



Hydrotropic agents for study of in vitro paclitaxel release from polymeric micelles

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Abstract

A new experimental method for in vitro release studies of poorly soluble drugs from polymeric micelle systems was developed using a hydrotropic agent, sodium salicylate. It is difficult to maintain a good sink condition for poorly water-soluble drugs, such as paclitaxel (PTX), because of their low aqueous solubility. In this study, a good sink condition for PTX was achieved by using aqueous sodium salicylate solution which solubilized more than 10 times the total amount of PTX incorporated in polymeric micelles. Sodium salicylate at 1 M concentration increased the aqueous PTX solubility by 100 times without destroying the micellar structure of poly(ethylene glycol)-block-poly(phenylalanine) (PEG-b-PPhe) copolymer. PTX was continuously released from PEG-b-PPhe micelles in the hydrotropic release medium. The hydrotropic solution presents a simple method for studying in vitro release behavior of poorly soluble drugs from polymeric micelles in aqueous media.

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1. Introduction

Studies on in vitro drug release profiles require a good sink condition. Maintaining a sink condition means keeping the drug concentration in a release medium low enough not to affect the concentration gradient for drug release. This causes a major constraint on conducting release experiments. As the volume of a release medium increases, the concentra-

tion of a drug to be measured decreases. The situation is more critical if the drug is hydrophobic, since the solubility of the drug in an aqueous medium may be extremely low and thus a very large volume may be needed to maintain a good sink condition. It has been recommended as a rule of thumb that the drug concentration in a release medium must be kept below 10% of saturation to maintain a sink condition [1].

Paclitaxel (PTX) is an effective anti-tumor agent with poor water-solubility. In vitro PTX release from polymeric matrices has been measured by including surfactants [2,3] or proteins [4] in release media and by using immiscible *n*-octanol/water phase systems [5]. Inclusion of surfactants in release media is cur-

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rently the most popular method. A small amount of surfactants in release media significantly increases the aqueous solubility of PTX, which makes it much easier to keep a good sink condition. The rate of in vitro PTX release from nano/micro-spheres was measured in PBS containing 0.1% Tween 80 [2,3]. The FDA guidance also allows use of a surfactant such as sodium dodecyl sulfate (SDS) for water-insoluble or sparingly water-soluble drugs [6]. Liggins and Burt [4] added 0.4% albumin to a PBS buffer medium to increase the aqueous PTX solubility. However, the PTX solubility in the albumin-containing medium did not seem to be high enough to maintain a good sink condition in the study. Concentrations near saturation were encountered in the first 3 days of the study. It was expected that a larger amount of PTX would be released over this time period if a sink condition had been maintained. Jackson et al. [5] used an immiscible *n*-octanol/water system as a release medium for the determination of in vitro release of PTX from cross-linked hyaluronic acid films. The PTX solubility in *n*-octanol is over 5 mg/ml, which is more than four orders of magnitude higher than the solubility in PBS. The PTX released in an aqueous phase partitions into the *n*-octanol phase.

Experimental difficulty of the in vitro release study of poorly soluble drugs such as PTX is even more critical for polymeric micelle systems. It is quite formidable to apply the above methods to polymeric micelle systems without extensive modifications. Addition of surfactants in release media might have a significant effect on a micellar structure and distort the release profiles. *n*-Octanol can also destroy a micellar structure upon contact with micelles. Furthermore, our preliminary studies showed that PTX was quite unstable and easily degraded in *n*-octanol, indicating that *n*-octanol is not suitable as the PTX release medium (data not shown). Although a number of papers on PTX delivery using polymeric micelles have been published [7–16], there has been no report on the in vitro PTX release profile from polymeric micelles.

Here, we suggest a new experimental method for the determination of in vitro PTX release from polymeric micelles using a hydrotropic agent. Our previous studies identified a number of hydrotropic agents that increased the water-solubility of PTX by orders of magnitude [17]. The most effective hydro-

tropic agents for PTX were *N,N*-diethylnicotinamide, *N*-picolylnicotinamide, *N*-allylnicotinamide, and sodium salicylate. *N,N*-Diethylnicotinamide was used effectively to create a sink condition for the release of PTX from PLGA matrix [18]. In this study, we used sodium salicylate to create a sink condition for PTX released from polymeric micelles. Block copolymers composed of poly(ethylene glycol) (PEG) and poly(phenylalanine) (PPhe) were synthesized to form micelles in water. Effects of a hydrotropic agent on the aqueous PTX solubility and the micellar structure of the PEG-*b*-PPhe copolymer were studied. The in vitro release behavior of PTX from the block copolymer micelles was studied in an aqueous solution containing sodium salicylate as a release medium.

