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Research Paper

Local Delivery of Indomethacin to Arthritis-Bearing Rats through Polymeric Micelles Based on Amphiphilic Polyphosphazenes

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Purpose. Preparation, *in vitro* and *in vivo* evaluation of indomethacin-loaded polymeric micelles based on amphiphilic polyphosphazene.

Methods. Amphiphilic polyphosphazenes (PNIPAAm/EAB-PPPs) with poly (*N*-isopropylacrylamide) (PNIPAAm) and ethyl 4-aminobenzoate (EAB) as side groups were synthesized through thermal ring-opening polymerization and subsequent substitution reactions. Indomethacin (IND) loaded polymeric micelles based on PNIPAAm/EAB-PPPs were prepared by dialysis procedure. *In vitro* IND release kinetics was investigated in 0.1 M PBS (pH 7.4), while *in vivo* pharmacokinetics was performed in Sprague–Dawley rats. *In vivo* pharmacodynamic study was carried out based on two animal models, i.e. carrageenan-induced acute paw edema and complete Freund's adjuvant (CFA) induced ankle arthritis model.

Results. Drug loading capacity of micelles based on this type of amphiphilic copolymers was mainly determined by copolymer composition and the chemical structure of drug. In addition to the compatibility between drug and micellar core, hydrogen bonding interaction between drug and hydrophilic corona may significantly influence drug loading as well. *In vitro* drug release in PBS suggested that there was no significant difference in release rate between micelles based on copolymers with various EAB content. Compared with the rats administered with free IND aqueous solution, IND concentration in rats' plasma showed a prolonged maintenance in experimental group treated with IND-loaded polymeric micelles. *In vivo* pharmacodynamic study indicated that sustained therapeutic efficacy could be achieved through topical injection of the aqueous solution of IND-loaded micelles. Local delivery of IND can avoid the severe gastrointestinal stimulation, which was frequently associated with oral administration as evidenced by ulceration evaluation.

Conclusions. The promising results of current preliminary study suggest that this type of amphiphilic copolymers could be used as injectable drug carriers for hydrophobic drugs.

KEY WORDS: amphiphilic copolymers; arthritis; indomethacin; local drug delivery; polymeric micelles.

INTRODUCTION

Arthritis is a widespread, chronic inflammatory and potentially disabling disease. Rheumatoid arthritis (RA) and osteoarthritis are the two common forms of arthritis. The impact of arthritis on pain, disability, and quality of life results in a considerable burden to the individual, health services, and society. Among these two common forms, RA is a leading cause of disability and primarily characterized by symmetric, erosive synovitis and sometimes multisystem

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ABBREVIATIONS: AET·HCl, 2-aminoethanethiol hydrochloride; AUC, area under the concentration–time curve; AIBN, 2, 2′-azobisisobutyronitrile; AUMC, area under the first moment of the plasma concentration–time curve; CFA, complete Freund's adjuvant; CMC, critical micelle concentration; DMS, dexamethasone; DMAc, dimethylacetamide; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulphoxide; DSC, differential scanning calorimeter; EAB, ethyl 4-aminobenzoate; GPC, gel permeation chromatography; HPLC, high-performance liquid chromatography; IBU, ibuprofen; IND, indomethacin; KET, ketoprofen; LCST, lower critical solution temperature; MRT, mean residence time; MPG, medroxyprogesterone acetate; NAP, naproxen; PBS, phosphate buffered solution; PNIPAAm, poly (*N*-isopropylacrylamide); PNIPAAm/EAB-PPP, amphiphilic polyphosphazene with PNIPAAm and EAB as side groups; PNS, predinisone acetate; RA, rheumatoid arthritis; SD, standard deviation; THF, tetrahydrofuran.

involvement and often results in pain, stiffness, and swelling of joints (1,2). In the late stages of RA, joint destruction, deformity, ankylosis and a significant decline in functional status development are often observed in many patients. Hence, there is a largely unmet medical need for its thorough treatment.

Drugs for the treatment of RA are available for delivery by both the oral and intraarticular routes, and these administration routes are comparably efficacious (3–5). However, oral administration is often related with severe adverse reaction, for example nonsteroidal anti-inflammatory drugs may cause gastrointestinal complications in a significant number of patients (4). Unlike other joint diseases, RA is locally restricted to one or a few joints, which provides a unique opportunity for local intraarticular treatment without the risk of systemic side effects (4). In addition, intraarticular administration would also permit the high-efficient delivery of drugs with low oral bioavailability, including the delivery of recombinant proteins, therapeutic genes and inhibitory RNAs (3).

Currently available intraarticular treatment options for RA on the market include various glucocorticoid and hyaluronic acid formulations, which, however, provide only short-term pain relief or/and often do not provide adequate pain relief (4,6,7). To achieve long-term drug exposure, different established formulations such as suspensions and hydrogels, and also novel approaches such as liposomes and nano- or microparticles are currently in development (8–10). The development of novel drugs in combination with new formulations for intraarticular treatment of RA, represents a promising approach in this challenging research area. On the other hand, due to their excellent physicochemical characteristics, micelles formed by self-assembly of amphiphilic copolymers with hydrophilic and hydrophobic components for solubilization of poorly soluble drugs have attracted much attention in controlled drug delivery systems recently (11–13). As an exciting example, clinical trials of the polymeric micelles comprised of doxorubicin-conjugated poly (ethylene glycol)-poly (α, β-aspartic acid) block copolymer incorporating doxorubicin have begun based on the successful result of animal studies obtained in laboratory (14).

Recently, polymer-drug conjugates, another type of polymer based nano-formulations that has been widely studied during the past few decades (15), were investigated as novel delivery carrier for the therapy of RA, and some promising result has been obtained (16,17). Our research, however, has been concentrating on the nanocarriers assembled from amphiphilic graft polyphosphazenes. Due to the synthetic flexibility and versatile modification of poly (dichlorophosphazene), the final graft copolymers with special properties for drug delivery and other biomedical applications can be tailored conveniently (18,19). During the past few years, synthesis and self-assembly behavior study of amphiphilic polyphosphazenes with PNIPAAm and amino acid ester as side groups have been carried out in our laboratory (20–23). In this paper we report on drug loading capacity of polymeric micelles to various hydrophobic drugs, which were based on amphiphilic polyphosphazenes (PNIPAAm/EAB-PPPs) bearing poly (*N*-isopropylacrylamide) (PNIPAAm) and ethyl 4-aminobenzoate (EAB) as side groups. In addition, *in vitro* and *in vivo* indomethacin (IND) release dynamics, and intraarticular treatment of arthritis bearing rats through IND-loaded nanocarriers were also performed.

