, poly(ethylene glycol)/polylactone forms micelles with sizes of approximately 100 nm (15). To achieve long blood circulation half-lives, particles should be small enough to avoid mechanical clearance by filtration or in the spleen (29), as the reticuloendothelial system (RES) uptake generally increases with increasing a particle size. Thus, a drug delivery systems with sizes smaller than 200 nm is desired for a long-circulating drug carrier (4,29). In addition, Otsuka *et al.* suggested that polymeric micelles with a size of 30–50 nm in diameter are favorable for extravasation to achieve the enhanced permeation and retention effect (EPR effect) (13).

The micellization behavior of IMC-loaded micelles in aqueous media was measured by DLS (8,44). After incorporation of IMC into $M_{12}P_5$ and $M_{12}P_2$ micelles, the average number-weighted diameter slightly increased compared to unloaded micelles (Table II) but the size distribution remained identical to unloaded micelles. Even though secondary aggregation (~90 nm) was minimal (less than 0.1%), a bimodal size distribution in all IMC-loaded micelles was observed. The smaller particles (~15 nm) were attributed to micelles, whereas the larger particles (~90 nm) are likely micelles that further associated (20,45). As suggested by Kataoka *et al.,* intermicellar association might occur by intermicellar hydrophobic-hydrophobic interactions or van der Waals interactions between cores due to insufficient isolation of the hydrophobic inner core from an outer aqueous environment by the hydrated shell (8,24). Notably, larger diameters of secondary aggregates were observed for higher IMC:polymer ratios, implying more intense precipitation or association of polymeric micelles (8).

TEM micrographs revealed that the unloaded AScM polymeric micelles (Fig. 2) as well IMC-loaded AScM polymeric micelles (data not shown) have a regular spherical shape with nano-size range, thus confirming results from DLS measurements.

The influence of indomethacin loading content on the micelle sizes of $M_{12}P_5$ and $M_{12}P_2$ was also investigated by DLS and TEM. As shown in Table II, the size range of indomethacin-loaded $M_{12}P_5$ and $M_{12}P_2$ micelles was 12.3–16.2 and 8.2–12.6 nm, respectively, indicating that IMC-loading did not significantly affect the micelle size ($p > 0.05$).

In summary, DLS and TEM measurements revealed that unloaded and IMC-loaded $M_{12}P_5$ and $M_{12}P_2$ micelles have sizes of <20 nm with a narrow distribution. The smaller size may have several advantages. First, the narrow size distribution is similar to that of viruses and lipoproteins and may be a critical factor in determining their *in vivo* distribution, based on the enhanced permeation and retention effect (EPR effect) (6,9). Second, $M_{12}P_5$ and $M_{12}P_2$ may be accommodated within endocytosis vesicles and thus enter into target cells via endocytosis because the micelles are smaller than 100 nm (5,9). Third, AScMs polymeric micelles may be sterilized simply by filtration to remove larger particles while the AScMs pass through the filter, obviating the need for aseptic processing. Last, pharmaceutical applications that require faster drainage from subcutaneous injection site may benefit from particles of this size range because of limited concern for polymeric micelles causing capillary embolisms (5,9).

In Vitro Release of IMC

Drug release from polymeric micelles is a rather complicated process and can be affected by many factors, including polymer degradation, molecular weight, crystallinity, glass transition temperature, binding affinity between the polymer and the drug (23). According to Hu *et al.,* the way in which drug is distributed in the polymer, also affects the release characteristics (23). Generally, micelle-incorporated drugs are slowly released from an intact micelles (5,6,9). However, drug molecules located within or adjacent to the corona can be quickly released, and thus be responsible for the "fast release" component of the net release curve (9). The phase state of the drug can also be important for its association with a micelle; if the drug is not dissolved in the core, but exists as a separate phase inside the core, this property can hinder drug release from the micelle (5,9,23).