2. Materials and methods

2.1. Materials

PTX (anhydrous form) was supplied by Samyang Genex (Taejeon, South Korea). Bis(trichloromethyl) carbonate (triphosgene), *L*-phenylalanine, and sodium salicylate were purchased from Aldrich (Milwaukee, WI). The reagents were used without further purification. Tetrahydrofuran (THF) and *N,N*-dimethylformide (DMF) were dried and distilled immediately before use. α -Methoxy- ω -aminopoly(ethylene glycol) (PEG; molecular weight 5000, amino content 98%) was purchased from Nektar Therapeutics (San Carlos, CA).

2.2. Synthesis of a block copolymer

2.2.1. *N*-carboxyanhydride of *L*-phenylalanine (Phe-NCA)

Synthesis of *N*-carboxyanhydride of *L*-phenylalanine (Phe-NCA) was carried out by the Fuchs-Farthing method using triphosgene [19]. *L*-Phenylalanine (5 g, 30.3 mmol) was suspended in 50 ml of THF and heated to 40 °C in a nitrogen atmosphere. A solution of 3 g (12.1 mmol) of triphosgene dissolved in THF was added dropwise to the stirred reaction mixture. A stream of dry nitrogen was periodically bubbled through the reaction mixture to remove HCl. After 3 h, the reaction mixture was filtered to remove any

insoluble materials and the filtrate was poured into 300 ml of hexane. The resulting suspension was stored at $-20\text{ }^{\circ}\text{C}$ overnight to assure complete crystallization. For further purification, the obtained Phe-NCA was recrystallized three times from a mixture of THF/*n*-hexane and dried at room temperature in a vacuum. The yield was 4.88 g (84.3%). The melting point of Phe-NCA ($95\text{--}96\text{ }^{\circ}\text{C}$) corresponded to the literature data [20,21] and the $^1\text{H-NMR}$ spectra agreed with the expected structure and purity.

2.2.2. Poly(ethylene glycol)-block-poly(L-phenylalanine) copolymer (PEG-*b*-PPhe)

PEG (1.0 g) was dissolved in 10 ml of DMF. Then a solution of Phe-NCA (0.35 g) in 4 ml of DMF was added to the solution of PEG. The reaction mixture was stirred for 24 h at $40\text{ }^{\circ}\text{C}$ under a dry nitrogen atmosphere and then precipitated with an excess of diethyl ether. The precipitate was dissolved in 10 ml of chloroform and then reprecipitated into an excess of diethyl ether. The yield was 1.06 g (84.0%). The composition of PEO-*b*-PPhe was determined by 300 MHz $^1\text{H-NMR}$ (Bruker ARX300, Billerica, MA) in DMSO- d_6 .

2.3. Solubility study

Excess PTX was added to screw-capped vials containing a fixed volume (2 ml) of sodium salicylate solutions. The mixture was stirred using a magnetic stirring bar for 24 h at $37\text{ }^{\circ}\text{C}$. An aliquot of the sample was collected and filtered through a $0.2\text{-}\mu\text{m}$ nylon membrane. The filtrate was diluted with acetonitrile (1:1), and the concentration of PTX was measured by HPLC as described below.

2.4. Paclitaxel loading into block copolymer micelles

PTX was incorporated into PEG-*b*-PPhe micelles by a solid dispersion method [7,8]. PTX (30 mg) and PEO-*b*-PPhe (270 mg) were dissolved in 4 ml of acetonitrile. After 30 min of stirring, acetonitrile was evaporated under reduced pressure at $60\text{ }^{\circ}\text{C}$ to obtain a transparent gel-like matrix. Addition of 100 ml water at $60\text{ }^{\circ}\text{C}$ to the gel-like matrix resulted in formation of PTX-incorporated micelles. The solution was filtered through a $1.0\text{-}\mu\text{m}$ filter and freeze-dried. The loading amount was measured

by HPLC as described below after dissolving the PTX-incorporated block copolymer micelles with acetonitrile.

