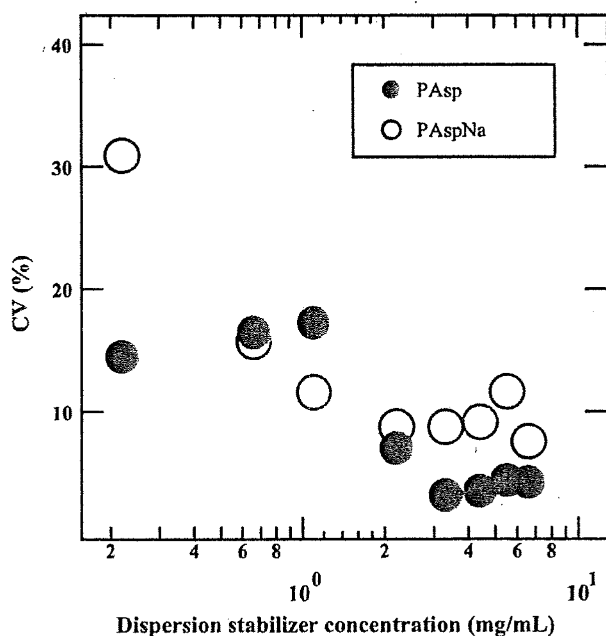

Fig. 7 Effect of dispersion stabilizer concentration on CV. Volume fraction of EtOH = 60 vol%

Both of the particle numbers decreased with time until the particle diameter became constant.

These results show that the number of particles synthesized with PAsp was higher than that synthesized with PAspNa. This is because the increase of hydrophobicity of dispersion stabilizer provided more loci for polymerization in the reaction solution. Since PAsp was more hydrophobic than PAspNa, the affinity of PAsp to EtOH was higher than that to PAspNa. These results also lead to the fact that higher polymerization rate used PAsp as a dispersion stabilizer.

Effect of EtOH volume fraction on average diameter and CV

Figure 4 shows the effect of the EtOH volume fraction in a polymerization medium on the average diameter when using PAspNa or PAsp. The EtOH volume fraction was between 0 and 70 vol.% with 3.4 mg/ml PAspNa or PAsp. The feed styrene monomer was completely dissolved in the EtOH/water solvent in which the EtOH content was above 40 vol.%, and the solution was homogeneous before polymerization.

The particle diameter obtained with PAspNa or PAsp increased with increasing EtOH volume fraction. There are two reasons for this result. One is that the amount of dissolved styrene was increased during EtOH volume fraction between 0 and 40 vol.%. Another reason is the critical chain length of styrene in precipitation increased with increasing EtOH volume fraction. The increasing critical chain length of styrene caused the reduction of the number of particle nuclei.

However, at higher EtOH volume fraction, microspheres synthesized with PAsp were smaller than those with PAspNa. This result concerned with the length of polymer chains in the reaction solvent. The solubility of PAsp into water was decreased as compared with that of PAspNa. In fact, however, PAsp dissolved in reaction solvent well. At the same time, PAsp had an affinity to organic solvents such as methanol and EtOH. Therefore, it was thought that PAsp solvated under condition of more spread own polymer chain at higher EtOH volume fraction. Thus, PAsp preserved the dispersion stability at higher EtOH volume fraction.

Figure 5 shows the effect of EtOH volume fraction in a polymerization medium on CV with PAspNa or PAsp. Polystyrene microspheres up to CV 10% were synthesized with PAspNa or PAsp under most conditions. At above 40 vol.% of EtOH volume fraction, the CV of prepared microspheres with PAsp was lower than those with PAsp. For the reason given above, PAsp preserved the higher dispersion stability under EtOH-rich conditions. Furthermore, the higher CV with PAspNa at 40 vol.% of EtOH was avoided using PAsp. Bimodal distribution of the particle diameter was observed when we prepared microspheres with PAsp at EtOH volume fraction of 40 vol%. We are not sure yet of the reason for the mechanism of the formation.