MATERIALS AND METHODS

Materials

Hexachlorocyclotriphosphazene (Strem Chemicals) was purified by recrystallization from hexane and subsequent sublimation at 80–90°C. *N*-isopropylacrylamide (Acros Organics) was recrystallized twice from hexane before use. 2,2′-azobisisobutyronitrile (AIBN) was purified by recrystallization in a benzene/hexane mixture and in ethanol, respectively. 2-Aminoethanethiol hydrochloride (AET·HCl), ethyl 4-aminobenzoate (EAB) and aluminum chloride (99%) obtained from Acros Organics were used as received. Ibuprofen (IBU), ketoprofen (KET), naproxen (NAP) and indomethacin (IND) were kindly supplied by Juhua Group Pharmaceutical Factory (Zhejiang, China). Dexamethasone (DMS), medroxyprogesterone acetate (MPG) and prednisone acetate (PNS) were purchased from Zhejiang Xianju Pharmacy Ltd (Zhejiang, China). The other reagents were commercially available and used without further purification.

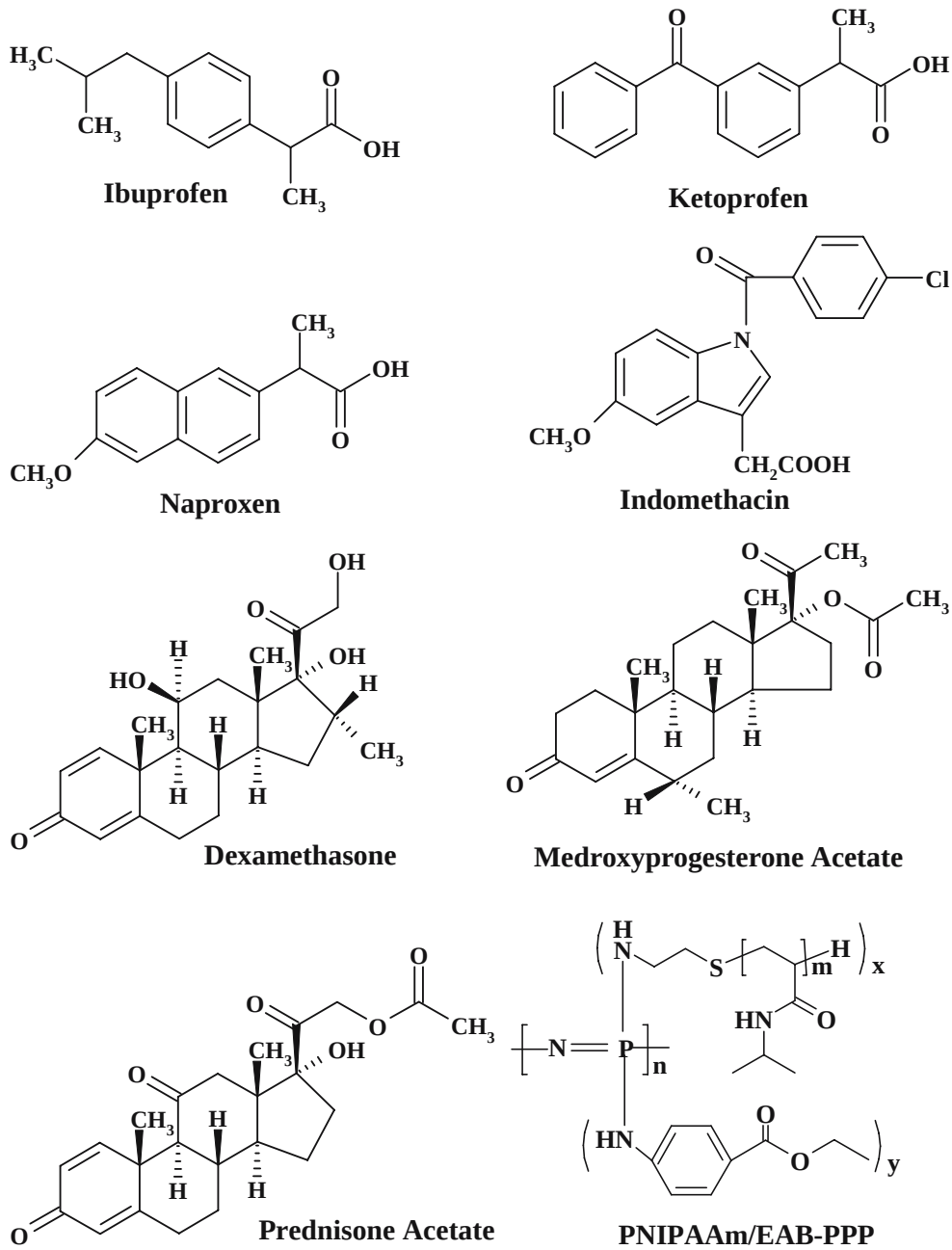
Copolymer Synthesis and Characterization

Oligo-PNIPAAm with a terminal amino group was synthesized by free-radical polymerization using AIBN and AET·HCl as initiator and chain transfer reagent respectively (24). Poly (dichlorophosphazene) was synthesized by thermal ring-opening polymerization, 5.0 % aluminum chloride was used as catalyst (25). Copolymers with PNIPAAm as hydrophilic segment and EAB as hydrophobic group (PNIPAAm/EAB-PPPs) were synthesized as described previously (21).

The molecular weights of both oligo-PNIPAAm and PNIPAAm/EAB-PPPs were determined using a gel permeation chromatography (GPC) equipped with a Waters 515 HPLC Pump and a Waters 2410 refractive index detector. THF was used as solvent with a flow rate of 1.5 ml/min at 40°C and narrow dispersed polystyrene as calibration standards. Copolymer composition, i.e. the molar ratio of PNIPAAm to EAB was calculated according to the UV absorbance of EAB group at 284 nm (21). The lower critical solution temperature (LCST) of oligo-PNIPAAm and copolymer solutions was measured by turbidity method. UV-Visible Spectrophotometer (Beijing Puxi General Instruments Co., Beijing, China) with a TC-1 temperature control was used to trace the phase transition by monitoring the temperature dependent transmittance at 500 nm. The polymer concentration was 0.2 wt%, and the heating rate was 0.1°C per 5 min. The critical micelle concentration (CMC) of copolymers was determined by fluorescence technique using pyrene as probe (20). Fluorescence measurements were conducted using a F-4500 fluorescence spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan).