Figure 3 shows *in vitro* cumulative release profiles of

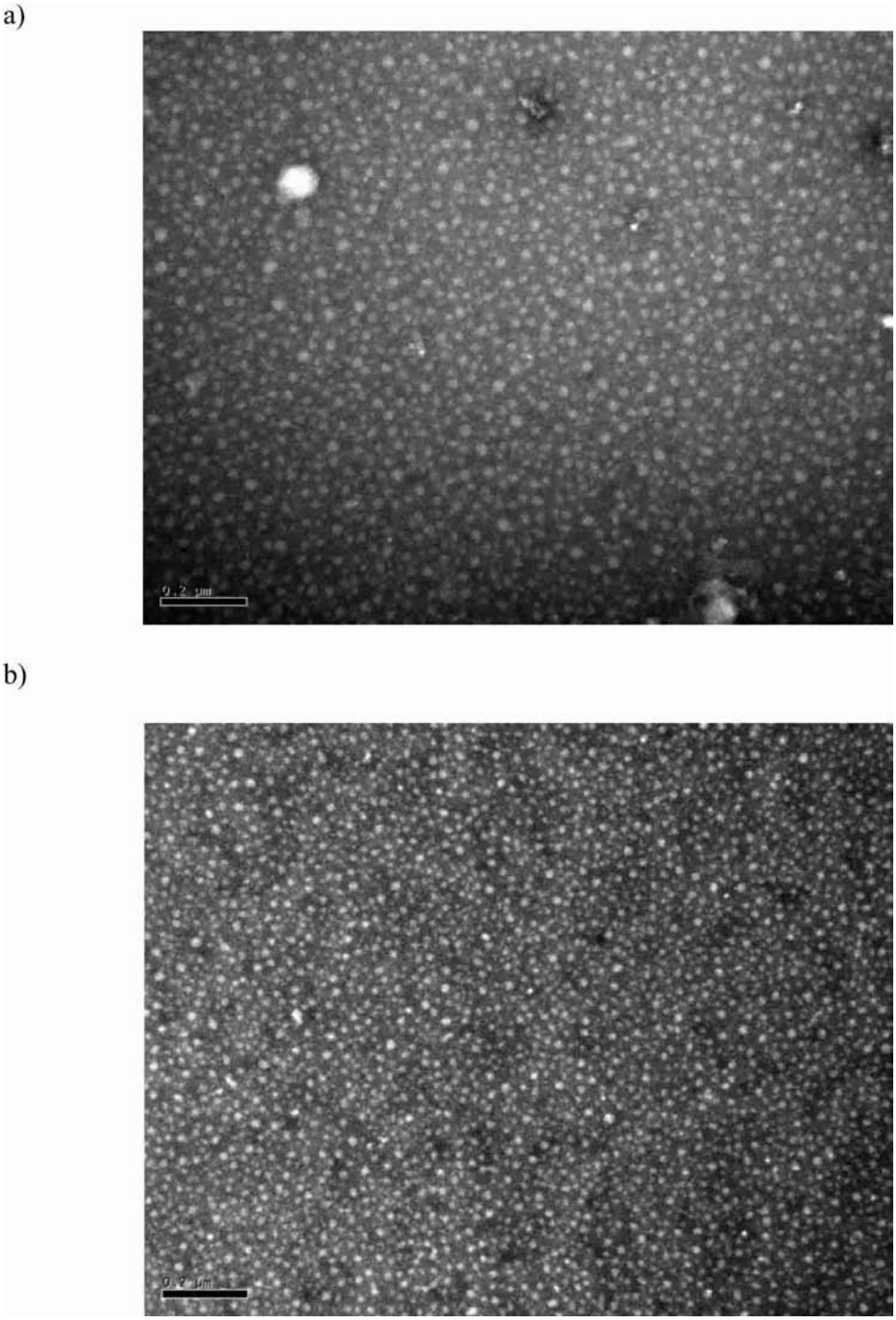

Fig. 2. TEM micrographs of polymeric micelles of (a) $M_{12}P_5$ (200 μm) and (b) $M_{12}P_2$ (100 μM).

indomethacin from $M_{12}P_5$ and $M_{12}P_2$ micelles prepared by using the 0.1:1 drug:polymer ratio. As shown in Fig. 3, IMC release from $M_{12}P_5$ and $M_{12}P_2$ micelles was slow and showed sustained release characteristics over 24 h ($p < 0.05$) relative to the control (free indomethacin). Notably, IMC release from $M_{12}P_5$ micelles (Fig. 3) was slower then from $M_{12}P_2$ micelles ($p < 0.05$). The observed effect can be explained by the higher IMC loading in $M_{12}P_5$ micelles (5.3 wt%) relative to IMC loading in $M_{12}P_2$ micelles (2.8 wt%) (46–48). The higher IMC concentration within the polymeric micelles should enhance interactions between the hydrophobic IMC and the hydrophobic blocks of AScMs, which overall decreases IMC release rates (1,47).

Drug-loading studies revealed that similar to other PEG-based amphiphilic block copolymers, indomethacin was slowly released (<50%) from AScMs during the 24 h experiment (15,29). Thus, slow release of hydrophobic drugs from AScM-based polymeric micelles could allow accumulation of polymeric micelles at targeted sites with minimal drug loss and localized drug release (5).