Table 2 The conditions for preparing monodisperse microspheres in EtOH/water-mixed solution with PAA, PAspNa, or PAsp as a dispersion stabilizer

Ingredients	PAA (M_w, 2.0×10^5) (non-biodegradable)	PAspNa (M_w, 6.0×10^4) (biodegradable)	PAsp (M_w, 4.25×10^4) (biodegradable)
Ethanol volume fraction in polymerization solvent (vol.%)	70	60	60
Concentration of dispersion stabilizer (mg/ml)	10.0	3.33	3.33
Concentration of monomer (mmol/ml)	1.93	2.89×10^{-1}	2.89×10^{-1}
Average diameter (μm)	1.80	1.69	0.91
CV (%)	5.8	7.7	1.7

Effect of dispersion stabilizer concentration on average diameter and CV

Figure 6 shows the effect of dispersion stabilizer concentration on particle diameter. In this study, the volume fraction of EtOH was fixed at 60 vol.%. The dispersion stabilizer concentration was varied from 2.2×10^{-1} to 6.7 mg/ml. This figure shows that the particle diameter decreased from approximately 2.0 to 1.4 μm by increasing the concentration of PAspNa. On the other hand, by changing the concentration of PAsp, the particle diameter decreased from approximately 1.5 to 0.9 μm. This is because the high initial stabilizer concentration causes the increasing amounts of stabilizer related to form particle nuclei in the initial stage of polymerization. Another reason is the enhancement of stabilizer absorbed on the nuclei and protected against aggregation processes in polymerization. In addition, smaller microspheres were prepared with PAsp as compared with PAspNa. This result indicated the PAsp worked as a dispersion stabilizer more effectively than PAspNa at 60 vol.% of EtOH.

Figure 7 shows the effect of dispersion stabilizer concentration on CV. The volume fraction of EtOH was 60 vol.%. The CV decreased with increasing dispersion stabilizer concentration. The monodisperse microspheres were prepared at the concentration more than 2.2 mg/ml. Thus, Figs. 6 and 7 indicate that we can control the particle diameter of monodisperse microspheres in the case of applying appropriate dispersion stabilizer to a reaction solution.

The effect of the relation between dispersion stabilizer and reaction solvent on forming monodisperse microspheres

Table 2 shows the conditions for preparing monodisperse microspheres in EtOH/water-mixed solution with several dispersion stabilizers [1]. The monodisperse microspheres with similar size were prepared using PAA or PAspNa as previously reported. PAspNa is biodegradable, and the concentration for preparing monodisperse microspheres is about one third of that of PAA.

In this study, we applied PAsp as a dispersion stabilizer to dispersion polymerization. Monodisperse microspheres were prepared at higher EtOH volume fraction, and the CV obtained was lower than that with PAspNa. Furthermore, smaller microspheres than that with PAspNa were prepared at higher EtOH volume fraction. However, at less than 40 vol.% of EtOH volume fraction, it was difficult to prepare monodisperse microspheres with PAsp. This suggested that, monodisperse microspheres were obtained when using the dispersion stabilizer having an affinity to a reaction solvent.

Conclusion

We synthesized PAsp by ion exchange of PAspNa. We applied PAsp as a dispersion stabilizer to dispersion polymerization. The monodisperse microspheres were obtained with PAsp as a dispersion stabilizer. It suggested that PAsp acts as a dispersion stabilizer for styrene polymerization. In addition, the polymerization rate of styrene with PAsp was higher than that with PAspNa. The particle diameter with PAsp increased as the EtOH volume fraction increased. At high EtOH volume fraction, microspheres prepared with PAsp were smaller than those with PAspNa. Consequently, we obtained monodisperse microspheres by using PAsp at higher EtOH volume fraction.