Preparation and Characterization of Drug-Loaded Micelles Based on PNIPAAm/EAB-PPPs

Drug containing polymeric nanocarriers were prepared by dialysis method. Briefly, copolymer and drug of appropriate weight ratio were co-dissolved in organic solvent with final



Scheme 1. The chemical structure of various drugs and amphiphilic polyphosphazene with PNIPAAm and EAB as side groups.

Table I. The Physicochemical Properties of Copolymers

Polymer	Molar Ratio		M_n	M_w	LCST/°C	CMC (g/l)
	PNIPAAm *x*	EAB *y*				
PNIPAAm			1,900[a] 1,400[b]	2,200	33.8	
PNIPAAm/EAB-PPP-1	1.80	0.20	22,000	43,000	32.6	0.037
PNIPAAm/EAB-PPP-2	1.00	1.00	19,000	38,000	36.0	0.028
PNIPAAm/EAB-PPP-3	0.68	1.32	12,000	21,000	35.0	0.015

[a] Obtained by GPC.
[b] Determined by elemental analysis.

Table II. The Effect of Co-solvents on Drug Loading and Micelle Yield

PNIPAAm/EAB-PPP-1 (mg)	IND (mg)	Co-solvent	Drug Loading (wt%)	Micelle Yield (%)
50	10	DMSO	8.6 ± 1.6	62.1 ± 6.4
		DMF	7.6 ± 1.8	62.2 ± 5.9
		DMAc	9.7 ± 1.7	70.2 ± 6.5
		THF	12.3 ± 2.5	63.5 ± 5.4
		Acetonitrile	7.2 ± 1.2	67.5 ± 5.8

polymer concentration of 10 mg/ml. This solution was placed into the dialysis tube (MWCO: 12 kDa) for dialysis against distilled water for 24 h at 15°C. The outer solution was exchanged at appropriate intervals. After filtered through a 0.8 μm microporous membrane, the dialysis solution was freeze-dried to obtain the resultant drug-loaded micelles.

Reversed-phase high-performance liquid chromatography (HPLC) was performed to quantify the weight content of various drugs in the resultant polymeric micelles. Agilent 1100 HPLC system equipped with a 5 μm ODS C_{18} 250 × 4.6 mm column was adopted. Mobile phase consisting of 6 μM phosphoric acid-acetonitrile (45:55, *v*/*v*) was adopted for IBU, KET, NAP and IND. And the column temperature was 40°C. The flow rate was 1.5, 1.5, 1.5 and 2.0 ml/min, while detection wavelength was 215, 245, 245 and 245 nm for IBU, KET, NAP and IND respectively. On the other hand, mobile phase was composed of methanol and water with a volume ratio of 70:30, and the flow rate was 1.0, 1.5 and 1.0 ml/min for DMS, MPG and PNS respectively. The column temperature was 25°C, and the effluent was detected at 240 nm.

Thermal analysis of IND-loaded micelles and copolymer was carried out using a differential scanning calorimeter (Model DSC Q100, New Castle, USA). Samples were accurately weighed and heated in closed aluminium crimped cells at a rate of 10°C/min in the range of 50 to 200°C under a nitrogen flow of 40 ml/ min.

In Vitro Drug Release Study

Ten milligrams lyophilized IND-loaded polymeric micelles dissolved in 2 ml 0.1 M PBS (pH 7.4) was contained in dialysis tubing with a MWCO of 3, 500, which was placed into 40 ml PBS (0.1 M, pH 7.4). At appropriate intervals, 4.0 ml was removed from the outer aqueous solution and replaced by fresh release medium. The released drug was quantified spectrophotometrically at 320 nm. The test was performed at 37°C in an incubator-shaker at 50 rpm.

In Vivo Pharmacokinetics Study

All the animal care and experimental protocols were complied with the Animal Management Rules of the Ministry of Health of the People's Republic of China (Document No.55, 2001) and the guidelines for the Care and Use of Laboratory Animals of the Third Military Medical University. Twelve Sprague–Dawley male rats (weighted from 200 to 250 g) were randomly divided into two groups of six animals each. One group of IND-loaded micelles in 1.0 ml of 0.1 M PBS (pH 7.4) and one group of free IND in 0.1 M PBS (pH 7.4) for comparison were administered subcutaneously at a dose of 5.0 $mg \cdot kg^{-1}$ to abdominal regions of rats. Blood samples were collected at specific time points postdose. The plasma was isolated and frozen prior to further processing.

Previously published method with some modifications was adopted to determine indomethacin levels in plasma (26). To a 100 μl aliquot of plasma, 100 μl of internal standard solution (mefenamic acid in acetonitrile) and 400 μl of acetonitrile were added. Samples were mixed by vortex for 60 s and centrifuged at 8,000 rpm for 5 min. The precipitate was discarded and the supernatant was evaporated to dryness under nitrogen gas. The residue was dissolved in 100 μl of mobile phase consisting of 6 μM phosphoric acid-acetonitrile (45:55, *v*/*v*) and 50 μl aliquot was injected onto the Agilent 1100 HPLC system (5 μm ODS C_{18} 250 × 4.6 mm column). The flow-rate was 2.0 ml/min and the column effluent was monitored at 245 nm. The column temperature was kept at 40°C.

Table III. The Effect of Drug Structure on Its Loading

Copolymer	Drug	Theoretical Drug Loading[a] (wt%)	Drug Loading (wt%)[b]
PNIPAAm/EAB-PPP-1	IBU	34.8	13.5 ± 1.8
	KET	34.8	11.2 ± 2.0
	NAP	34.8	14.1 ± 1.6
	IND	34.8	12.8 ± 2.5
	DMS	34.8	0.4 ± 0.05
	MPG	34.8	0.3 ± 0.05
	PNS	34.8	0.5 ± 0.1
PNIPAAm/EAB-PPP-1	IND	37.5	12.0 ± 1.5
PNIPAAm/EAB-PPP-2	IND	37.5	16.5 ± 2.0
PNIPAAm/EAB-PPP-3	IND	37.5	20.6 ± 1.8

[a] Theoretical drug loading can be calculated by division of the drug weight with the total weight of drug and copolymer.
[b] Drug loading is defined as the drug content in the resultant drug-loaded micelles.