2.5. In vitro paclitaxel release from block copolymer micelles

In vitro release profiles of PTX from polymeric micelles were investigated in an aqueous medium containing sodium salicylate. The freeze-dried PTX-containing polymeric micelles were weighed, dissolved in 1 ml of water, and introduced into a dialysis membrane bag (MWCO = 6000–8000 Da, Spectrum Lab., Rancho Dominguez, CA). The release experiment was initiated by placing the end-sealed dialysis bag in 20 ml of 1 M sodium salicylate solution at $37\text{ }^{\circ}\text{C}$. The release medium was stirred at a speed of 100 rpm. At predetermined time intervals, samples (2 ml) were withdrawn and replaced with an equal volume of fresh medium (1 M sodium salicylate). The concentration of PTX in the samples was measured by HPLC as described below. For comparison, PTX release from bulk powders placed in a dialysis bag was conducted under the same conditions.

2.6. Dynamic light scattering measurement

The particle size distribution of the block copolymer micelle was measured by dynamic light scattering (Precision Detectors PDDLs Light Scattering System, Precision Detectors, Bellingham, MA) tuned at a wavelength of 800 nm. Sample solutions passing through a $0.45\text{-}\mu\text{m}$ filter were transferred into the light scattering cells. The intensity autocorrelation was measured at a scattering angle of 90° at $25\text{ }^{\circ}\text{C}$. The CONTIN algorithms were used in the Laplace inversion of the autocorrelation function to obtain size distribution of micelles. The mean diameter was evaluated from the Stokes–Einstein equation.

2.7. HPLC analysis of paclitaxel

An isocratic reverse-phase HPLC was performed on an Agilent 1100 series HPLC system (Agilent Technologies, Wilmington, DE) using a Symmetry column (Waters, Milford, MA) at $25\text{ }^{\circ}\text{C}$. The mobile

phase consisted of acetonitrile–water (45:55 v/v) with a flow rate of 1.0 ml/min. A diode array detector was set at 227 nm and linked to ChemStation software for data analysis. The PTX concentrations in the samples were obtained using a calibration curve.

3. Results and discussion

3.1. Poly(ethylene glycol)-poly(phenylalanine) block copolymer (PEG-b-PPhe) micelles

PTX is extremely lipophilic and sparingly water-soluble, due to its cyclic components which are extremely hydrophobic. To make the optimum polymeric micelles as a micro-reservoir for PTX, we prepared an amphiphilic block copolymer to give a core composed of strong hydrophobic aromatic rings.

PEG-b-PPhe copolymers were synthesized by ring opening polymerization of Phe-NCA with PEG having a methoxy group at one terminal and a primary amino group at the other terminal, as shown in Fig. 1 [21–23]. The primary amino group attacks exclusively the carbonyl carbon (C-5) of Phe-NCA to initiate the polymerization. As the primary amino group is more nucleophilic than the active chain ends, initiation is faster than propagation. Therefore, all PEG should be incorporated into copolymerization [21–23]. The composition of the block copolymer was determined by the intensity ratio of the $^1\text{H-NMR}$ peaks of the methylene pro-

Table 1

Characteristics of poly(ethylene glycol)-block-poly(phenylalanine) (PEG-b-PPhe) micelles

M_n of PEG	M_n of PPA	Mean diameter (nm)		CMC ^a (μg/ml)	Loading amount (wt.%)	Loading efficiency (%)
		Before PTX loading	After PTX loading			
5000	1130	51.4 ± 4.0	55.1 ± 3.1	0.25	8.8	88

^a Critical micelle concentration (CMC) was determined by a fluorescence technique [26].

tons (OCH_2CH_2 , $\delta = 3.5$ ppm) of the PEG chain and the protons of the benzyl group (C_6H_5 , $\delta = 7.2$ ppm) of the PPhe chain. The molecular weight of the PPhe block was 1130 g/mol (Table 1). The size of the block copolymer micelles and their distribution in an aqueous medium were measured by dynamic light scattering. Fig. 2 shows the particle size distribution of the micelles. PEG-b-PPhe formed stable micelles in water with the mean diameter of 51.4 ± 4.0 nm.