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SHORT COMMUNICATION

Synthesis of polyaspartate macromonomer having a vinyl end group and application to dispersion copolymerization of styrene

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Abstract Sodium polyaspartate (PAspNa) macromonomer with an acryloyl end group was synthesized for dispersion polymerization. At first, a poly(succinimide) (PSI) derivative with a hydroxyphthalimide end group was synthesized by polycondensation of L-aspartic acid and 4-hydroxyphthalic acid. Then, the PSI derivative was end-capped with an acryloyl group by a reaction with acrlyloyl chloride. Finally, a PAspNa derivative with a vinyl end group was synthesized by a hydrolysis of succinimide units by sodium hydroxide. The synthesized macromonomer was applied as a polymerizable stabilizer in dispersion copolymerization of styrene in a mixture of ethanol and water. The PAspNa macromonomer acted as an effective stabilizer and gave sub-micron-sized polymeric particles in dispersion polymerization in polar medium.

Keywords Macromonomer · Polyaspartate · Particle · Dispersion polymerization

Introduction

Particles having a hydrophobic core and a hydrophilic layer are called hairy particles. A layer of concentrated polymer chains is formed on these particle surfaces and provides high stability and functionality. Hairy particles with well-designed functionality are promising for the applications such as affinity particles, drug delivery carriers, and catalyst. Therefore, the design of functionality of the hairy chains is a key factor for the particle usage in these applications.

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Generally, there are two methods to prepare hairy particles from existing core particles: (1) "grafting onto" method [1–3], where end-functionalized polymer chains are coupled with core particles, and (2) "grafting from" method [4–11], where hairy chains are grown from the surface of core particles modified with initiators. Especially, surface-initiated living radical polymerization has been used in recent years by a lot of researchers to prepare hairy particles because this method produces hairy chains with controlled chain length on the particle surface. However, multistep reactions are required to produce the core particles modified with initiators.

Heterogeneous polymerization with macromonomer is an alternative for preparing hairy particles. Heterogeneous polymerization, such as emulsion polymerization and dispersion polymerization, is a one-pot polymerization method to obtain polymeric particles in nano- to micro-scale, and the particle diameter can be controlled by changing the reaction parameters. Polymeric particles prepared by dispersion copolymerization [12–27] or emulsion copolymerization [26–34] with macromonomer have high stability and functionality derived from anchoring of hydrophilic polymer chains on the particle surface. Almost all studies used the macromonomer based on poly(ethylene oxide) (PEO) in the heterogeneous polymerization [13, 17, 20–22, 24–26, 29, 31–34] because of the high solubility in various solvents. However, the functionalization of PEO chains is difficult owing to chemical stability of the ethylene oxide units. Thus, the heterogeneous polymerization with PEO macromonomer is not suitable to design the functionality of hairy chains.

Sodium polyaspartate (PAspNa), a hydrophilic biodegradable polymer, can easily accept the introduction of

functional groups into the side chains. PAspNa is derived from poly(succinimide) (PSI), product from the polycondensation of L-aspartic acid, by hydrolysis with sodium hydroxide [35]. PSI reacts with various amine compounds without any coupling agent. Therefore, PAspNa derivatives with various functional pendant groups are easily designed [35–38].

In this study, we synthesized PAspNa macromonomer with an acryloyl end group and applied to prepare functionalized hairy particles. The outline of the synthesis route is shown in Scheme 1. At first, a PSI derivative with a hydroxyphthalimide end group (1) was synthesized by polycondensation of L-aspartic acid and 4-hydroxyphthalic acid. Then, 1 was end-capped with an acryloyl group to obtain a PSI derivative with a vinyl end group (2). Finally, PAspNa macromonomer (3) was synthesized by a hydrolysis of 2. The synthesized PAspNa macromonomer was used as a polymerizable stabilizer in dispersion copolymerization of styrene to prepare the polymeric particles. A PAspNa derivative without vinyl end group was also synthesized in order to compare the results obtained between macromonomer and nonpolymerizable stabilizer.