Table IV. The Effect of the Initial Drug Feed on Drug Loading

Copolymer (mg)	Theoretical IND Loading (wt%)	Drug Loading (wt%)	Entrapment Efficiency (%)[a]
PNIPAAm/EAB-PPP-1	16.7	8.8 ± 1.6	52.7 ± 9.6
	33.3	15.6 ± 2.5	46.8 ± 7.5
	50.0	28.9 ± 3.6	57.8 ± 7.2
	66.7	40.6 ± 3.8	60.9 ± 5.7

[a] Entrapment efficiency is defined as the ratio of drug loading to theoretical drug loading.

Carrageenan-Induced Paw Edema

Fifty six Sprague–Dawley male rats were randomly divided into seven groups: negative control, no drug and copolymer was administered in this group; copolymer control group; three groups for IND-loaded polymeric micelles of lower dosage (0.5 $mg{\cdot}kg^{-1}$), medium dosage (1.5 $mg{\cdot}kg^{-1}$) and higher dosage (4.5 $mg{\cdot}kg^{-1}$) respectively; one group for free IND at a dose of 1.5 $mg{\cdot}kg^{-1}$ in 5% NaOH; and one positive control group administrated orally (5.0 $mg{\cdot}kg^{-1}$ IND suspension). The positive control group was administered via gastric gavage, while other groups were administered by subcutaneous injection of 0.1 ml of the corresponding aqueous solution in the right-back paw of rats. Post-administration of one hour, 0.1 ml of 1% carrageenan in 0.9% sodium chloride aqueous solution was injected subcutaneously at the same site (27). The right-back paw volume was measured by a transducer-linkered plethysmometer (Equipment Factory in Academy of Medical Sciences of Shandong Province, China) at predetermined time after inflammation. Following immersion of the paw in the Perspex cell up to the inscribed line, a direct measurement of the displacement volume was recorded. The swelling degree was expressed as the difference of paw volume before and after inflammation.

Complete Freund's Adjuvant (CFA) Induced Ankle Joint Arthritis

Fifty six Sprague–Dawley male rats were randomly divided into seven groups: normal control group, in which no drug/copolymer or CFA was administered; copolymer control group, in which copolymer and CFA was administered; three groups for IND-loaded polymeric micelles of lower dosage (0.5 $mg{\cdot}kg^{-1}$), medium dosage (1.5 $mg{\cdot}kg^{-1}$) and higher dosage (4.5 $mg{\cdot}kg^{-1}$) respectively; one group for free IND aqueous solution at a dosage of 1.5 $mg{\cdot}kg^{-1}$ in 5% NaOH; and one positive control group with oral administration (5.0 $mg{\cdot}kg^{-1}$ IND suspension). Only 0.1 ml of sodium chloride aqueous solution (0.9%) was injected into the ankle joint of right-back paw of rat in the normal control group, while 0.1 ml CFA was injected into the same site in other groups. All the rats were fed normally after inflammation. On the day 8, copolymer control group and IND-treated groups were administered by intraarticular injection, while the positive control group was administered via gastric gavage that was performed one time each day for 7 days. The changes of body weight, swelling of right-back paw and the stimulation to the stomach were determined. The paw edema intensity was expressed as the volume change before and after inflammation.

Statistical Analysis

All data were expressed as mean±standard deviation (SD), and the differences between experimental groups and control group (each experimental group compared with control group) were compared using Student's t test. The acceptable level of statistical significance was set at a probability of less than 0.05.

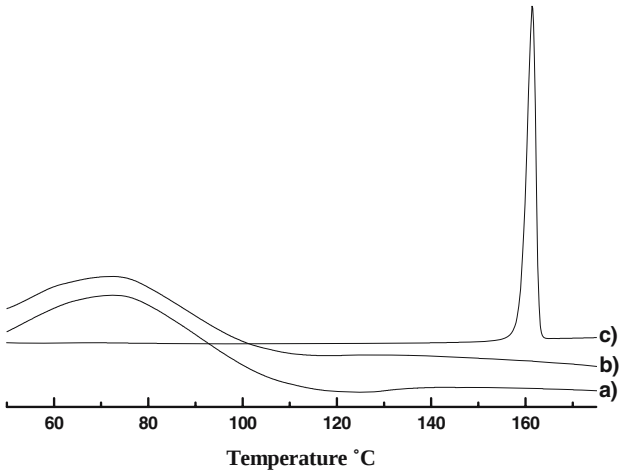


Fig. 1. DSC curves of (*a*) PNIPAAm/EAB-PPP-1 based micelles with 8.8% IND, (*b*) PNIPAAm/EAB-PPP-1 micelles containing 28.9% IND, and (*c*) IND.

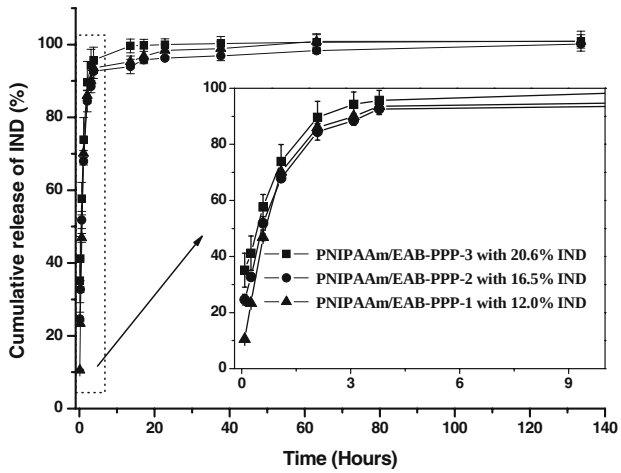


Fig. 2. *In vitro* release behaviors of IND-loaded polymeric micelles based on copolymers with different EAB contents.

Table V. The Pharmacokinetic Parameters of IND after Subcutaneous Administration of the Same Dose of Aqueous Solution of Free Drug and Micelles Respectively

Parameters	IND in 0.1 M PBS (pH 7.4)	IND-Loaded PNIPAAm/EAB-PPP-1 Micelles
T_{max}(h)	0.7 ± 0.2	1.0 ± 0.2
C_{max}(μg/ml)[a]	23.0 ± 3.8	17.9 ± 2.9
$AUC_{(0-t)}$ (μg·h/ml)[a]	172.7 ± 33.5	234.9 ± 51.3
$AUMC_{(0-t)}$ (μg·h^2/ml)[a]	1,549.2 ± 156.4	3,058.6 ± 357.7
$MRT_{(0-t)}$ (h)[a]	9.0 ± 2.2	13.0 ± 4.6

[a] Indicate significant difference ($p < 0.05$).