3.2. Paclitaxel loading into PEG-b-PPhe micelles

PTX was incorporated into the block copolymer micelles by the solid dispersion method. The loading amount and efficiency was measured by HPLC after dissolving PTX-loaded micelles with acetonitrile. The loading amount was 8.8 wt.% and the loading efficiency was quite high at 88% (Table 1), indicating that PEG-b-PPhe makes a core structure as a micro-reservoir for PTX. The average size of the

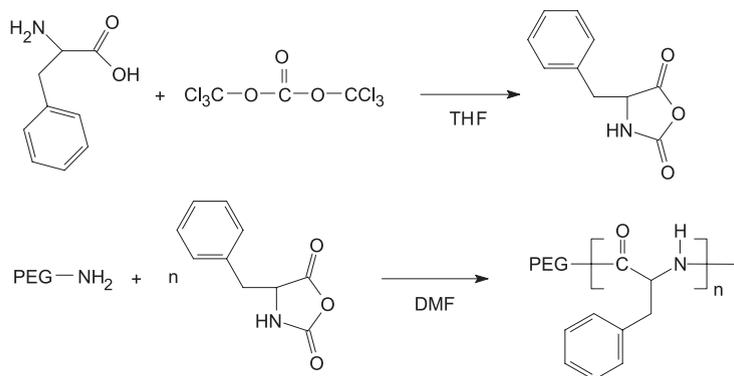


Fig. 1. Synthesis of poly(ethylene glycol)-block-poly(phenylalanine) (PEG-b-PPhe) copolymers.

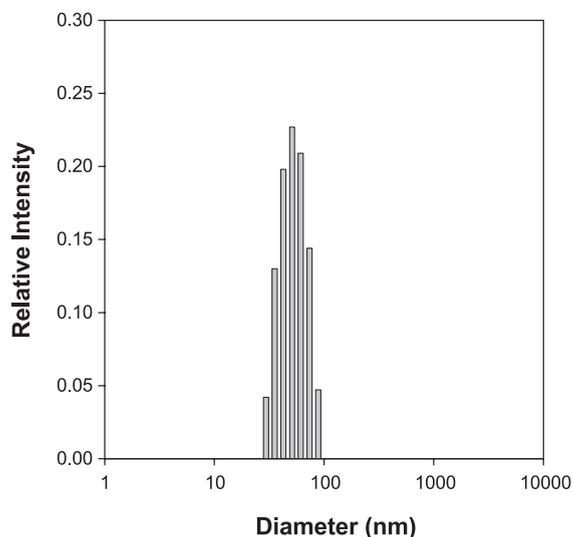


Fig. 2. Particle size distribution of poly(ethylene glycol)-block-poly(phenylalanine) (PEG-b-PPhe) copolymer micelles measured by light scattering.

micelle was slightly increased from 51.4 to 55.1 nm after PTX loading.

3.3. Effect of a hydrotropic agent on a PEG-b-PPhe micellar structure

Hydrotropic agents have been used to enhance aqueous solubility of hydrophobic drugs [17,24]. In many instances, the aqueous solubility was increased by orders of magnitude simply by mixing with hydrotropic agents in water. Hydrotropy is a collective molecular phenomenon describing an increase in the aqueous solubility of a sparingly water-soluble drug by addition of a relatively large amount of a second solute. Hydrotropic agents self-associate into loose non-covalent assemblies of non-polar microdomains to solubilize hydrophobic solutes. However, the detailed mechanisms of hydrotropy have not been fully understood.

The aqueous solubility of PTX is 0.3 $\mu\text{g/ml}$ at 37 $^{\circ}\text{C}$ [17]. Fig. 3 shows increased aqueous solubility of PTX by sodium salicylate, a hydrotropic agent chosen in this study. The PTX solubilities at 1 M and 3.5 M sodium salicylate were 28.1 $\mu\text{g/ml}$ and 5.54 mg/ml, respectively. The effect of sodium salicylate on PEG-b-PPhe copolymer micelles in

aqueous media was studied by dynamic light scattering analysis, and compared with that of SDS treatment of the micelle solution. Fig. 4A shows the size distribution of the PEG-b-PPhe micelles at 1.0 M (16.0 g/dl) sodium salicylate. The size distribution of the micelles in the 1.0 M sodium salicylate was not significantly changed from that of the micelles in pure water (Fig. 2). Fig. 4B–D shows changes in the size distribution of micelles by SDS treatment at different concentrations. Addition of a small amount of SDS (to make the final concentration of 0.025 g/dl) greatly affected the size distribution of micelles (Fig. 4B). Increase in the SDS concentration to 0.1 and 0.2 g/dl further altered the size distribution of the micelles. These concentrations were lower than the critical micelle concentration (CMC) of SDS in water, which is around 0.25 g/dl. As shown in Fig. 4B–D, new larger aggregates appeared in the presence of SDS below its CMC. The size and amount of the aggregates increased as the concentration of SDS increased. It appears that the SDS molecules interacted with the block copolymer micelles to destroy the micellar structure of PEG-b-PPhe, leading to formation of large aggregates with a diameter up to 1 μm .