Experimental

Materials

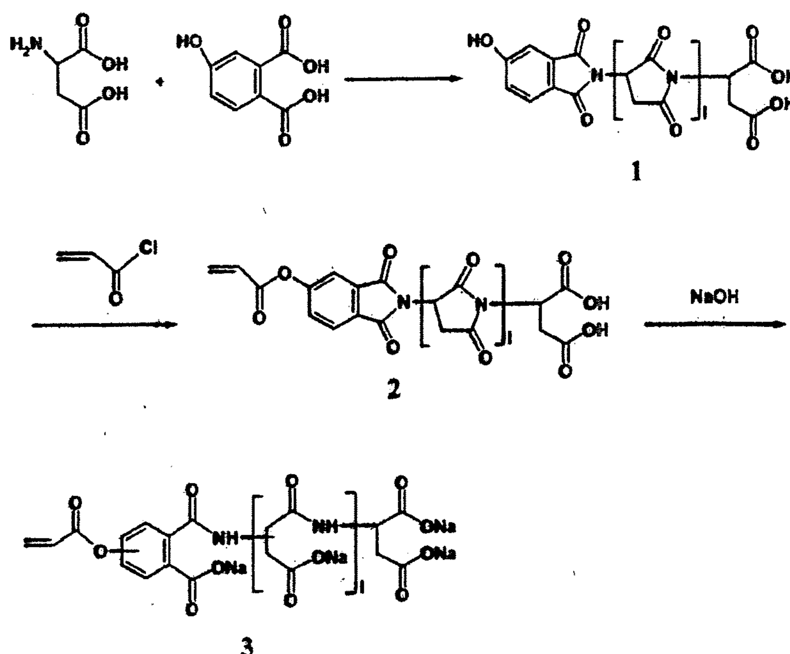
All materials were obtained from Wako Pure Chemical Industries. *N,N*-Dimethylformamide (DMF) was dehydrated by adding dried molecular sieves. Styrene was purified by distillation under reduced pressure. 2,2′-Azobisisobutyronitrile (AIBN) was purified by recrystallization from ethanol. Other materials were used without further purification. Water was purified by a Millipore Milli-Q purification system.

Synthesis of PSI derivative with a hydroxyphthalimide end group (1)

A typical procedure for the synthesis of 1 is as follows: 23.4 g of L-aspartic acid (Asp), 1.19 g of 4-hydroxyphthalic acid (HPA), 9.94 g of phosphoric acid, and 1.81 g of water were mixed in a flask. The flask was placed in a rotary evaporator and heated at 453 K for 7 h under reduced pressure. The product was dissolved in 300 ml of DMF, and the solution was poured into 2 l of methanol. The precipitate was washed three times with methanol and three times with water. The resultant polymer was dried in a vacuum at 313 K to obtain the 1 (15.0 g, 83%).

Synthesis of PSI derivative with a vinyl end group (2)

1 (3.0 g) was dissolved in 30 ml of dry DMF, and more than ten excess of acryloyl chloride (AC) and more than ten excess of triethylamine (TEA) were added to the solution. The mixture was stirred at room temperature for 24 h and then poured into 300 ml of methanol. The precipitate was washed six times with methanol and three times with water. The resultant polymer was dried in a vacuum at room temperature to obtain the 2 (2.68 g, 88%).



Scheme 1 Synthesis of PAspNa derivative with a vinyl end group

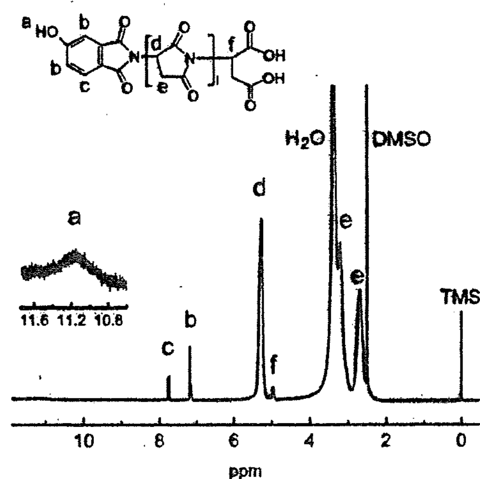


Fig. 1 ^{1}H NMR spectrum of 1 in DMSO-d_6

Synthesis of PAspNa derivative with a vinyl end group (3)