Evaluation of Gastric Ulceration

On the 14th day, IND and IND-loaded polymeric micelles (0.5, 1.5 and 4.5 mg·kg^{-1}) were administered to rats of corresponding groups by gastric gavage or intraarticular injection respectively. They were fasted for 24 hours, while water was freely available. Then, all the rats were sacrificed by dislocating neck vertebra. The rats' pylori were ligated, 10 ml of 0.5% neutral solution of formalin was infused into stomach through their esophagus, and then the stomachs were put into formalin solution (10%) after preventriculus ligature. After 10 min, the greater curvature of stomach was split, and the number of hemorrhage points and ulcers were counted by a ten times-magnifier. The grade of lesion was scored on a scale of four grades: grade 0, no lesion; grade 1, light petechia; grade 2, diffuse bleeding or aphtha (<2 mm); grade 3, two or more aphtha (<2 mm); grade 4, one or more ulcer (>2 mm).

RESULTS AND DISCUSSION

Synthesis of Copolymers

Amphiphilic polyphosphazenes with hydrophilic PNIPAAm and hydrophobic EAB as side groups were synthesized according to our previously reported method (21,23). The chemical structure of copolymer is illustrated in Scheme 1. The number of structural unit of PNIPAAm, i.e. the value of *m* in Scheme 1, is about 11.6, which was calculated according to the number average molecular weight of PNIPAAm determined through elemental analysis. Other physicochemical properties of copolymers are listed in Table I.

Preparation of Drug-Loaded Polymeric Micelles

Incorporation of hydrophobic drugs into polymeric micelles was carried out by a dialysis method. Several organic solvents including dimethyl sulphoxide (DMSO), *N*,*N*-dimethylformamide (DMF), dimethylacetamide (DMAc), THF and acetonitrile were selected because these solvents can dissolve both IND and copolymer and are miscible with water which was used as dialysate. As listed in Table II, when DMAc or THF was employed as co-solvent, PNIPAAm/EAB-PPP-1 based micelles with relatively higher IND loading and yield were obtained. Since no IND precipitate was observed during the whole dialysis procedure, micelle formation and drug incorporation into the polymeric micelle might occur simultaneously. Generally, interactions, mainly hydrophobic interactions, among the hydrophobic chain, drug, and solvent may be an important factor to control the drug incorporation process. In addition, large particles resulted from the inter-micellar aggregation were removed by extrusion through a 0.8 μm diameter membrane. All these combinational factors should be responsible for the loading results presented in Table II. Considering its excellent solubility to all the drugs employed in this study, DMAc was used as co-solvent to prepare drug containing micelles in the following experiments.

Seven drugs including IBU, KET, NAP, IND, DMS, MPG and PNS (Scheme 1), which exhibit various chemical structures, were used to elucidate the effect of drug type on its encapsulation into polymeric micelles based on PNIPAAm/EAB-PPP-1. As can be clearly seen in Table III, drug loading was very lower for DMS, MPG and PNS, while it was relatively higher for drugs such as IBU, KET, NAP and IND. According to the previous reports on the incorporation of hydrophobic compounds into micelles, loading efficiency is closely correlated with molecular volume of solubilizates, Van der Waals dispersion forces, hydrogen bonding and dipole–dipole interactions between hydrophobic segment and solubilizates (28,29). As for drug molecules under investigation, from their chemical structures as illustrated in Scheme 1, one can expect that polycyclic molecules

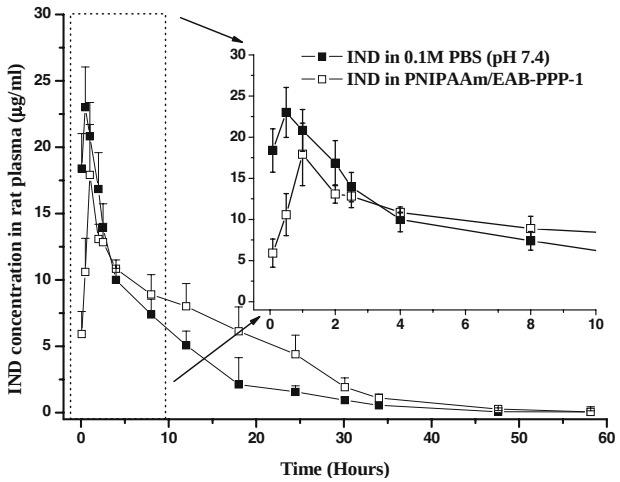


Fig. 3. Concentration–time curves of IND-loaded PNIPAAm/EAB-PPP-1 micelles and 0.1 M PBS (pH 7.4) solution containing IND after subcutaneous injection of the same dose.

Table VI. Treatment of Acute Carrageenan-Induced Paw Edema with Various Protocols

Treatment Group	IND Dosage (mg/kg)	Initial Paw Volume (ml)	Swelling Degree (ml)[a]			
			1 h[b]	3 h	5 h	7 h
Negative control		0.92 ± 0.02	0.38 ± 0.05	0.53 ± 0.06	0.59 ± 0.05	0.60 ± 0.05
Copolymer control		0.94 ± 0.13	0.41 ± 0.05	0.55 ± 0.09	0.60 ± 0.10	0.62 ± 0.10
IND loaded	4.5	0.93 ± 0.07	0.36 ± 0.06	0.43 ± 0.07[c]*	0.37 ± 0.07*	0.33 ± 0.05*
copolymer	1.5	0.96 ± 0.05	0.38 ± 0.09	0.45 ± 0.11**	0.41 ± 0.09*	0.36 ± 0.07*
micelles	0.5	0.93 ± 0.18	0.41 ± 0.05	0.46 ± 0.11	0.45 ± 0.07*	0.50 ± 0.05*
IND in 5% NaOH solution	1.5	0.95 ± 0.05	0.39 ± 0.04	0.50 ± 0.09	0.51 ± 0.08**	0.51 ± 0.08**
IND by oral administration	5.0	0.91 ± 0.07	0.39 ± 0.05	0.46 ± 0.04	0.50 ± 0.09**	0.52 ± 0.09**