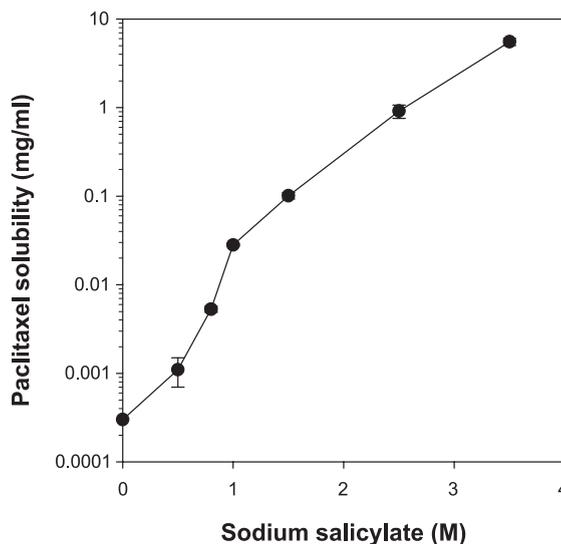


Fig. 3. Aqueous solubility of paclitaxel as a function of the molar concentration of sodium salicylate.

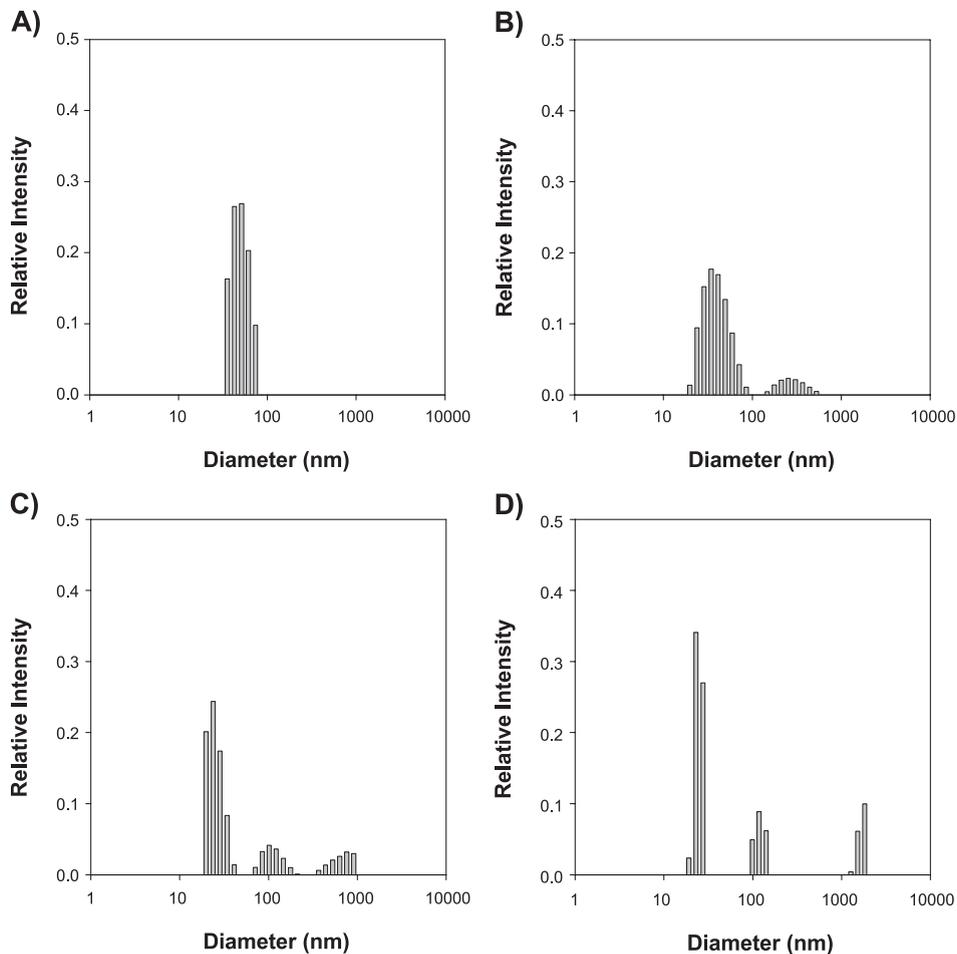


Fig. 4. Particle size distributions of poly(ethylene glycol)-block-poly(phenylalanine) copolymer micelles in (A) 1.0 M (16.0 g/dl) sodium salicylate solution; (B) 0.025 g/dl sodium dodecyl sulfate solution; (C) 0.10 g/dl sodium dodecyl sulfate solution; (D) 0.20 g/dl sodium dodecyl sulfate solution.

Changes in the mean diameter and concentration of the micelles as a function of the sodium salicylate concentration are shown in Figs. 5 and 6. The diameter of the block copolymer micelle was not significantly changed by the addition of sodium salicylate up to 1.25 M. However, the diameter markedly increased at 1.5 M sodium salicylate and micelles disappeared over 2.0 M sodium salicylate. The scattering photon counting number remained close to its original value in pure water as the concentration of sodium salicylate increased up to 1.0 M, indicating that the number of micelles was not decreased by the addition of sodium salicylate up