2 (2.5 g) was dispersed in 100 ml of water, and 1 N sodium hydroxide (NaOH) solution was added dropwise so as not to exceed pH 10 in the solution. The solution was then neutralized by 1 N hydrochloric acid solution and concentrated under reduced pressure. The solution was recrystallized from methanol. The precipitate was dried in a vacuum to obtain the 3 (3.48 g, 99%).

Synthesis of a PAspNa derivative without a vinyl end group (3′)

3′ was synthesized from 1 and 1 N NaOH solution using the same method for 3 (3.53 g, 99%).

Dispersion copolymerization

Dispersion copolymerization was carried out in a reactor equipped with a reflux condenser and a magnetic stirrer and placed in an oil bath equipped with a temperature control. A typical procedure for dispersion copolymerization of styrene with 3 is presented below: 0.107 g of AIBN and 1.34 g of styrene were dissolved in ethanol 27 ml, and it was added into 18 ml of aqueous solution containing of 0.10 g of 3. The mixture was polymerized in the reactor at 343 K for 6 h under nitrogen atmosphere. The resultant particles were refined by centrifugating washes with water three times.

Measurements and characterization

^{1}H NMR spectra were measured using a NMR spectrometer (JEOL AL300 SC-NMR). Weight-average molecular weight (M_w) and molecular weight distribution (M_w/M_n) of a PSI derivative were determined by a gel permeation chromatography (TOSOH HLC-8120 GPC system) using polystyrene standards with DMF as an eluant. The flow rate and the column temperature were 0.6 ml/min and 313 K, respectively. The particle diameter and the diameter distribution were determined by scanning electron microscopy (SEM, Hitachi S-4700). The number-average particle diameter was obtained by counting 200 particles in SEM photographs. Coefficient of variation of the particle diameter was calculated using the following equation:

$$CV(\%) = \frac{\text{Standard derivation } (\mu m)}{\text{Number} - \text{average particle diameter } (\mu m)} \times 100$$

Styrene monomer conversion was calculated from the unreacted styrene monomer concentration, which is measured by high-performance liquid chromatography. Small amount of resultant latex was added to methanol with 4-*t*-butylpyrocatechol to terminate polymerization. These solutions were centrifuged at 30,000 rpm for 15 min to remove the particles. Unreacted styrene concentration in the supernatant was measured by high-performance liquid

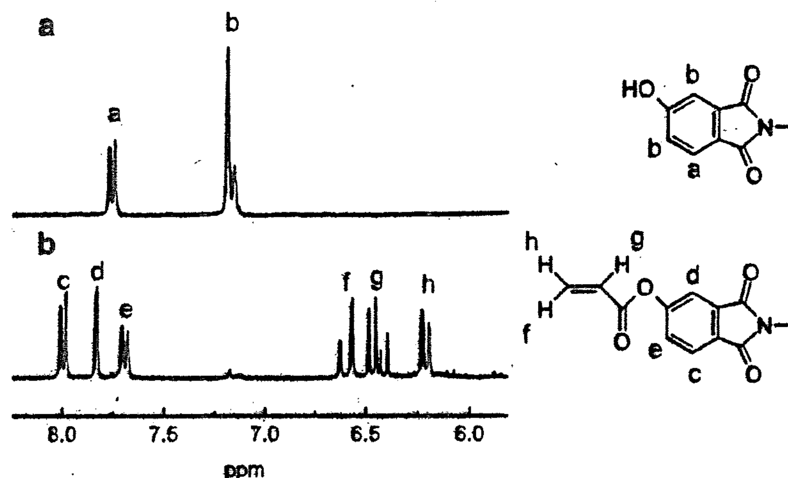
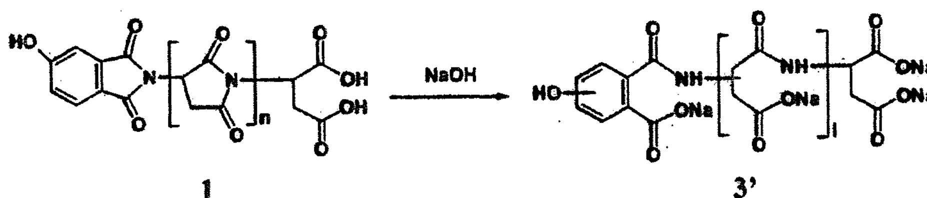