[a] Swelling degree was expressed as the difference in paw volume before and after inflammation.
[b] The time point after subcutaneous injection of carrageenan aqueous solution.
[c] All the statistical analysis was made in comparison with copolymer control group.
*$p < 0.01$
**$p < 0.05$

such as DMS, MPG and PNS might occupy relatively larger three-dimensional space in polymeric micelles, that is, these drugs might have relatively larger molecular volume compared with drugs like IBU, KET, NAP and IND. This might partly explain the very lower loading level of these drugs in polymeric micelles. Additionally, the latter are highly hydrophobic steroidal compounds, suggesting these drugs are not compatible with the micellar cores constructed from EAB groups of PNIPAAm/EAB-PPPs, which might be also responsible for their poor encapsulation. In addition, drugs like IBU, KET, NAP and IND are more hydrophilic compounds, which lead to their significant solubilization. Another factor that can contribute to the relatively high efficiency encapsulation of these drugs should be the hydrogen bonding interaction of amide groups of PNIPAAm and carboxylic acid group of drug since this strong noncovalent interaction between the amide groups in PNIPAAm chains and carboxyl group has been well elucidated (24). Most recently, hydrogen bonding enhanced drug loading was also reported by Benahmed *et al.* (30) for polymeric micelles based on PVP-b-PDLLA and IND. Additionally, in a separate study, it was found that IND loading level increased significantly as the EAB content in copolymer enhanced (Table III). Attention, however, should be paid when compare the drug loading levels that correspond to theoretical drug loading of 34.8 and 37.5%, lower level was observed for the latter in spite of its slightly higher initial feed. This might be attributed to the batch-to-batch deviation since these loading experiments were performed in different batches. In general, these results suggest that IND can be effectively loaded into micelles based on PNIPAAm/EAB-PPPs.

Based on above results, we selected IND to investigate the initial drug feed on the final drug loading. As presented in Table IV, actual drug loading increased accordingly concomitant with increase in drug feed from 16.7 to 66.7%. This result further suggested that polymeric micelles based on PNIPAAm/EAB-PPPs are more suitable carriers for relatively hydrophilic compounds such as IND. For instance,

Table VII. The Effect of Various Therapeutic Protocols on the Edema Intensity of Rats Bearing CFA-Induced Adjuvant Arthritis

Treatment Group	IND Dosage (mg/kg)	Increase in Body Weight (g)	Paw Volume before Inflammation (ml)	The Paw Edema Intensity after Inflammation (ml)					
				1d[a]	5d	8d	10d	12d	15d
Normal Control		58.61 ± 6.70	1.13 ± 0.10	0.01 ± 0.01	0.02 ± 0.01	0.04 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.11 ± 0.01
Copolymer Control		46.94 ± 8.25	1.18 ± 0.06	0.86 ± 0.08	0.76 ± 0.05	0.85 ± 0.08	0.81 ± 0.06	0.69 ± 0.06	0.78 ± 0.06
Free IND	1.5	53.99 ± 7.70	1.16 ± 0.06	0.85 ± 0.08	0.81 ± 0.06	0.84 ± 0.07	0.65 ± 0.05*	0.72 ± 0.04	0.69 ± 0.02**[b]
IND Suspension	5.0	8.01 ± 7.93*	1.14 ± 0.10	0.91 ± 0.07	0.75 ± 0.05	0.72 ± 0.13	0.51 ± 0.08*	0.60 ± 0.09**	0.39 ± 0.06*
Copolymer	4.5	53.63 ± 9.03	1.14 ± 0.08	0.90 ± 0.06	0.79 ± 0.07	0.81 ± 0.05	0.37 ± 0.13*	0.42 ± 0.12*	0.29 ± 0.10*
Micelles	1.5	62.08 ± 9.48	1.12 ± 0.09	0.83 ± 0.04	0.73 ± 0.12	0.81 ± 0.04	0.45 ± 0.03*	0.38 ± 0.07*	0.34 ± 0.04*
	0.5	49.30 ± 8.46	1.16 ± 0.06	0.81 ± 0.04	0.74 ± 0.04	0.74 ± 0.03	0.47 ± 0.05*	0.57 ± 0.10**	0.44 ± 0.04*

[a] Time point after CFA administration.
[b] All the statistical analysis was made in comparison with copolymer control group.
*$p < 0.01$
**$p < 0.05$

Table VIII. Gastric Ulceration Induced by IND Administration

Treatment Group	IND Dosage (mg/kg)	The Number of Hemorrhage Point	The Number of Ulceration	Average Trauma Grade
Orally administered IND	5.0	15.87 ± 2.47	2.75 ± 1.03	2.62 ± 0.86
IND loaded PNIPAAm/EAB-PPP-1 micelles	4.5	0	0	0
	1.5	0	0	0
	0.5	0	0	0

after it was encapsulated in polymeric micelles, the solubility of IND in water can be increased from 0.016 mg/ml (IND solubility at 37°C) to above 10 mg/ml.

The existence form of IND, i.e. it exists in the form of little crystal or molecularly disperses in the polymeric micelles, was partly confirmed by DSC analysis. If there are IND crystals in the drug-containing micelles, there should be an endothermal peak that corresponding to the melt point of IND in the DSC profile. On the other hand, the absence of endothermal peak suggests that IND might molecularly disperse in the drug-loaded micelles. The DSC curves of PNIPAAm/EAB-PPP-1 based micelles containing various amounts of IND and IND itself are presented in Fig. 1. While IND itself exhibits a melting peak at about 161°C, polymeric micelles with different contents of IND do not have the same endothermal peak, which was similar to the blank copolymer. This result indicated that IND might be molecularly dispersed in polymer matrix in the final drug-loaded micelles.

In Vitro and *In Vivo* Release Kinetics

Figure 2 shows the *in vitro* release profiles of IND from polymeric micelles based on copolymers with different compositions. It seemed that copolymer composition has no significant effect on IND release rate. Independent of various copolymers, above 95% IND in the micelles was released within the first 5 h although complete release was finished within 5 days. This might be due to the relatively high drug solubility of IND in release media employed in this study. According to our test, the solubility of IND in 0.1 M PBS (pH 7.4) is 2.75 mg/ml at 37°C.

The drug concentration–time curves of IND in rat plasma after subcutaneous administration of aqueous solutions of free IND and IND-loaded polymeric micelles are illustrated in Fig. 3. Within the early 3 h, IND concentration in the group administered with micelles was lower than that of group treated with free drug solution. After this stage, however, IND concentration in micelle group was higher than that of free drug group. The maximum concentration was shown at 0.7 and 1.0 h, and the corresponding values were 23.0 and 17.9 μg/ml for free drug solution and micelles, respectively. Selected pharmacokinetic parameters are listed in Table V. IND-loaded micelles showed higher MRT, AUC and AUMC than the free drug solution ($p < 0.05$). This might be due to the relatively slowed release rate of IND from polymeric micelles compared with free IND solution. In addition, it can be seen from Fig. 3 that IND was released completely within 50 h in *in vivo* release study, and this time period was short taking into account of *in vitro* release profiles. A possible reason might be based on the fact that there should be continuous exchange of body fluid at the injection site and frequent movement of local tissue at/or near the site where drug carriers exist as far as *in vivo* study was concerned, while obviously it was not the case for *in vitro* test.