to 1.0 M. However, the photon counting was considerably decreased at 1.25 M sodium salicylate, implying that some micelles had dissociated. At 1.0 M sodium salicylate, the mean diameter and photon counting remained constant for 4 weeks of experimental time period, indicating that the micelles are stable at 1.0 M sodium salicylate.

Currently, the most widely used method for increasing the aqueous PTX solubility is to add surfactants to the aqueous release media. However, this method is not applicable for polymeric micelle systems because even a small amount of surfactants could destroy their micellar structure and distort their

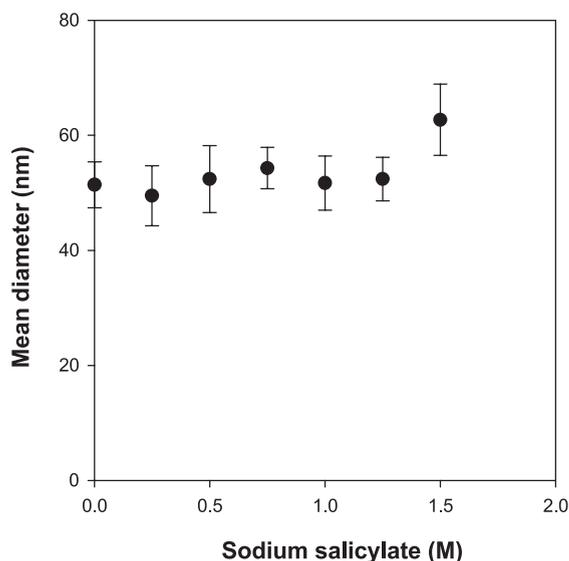


Fig. 5. Mean diameters of poly(ethylene glycol)-block-poly(phenylalanine) (PEG-b-PPhe) copolymer micelles at different sodium salicylate concentrations.

release profiles. A hydrotropic agent could be a good alternative to increasing the aqueous PTX solubility for in vitro PTX release studies from polymeric micelles.

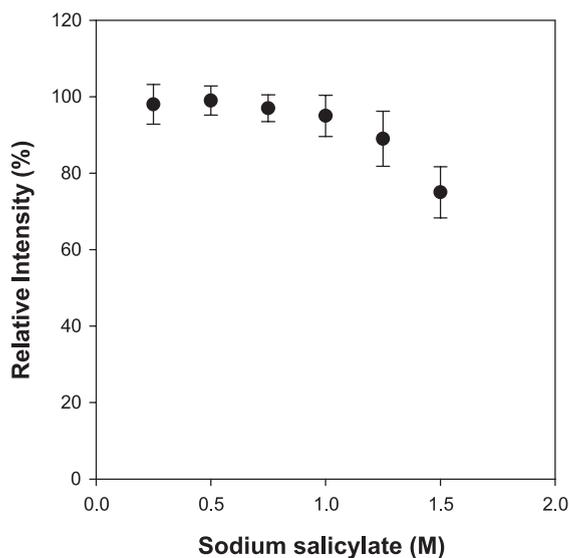


Fig. 6. Light scattering intensity of poly(ethylene glycol)-block-poly(phenylalanine) (PEG-b-PPhe) copolymer micelles measured at different sodium salicylate concentrations.