Fig. 2 ^{1}H NMR spectra 1 (a) and 2 (b) in DMSO-d_6

Scheme 2 Synthesis of PaspNa derivative without a vinyl end group



chromatography (SHIMADZU Prominence HPLC system) with a UV–VIS detector (SPD-M20A, λ=254 nm) with the mixture of methanol/water=7:3 (vol/vol) as an eluant. The column was a TSK-Gel ODS-80Ts QA (150×4.6 mm, TOSOH). The flow rate and the column temperature were 0.8 ml/min and 313 K, respectively.

Results and discussion

A PSI derivative with a hydroxyphthalimide end group (1) was synthesized by the bulk polycondensation of Asp and HPA in the presence of phosphoric acid. The polymer with M_w of 7,700 was obtained, and the distribution was broad (M_w/M_n=4.2). Figure 1 shows the ^{1}H NMR spectrum of 1 in deuterated dimethyl sulfoxide (DMSO-d_6). The peak at 4.9 ppm is assigned to a methine proton of the succinic acid end group, and the peaks at 7.2 and 7.8 ppm are assigned to aromatic protons of the phthalimide end group. The end functionality of 1 was estimated from the integration value ratio at 4.9 and 7.8 ppm. The number-average molecular weight (M_n) was calculated by the integration values of a peak at 4.9 and 5.3 ppm for a methine proton of the succinimide unit. The hydroxyphthalimide end functionality (f_{OH}) and M_n estimated by ^{1}H NMR spectrum were 98% and 3,400, respectively. A PSI derivative with a vinyl end group (2) was synthesized by the reaction of hydroxyphthalimide end group of 1 and AC in the presence of TEA. Expanded region of the ^{1}H NMR spectra of 1 and 2 are shown in Fig. 2. This figure shows that the peaks at 7.2 ppm disappeared as well as three peaks at 6.2~6.7 ppm and three peaks at 7.7~8.0 ppm in ^{1}H NMR spectrum of 2. These peaks were assigned to the vinyl and aromatic protons of the acryloxyphthalimide group, respectively. Vinyl end functionality (f_{vinyl}) of 2 was calculated by follow equation:

$$f_{vinyl} = \frac{I_{6.3}/3}{I_{6.3}/3 + I_{7.2}/2} \times f_{OH}$$

where I_i is the integration value of the peak at i ppm in ^{1}H NMR spectrum of 2. 2 with 91% of f_{vinyl} was obtained by the reaction with a large excess of AC. Hydrolysis of succinimide units in 2 by NaOH solution produced PAspNa macromonomer with a vinyl end group (3). The hydrolysis of succinimide units was confirmed by disappearance of the peak at 5.3 ppm and appearance of the peaks at 4.5 and 4.7 ppm for methine protons of an aspartic acid unit in ^{1}H NMR spectrum in deuterium oxide (data not shown). A PAspNa derivative without a vinyl end group (3′) was synthesized by the hydrolysis of 1 (Scheme 2).