Local Delivery of IND to Rats Bearing Paw Edema

Two *in vivo* models, i.e. carrageenan-induced acute arthritis (paw edema) and CFA-induced adjuvant arthritis, were employed to separately evaluate the short and long time period of therapeutic effect of IND-loaded polymeric micelles after topical drug administration. In the acute model, blank copolymer, free IND aqueous solution and IND-tablet suspension for oral administration were used as controls. In addition, negative control, in which no drug or copolymer was administered, was also provided. As presented in Table VI, although the swelling degree in the copolymer control group was slightly higher than that of negative control group, there is no statistical difference, suggesting that the local tissue almost has no inflammatory reaction to copolymer itself, which is very important for polymer used for local drug delivery. It can be also found from Table VI, IND-loaded micelles exhibited therapeutic efficacy at 5 h, 3 h and 3 h after inflammation at the doses of 0.5, 1.5 and 4.5 $mg \cdot kg^{-1}$ respectively. Compared with the groups treated with free IND aqueous solution and oral administration, micelles at doses of 4.5 or 1.5 $mg \cdot kg^{-1}$ displayed significant difference during the time period from the 3rd to the 7th hour post inflammation ($p < 0.05$–0.01). While for experimental group treated with drug-loaded micelles at 0.5 $mg \cdot kg^{-1}$, significant difference was only observed at the 5 h to 7 h period post inflammation, which was almost the same case as those groups administered with free IND and tablet suspension.

Intraarticular Delivery of IND to Arthritis Rats

In the case of study on adjuvant arthritis model, administration was performed through intra-ankle joint injection. After injection of CFA into the ankle joint, edema intensity will be decreased after the maximum edema maintained for 3–4 days, and then it will enhance again on days 8 to 10. As can be seen from the data presented in Table VII, free IND aqueous solution exhibited slight therapeutic effect. On the other hand,

micelles of various doses could significantly suppress retumefying, suggesting that the efficacy of IND encapsulated in micelles was enhanced compared with free IND solution at the same dose. As mentioned above, this adjuvant arthritis model enables us to evaluate the long term therapeutic effect of IND-loaded micelles. However, both *in vitro* and *in vivo* drug release study suggested that most of IND will be released within 5 (*in vitro*) or 30 hours (*in vivo*). Consequently, the prolonged therapeutic effect of IND-loaded micelles to CFA arthritis suggested that there should be a more sustained drug release in the inflamed site. As well known, one of pathological features of the rheumatic joint is the presence of low pH (31–33), which has been utilized sufficiently by Wang *et al.* (17) to design a pH-sensitive polymer-drug conjugate for the treatment of RA most recently. This decrease in pH compared with normal tissue may lead to a decrease in IND release rate considering the pH dependent release profiles of IND from polymeric micelles as clearly demonstrated by the research of La *et al.* (34).

On the other hand, although repeated oral administration of IND could also provide significant therapy, increase in body weight of rats in this group was dramatically lower than that of copolymer control group, which might be due to the fact that the stimulation of IND on gastrointestinal tract could influence the food taking. This was indeed the case. As shown in Table VIII, oral administration of IND resulted in significant formation of gastric ulceration, while no rats in micelles related groups were observed to exhibit ulceration.

CONCLUSIONS

Amphiphilic polyphosphazenes with PNIPAAm and EAB as side groups were synthesized and used as delivery carriers for hydrophobic drugs. The effects of co-solvent type in dialysis procedure, drug structure, initial drug feed and copolymer composition on the drug incorporation into polymeric micelles based on amphiphilic polyphosphazenes with PNIPAAm and EAB as side groups were investigated. When the dialysis method was used to prepare drug-loaded micelles, lower drug loading and entrapment efficiency were observed for highly hydrophobic drugs such as steroidal compounds, while relatively higher drug loading was found for non-steroidal anti-inflammatory drugs. In addition, the drug loading capacity could be enhanced concomitant with increase in EAB content in amphiphilic copolymers. Increasing initial IND feed could result in substantial increase in IND loading in the final micelles. Through loading into polymeric micelles, IND solubility in water can be enhanced dramatically. These results suggest that the drug loading ability of micelles based on this type of amphiphilic polyphosphazenes is mainly determined by copolymer composition and the chemical structure of drug. In addition to the compatibility between drug and micellar core, hydrogen bonding interaction between drug and hydrophilic corona may significantly influence drug loading as well.

In vitro drug release tests suggested that no significant difference in release rate was observed for micelles based on copolymers with various compositions. Compared with the free drug solution of the same dose, IND concentration in rat plasma showed a prolonged retention in micelle group. *In vivo* pharmacodynamic study based on both acute paw edema and adjuvant arthritis model indicated that sustained therapeutic efficacy could be achieved through local or intraarticular injection of IND-loaded micelles. Most importantly, local delivery of IND can avoid the severe gastrointestinal stimulation, which is associated with oral administration.