3.4. In vitro paclitaxel release from block copolymer micelles in a hydrotropic solution

Fig. 7 shows the cumulative PTX release profile from PEG-b-PPhe block copolymer micelles. PTX was continuously released from the block copolymer micelles in the aqueous medium containing 1 M sodium salicylate for 3 days. The cumulative release amount of PTX in 3 days was around 99%.

Maintaining a good sink condition for poorly water-soluble drugs has been one of the difficulties in designing in vitro release experiments. Continuous flow methods to provide infinite water to the release media have been designed to mimic a perfect sink condition [1,25]. In this method, the release media is removed and replaced infinitely rapidly and the instantaneous release rate is measured. The drug concentration is calculated from the integral of the release rate. However, this method is elaborate and not useful for poorly soluble drugs because of limitations in measurement of the drug concentration due to their low concentrations in the aqueous media.

We examined the PTX release behavior from polymeric micelles using *n*-octanol/water systems. However, polymeric micelles at the *n*-octanol/water interface lost their structure. Furthermore, PTX was degraded in *n*-octanol in a few hours, indicating that

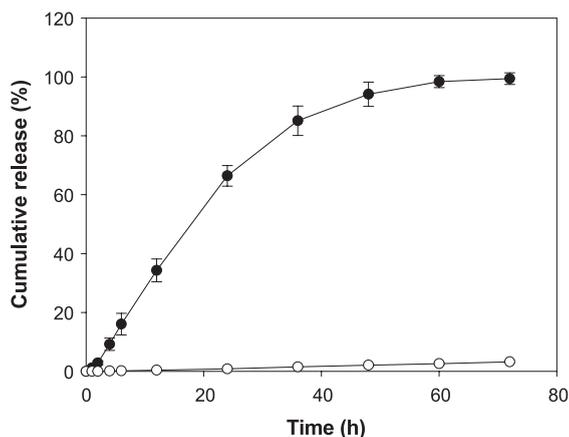


Fig. 7. A release profile of paclitaxel from poly(ethylene glycol)-block-poly(phenylalanine) (PEG-b-PPhe) copolymer micelles in 1.0 M sodium salicylate at 37 °C. (Closed circle: PTX release from the polymeric micelles. Open circle: PTX release from PTX powders. Cumulative release (%)=(Cumulative release amount/Loading amount) × 100 (%)).

n-octanol is not suitable for its release medium. Addition of a small amount of organic solvents, e.g., ethanol (5 v/v%), to the release media significantly altered the mean diameter of the micelles and their size distribution.

In this study, a good sink condition was achieved by using 1 M sodium salicylate solution that can solubilize more than 10 times the total amount of PTX incorporated in the micelles. Penetration of sodium salicylate into the dialysis bag may influence the release rate of PTX. To examine this possibility, release of PTX from the PTX particles across the dialysis membrane was carried out. Only 3.2% of PTX was released over a 72-h period, while almost all PTX was released from the micelle solutions during the same time period (Fig. 7). This result clearly indicates that the solubilizing effect of the release medium penetrating into the dialysis bag was minimal. Thus, the PTX release from the polymeric micelle solutions can be attributed to the solubilizing ability exhibited by the polymeric micelles.

Sodium salicylate, a hydrotropic agent, played an important role in examining the *in vitro* PTX release profiles from polymeric micelle systems. Sodium salicylate at 1.0 M of concentration increased the aqueous PTX solubility by two orders of magnitude without noticeable damage to the polymeric micelle systems. This experimental method using a hydrotropic agent provides an alternative tool for studying release of poorly soluble drugs from various polymeric micelle systems in aqueous solutions.

4. Conclusions

Sodium salicylate, a hydrotropic agent, significantly increased the aqueous PTX solubility without destroying the micelle structure of PEG-b-PPhe. This property made it easy to maintain a good sink condition for *in vitro* release studies of PTX from the polymeric micelle systems. PTX loaded into the PEG-b-PPhe micelles were continuously released from the polymeric micelles to the hydrotropic release medium during the 72-h period, while PTX was hardly dissolved and released from the solid PTX powders under the same condition. A hydrotropic agent is expected to be a useful tool for maintaining a good sink

condition for *in vitro* release studies of poorly soluble drugs from polymeric micelles.

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