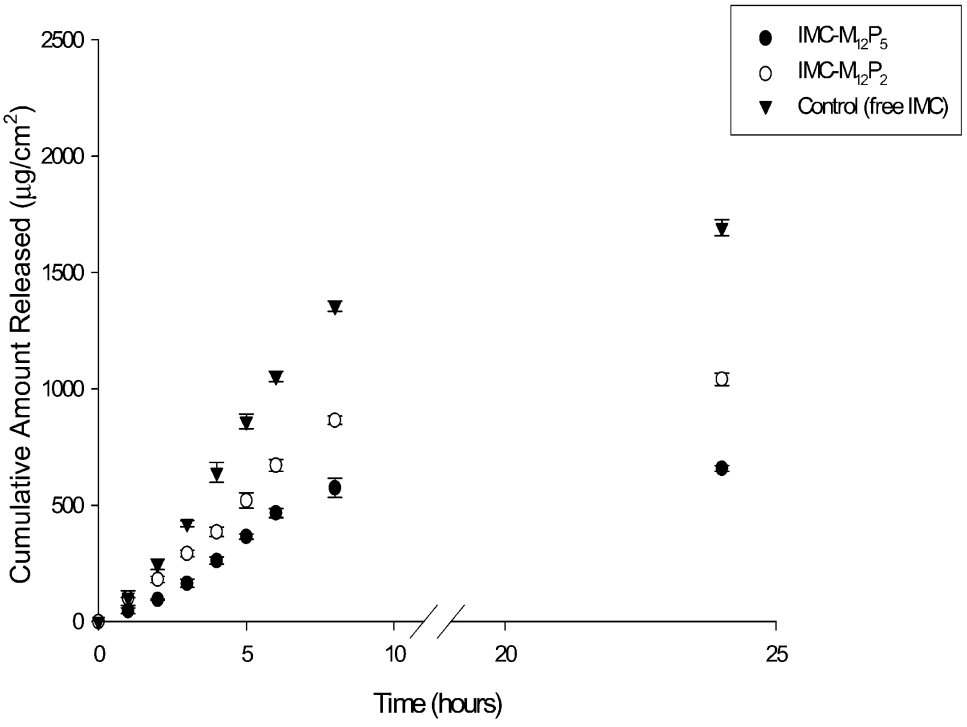

Fig. 3. Cumulative release profiles of indomethacin (IMC) from polymeric micelles of $M_{12}P_5$ and $M_{12}P_2$ as well as for IMC suspension, as control (n = 4).

In Vitro Cytotoxicity Test

For polymeric carriers and especially colloidal carriers for parenteral administration, the host response is typically negative against the carrier itself and to the drug/carrier conjugate (1). Thus, another critical criteria that must be satisfied for novel drug carriers is biocompatibility (1,5). As described in the "Materials and Methods" section, the biocompatibility of $M_{12}P_5$ and $M_{12}P_2$ polymeric micelles was evaluated *in vitro* with HUVECs and compared with two commercially available polymers, PEG and Pluronic P-85 (25,33). PEG was used as a control polymer because of its well-known ability to impart cellular stealth properties to surfaces and it is nontoxic (1). Pluronic P-85 block copolymer was chosen as an alternate polymer control because it self-assembles in aqueous solutions (CMC of 6.7×10^{-7} M), is widely used as structural component in micellar drug formulations such as water-in-oil, oil-in-water, and water-in-oil-in-water emulsions, and is a recognized pharmaceutical excipient listed in the U.S. and British pharmacopoeias (4,9),

In the cytotoxicity testing, the concentration of $M_{12}P_5$, PEG, and Pluronic P-85 polymer was varied from 0.0001 to 2 mM, whereas the concentration of $M_{12}P_2$ polymer was varied from 0.0001 to 0.5 mM due to solubility limitations. Figure 4 shows cell viability upon exposure to different polymer concentrations for 48 h. Cell viability was expressed relative to the control (no polymer added), which was normalized to 100% (33,48). The cytotoxicity studies demonstrated that cell viability decreased in proportion to $M_{12}P_5$, $M_{12}P_2$, PEG and Pluronic P-85 polymer concentration. More specifically, the cytotoxicity of $M_{12}P_5$ and PEG was negligible until 2 mM; cell survival fell to 60 and 64% at 48 h of incubation, respectively ($p < 0.05$). However, $M_{12}P_2$ and Pluronic P-85 concentrations of 0.5 mM caused significant cell death; cell survival fell to 34% and 48% at 48 h of incubation, respectively ($p < 0.05$). Overall, cytotoxicity testing revealed that toxicity of $M_{12}P_5$ is similar to the toxicity of PEG. In comparison, $M_{12}P_2$ and Pluronic P-85 exhibit higher cytotoxicity than $M_{12}P_5$ and PEG at the identical concentration (e.g., 0.5 mM). As the level of toxicity for $M_{12}P_5$ was similar to the well established biocompatible PEG and lower than the widely used Pluronic P-85 block copolymer, $M_{12}P_5$ may be considered biocompatible. As suggested by Otsuka *et al.,* PEG chains attached to a surface of micelles exhibit rapid chain motion an aqueous medium and have a large excluded volume (13). Moreover, the steric repulsion resulting from a loss of conformational entropy of the bound PEG chains upon the approach of foreign substance and low interfacial free energy of PEG in water may contribute to the extraordinary physiologic properties of carrier covered with PEG (e.g., $M_{12}P_5$) (13).