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REFERENCES

1. E. D. J. Harris. Rheumatoid arthritis: pathophysiology and implications for therapy. *New Engl. J. Med.* **322**:1277–1289 (1990).
2. T. Doanand and E. Massarotti. Rheumatoid arthritis: an overview of new and emerging therapies. *J. Clin. Pharmacol.* **45**:751–762 (2005).
3. J. Steinmeyerand and Y. T. Konttinen. Oral treatment options for degenerative joint disease-presence and future. *Adv. Drug Delv. Rev.* **58**:168–211 (2006).
4. N. Gerwin, C. Hops, and A. Lucke. Intraarticular drug delivery in osteoarthritis. *Adv. Drug Delv. Rev.* **58**:226–242 (2006).
5. S. Abramson. Drug delivery in degenerative joint disease: where we are and where to go?. *Adv. Drug Delv. Rev.* **58**:125–127 (2006).
6. C. T. Wang, J. Lin, and C. J. Chang. Therapeutic effects of hyaluronic acid on osteoarthritis of the knee. A meta-analysis of randomized controlled trials. *J. Bone Jt. Surg.* **86**:538–544 (2004).
7. X. Ayral. Injections in the treatment of osteoarthritis. *Best Pract. Res. Clin. Rheumatol.* **15**:609–626 (2001).
8. K. E. Brown, K. Leong, C. H. Huang, R. Dalal, G. D. Green, H. B. Haimes, P. A. Jimenez, and J. Bathon. Gelatin/chondroitin 6-sulfate microspheres for the delivery of therapeutic proteins to the joint. *Arthritis Rheum.* **41**:2185–2195 (1998).
9. M. Trif, C. Guillen, D. M. Vaughan, J. M. Telfer, J. M. Brewer, A. Roseanu, and J. H. Brock. Liposomes as possible carriers for lactoferrin in the local treatment of inflammatory diseases. *Exp. Biol. Med.* **226**:559–564 (2001).
10. E. Horisawa, T. Hirota, S. Kawazoe, J. Yamada, H. Yamamoto, H. Takeuchi, and Y. Kawashima. Prolonged anti-inflammatory action of dl-lactide/glycolide copolymer nanospheres containing betamethasone sodium phosphate for an intra-articular delivery system in antigen-induced arthritic rabbit. *Pharm. Res.* **19**:403–410 (2002).
11. C. Allen, D. Maysinger, and A. Eisenberg. Nano-engineering block copolymer aggregates for drug delivery. *Colloids Surf. B* **16**:3–27 (1999).
12. M. C. Jonesand and J. C. Leroux. Polymeric micelles-a new generation of colloidal drug carriers. *Eur. J. Pharm. Biopharm.* **48**:101–111 (1999).
13. K. Kataoka, A. Harada, and Y. Nagasaki. Block copolymer micelles for drug delivery: design, characterization and biological significance. *Adv. Drug Delv. Rev.* **47**:113–131 (2001).
14. Y. Kakizawaand and K. Kataoka. Block copolymer micelles for delivery of gene and related compounds. *Adv. Drug Delv. Rev.* **54**:203–222 (2002).
15. R. Duncan. The dawning era of polymer therapeutics. *Nat. Rev. Drug Discov.* **2**:347–360 (2003).
16. D. Wang, S. C. Miller, M. Sima, D. Parker, H. Buswell, K. C. Goodrich, P. Kopeckova, and J. Kopecek. The arthrotropism of macromolecules in adjuvant-induced arthritis rat model: a preliminary study. *Pharm. Res.* **21**:1741–1749 (2004).
17. D. Wang, S. C. Miller, X. M. Liu, B. Anderson, X. S. Wang, and S. R. Goldring. Novel dexamethasone-HPMA copolymer conjugate and its potential application in treatment of rheumatoid arthritis. *Arthritis Res. Ther.* **9**:R2, 2007 (2007).
18. Y. K. Chang, E. S. Powell, and H. R. Allcock. Environmentally responsive micelles from polystyrene-poly[bis(potassium carbox-

ylatophenoxy)phosphazenel block copolymers. *J. Polym. Sci. Pol. Chem.* **43**:2912–2920 (2005).
19. R. Song, Y. J. Jun, J. I. Kim, C. Jin, and Y. S. Sohn. Synthesis, characterization, and tumor selectivity of a polyphosphazene-platinum(II) conjugate. *J. Control. Release* **105**:142–150 (2005).
20. J. X. Zhang, L. Y. Qiu, K. J. Zhu, and Y. Jin. Thermosensitive micelles self-assembled by novel N-isopropylacrylamide oligomer grafted polyphosphazene. *Macromol. Rapid Commun.* **25**:1563–1567 (2004).
21. J. X. Zhang, L. Y. Qiu, Y. Jin, and K. J. Zhu. Physicochemical characterization of polymeric micelles constructed from novel amphiphilic polyphosphazene with poly(N-isopropylacrylamide) and ethyl 4-aminobenzoate as side groups. *Colloids Surf. B* **43**:123–130 (2005).
22. J. X. Zhang, L. Y. Qiu, and K. J. Zhu. Solvent controlled multimorphological self-assembly of amphiphilic graft copolymers. *Macromol. Rapid Commun.* **26**:1716–1723 (2005).
23. J. X. Zhang, L. Y. Qiu, Y. Jin, and K. J. Zhu. Multimorphological self-assemblies of amphiphilic graft polyphosphazenes with oligopoly (N-isopropylacrylamide) and ethyl 4-aminobenzoate as side groups. *Macromolecules* **39**:451–455 (2006).
24. G. Chen and A. S. Hoffman. Graft copolymers that exhibit temperature-induced phase transitions over a wide range of pH. *Nature* **373**:49–52 (1995).
25. Y. S. Sohn, Y. H. Cho, H. Baek, and O. S. Jung. Synthesis and properties of low molecular weight polyphosphazenes. *Macromolecules* **28**:7566–7568 (1995).
26. J. Sato, T. Amizuka, Y. Niida, M. Umetsu, and K. Ito. Simple, rapid and sensitive method for the determination of indomethacin in plasma by high-performance liquid chromatography with ultraviolet detection. *J. Chromatogr. B* **692**:241–244 (1997).
27. I. G. Otterness and M. L. Bliven. *Laboratory models for testing non-steroidal anti-inflammatory drugs*, Wiley, New York, 1985.
28. R. Nagarajan, M. Barry, and E. Ruckenstein. Unusual selectivity in solubilization by block copolymer micelles. *Langmuir* **2**:210–215 (1986).
29. J. B. Liu, Y. H. Xiao, and C. Allen. Polymer-drug compatibility: a guide to the development of delivery systems for the anticancer agent, ellipticine. *J. Pharm. Sci.* **93**:132–143 (2004).
30. A. Benahmed, M. Ranger, and J. C. Leroux. Novel polymeric micelles based on the amphiphilic diblock copolymer poly(N-vinyl-2-pyrrolidone)-blockpoly(D,L-lactide). *Pharm. Res.* **18**:323–328 (2001).
31. S. E. Andersson, K. Lexmuller, A. Johansson, and G. M. Ekstrom. Tissue and intracellular pH in normal periarticular soft tissue and during different phases of antigen induced arthritis in the rat. *J. Rheumatol.* **26**:2018–2024 (1999).
32. V. A. Bobkov, T. N. Brylenkova, and R. S. Moiseenko. Acid-base balance of the synovial fluid in patients with RA debute. *Terapevt. Arkh.* **72**:35–38 (2000).
33. J. R. Levick. Hypoxia and acidosis in chronic inflammatory arthritis:Relation to vascular supply and dynamic effusion pressure. *J. Rheumatol.* **17**:579–582 (1990).
34. S. B. La, T. Okano, and K. Kataoka. Preparation and characterization of the micelle-forming polymeric drug indomethacin-incorporated poly (ethylene oxide)-poly (β-benzyl L-aspartate) block copolymer micelles. *J. Pharm. Sci.* **85**:85–90 (1996).