CONCLUSIONS

Indomethacin-loaded $M_{12}P_5$ and $M_{12}P_2$ polymeric micelles were successfully prepared by the organic solvent/water (o/w) emulsion technique. For incorporation of IMC into AScM polymeric micelles, the drug:polymer ratio and PEG molecular weight of the amphiphilic scorpion-like macromolecules (AScMs) significantly influenced indomethacin loading and entrapment efficiency. For both AScMs polymers studied ($M_{12}P_5$ and $M_{12}P_2$), the highest drug loadings and entrapment efficiencies were achieved with a 0.1:1 drug:polymer ratio. Maximal IMC encapsulation efficiency in $M_{12}P_5$ micelles (72.3%) was much higher than in other PEG-based amphiphilic polymers (e.g., 42.2% in methoxy poly(ethylene glycol)/ε-caprolactone micelles). Dynamic light scattering and TEM experiments revealed that the size of indomethacin-loaded $M_{12}P_5$ and $M_{12}P_2$ micelles did not significantly change rela-

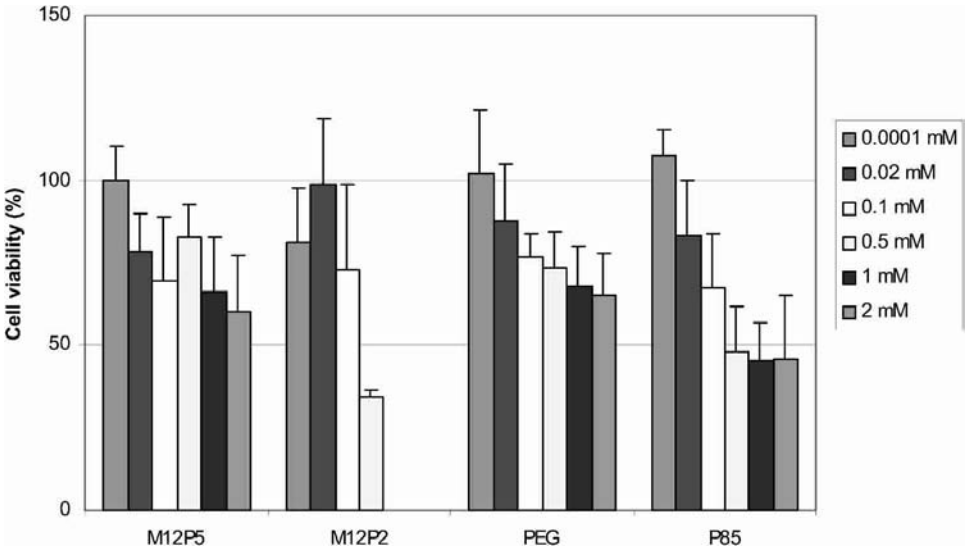

Fig. 4. Viability of HUVEC cells after 48 h exposure with varying concentrations of $M_{12}P_5$, $M_{12}P_2$, PEG (control) and Pluronic P-85 (control) polymers, respectively, (n = 6).

tive to unloaded micelles ($p > 0.05$). Polymeric micelles, with and without IMC, have diameters below 20 nm and therefore expected to show higher vascular permeability by diffusion mechanisms. Furthermore, the size range of AScM polymeric micelles is appropriate to evade renal excretion and nonspecific capture by reticuloendothelial systems. *In vitro* release studies showed that IMC release from $M_{12}P_5$ and $M_{12}P_2$ micelles was sustained during 24 h of the experiment ($p < 0.05$), compared to IMC alone. *In vitro* MTT-based cytotoxicity measurements revealed that $M_{12}P_5$ and $M_{12}P_2$ polymeric micelles did not induce remarkable cytotoxicity against HUVEC cells ($p < 0.05$) up to concentrations of 1 and 0.5 mM, respectively. Furthermore, the toxicity levels of $M_{12}P_5$ was similar to PEG and lower than Pluronic P-85 block copolymer, thus $M_{12}P_5$ is considered biocompatible. Overall, these results highlight the potential of these amphiphilic scorpion-like macromolecules as drug carriers for hydrophobic drugs. However, $M_{12}P_5$ polymeric micelles have better potential for sustained release of hydrophobic drugs (e.g., indomethacin), thus providing a convenient method of drug delivery while minimizing drug toxicity and maximizing drug effectiveness.

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