Dispersion (co)polymerization using 3 or 3′ as a dispersion stabilizer in a mixture of ethanol and water were carried out. By dispersion polymerization using 3′, polymer colloid was obtained in 78% conversion; however, much coagulum was also formed. On the other hand, polymer colloid with no coagulum was obtained in 88% conversion by dispersion copolymerization with 3. Figure 3 shows the SEM images of the particles prepared using macromonomer 3 and nonpolymerizable stabilizer 3′. Particle diameter of the particles prepared using 3′ and 3 were 2.33 and 0.407 μm, respectively. Macromonomers are chemically anchored on the particle surface during dispersion poly-

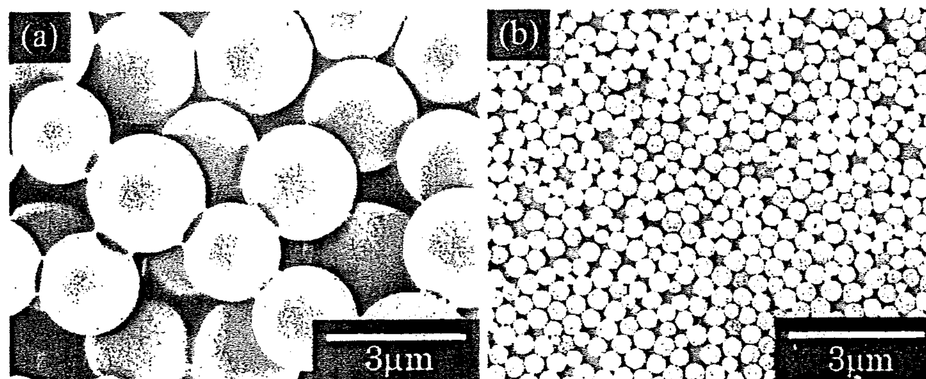


Fig. 3 SEM images of the particles prepared by dispersion (co)polymerization of styrene with PAspNa derivatives: a nonpolymerizable stabilizer (3′); b macromonomer (3). [3 or 3′]=2.22 g/l

merization and provide high dispersion stability [14]. Thus, the particles prepared using **3** were much smaller than those prepared using **3'**. The comparison results indicated that the PAspNa macromonomer is an effective stabilizer in dispersion polymerization in polar medium.

Conclusion

A PSI derivative with a hydroxyphthalimide end group was synthesized by bulk polycondensation of Asp and HPA in the presence of phosphoric acid. This polymer was reacted with AC in the presence of TEA and hydrolyzed by NaOH solution to obtain PAspNa macromonomer with an acryloyl end group. Sub-micron-sized polymeric particles were obtained by dispersion copolymerization of styrene and PAspNa macromonomer in a mixture of ethanol and water. These particles obtained were smaller than those prepared using a PAspNa derivative without polymerizable group as a dispersion stabilizer.

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Preparation of Monodisperse Polylactide Microspheres by Dispersion Polymerization Using a Polymeric Stabilizer with Hydroxy Groups

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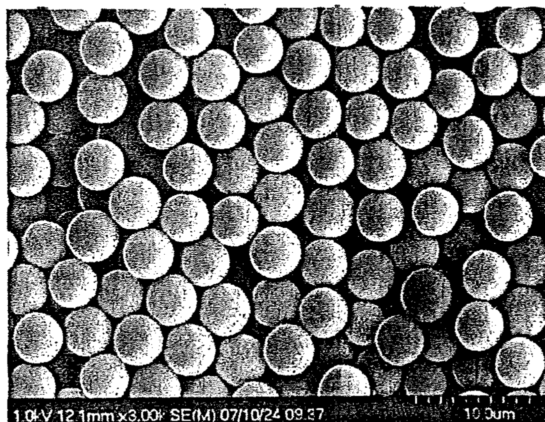
Monodisperse poly(D,L-lactide) (PDLLA) microspheres have been prepared by dispersion polymerization of D,L-lactide with a synthetic polymeric stabilizer. The polymerization is carried out in xylene/heptane (1:2, v/v) at 368 K for 3 h with poly[(dodecyl methacrylate)-*co*-(2-hydroxyethyl methacrylate)] (P(DMA-*co*-HEMA)). P(DMA-*co*-HEMA) has hydroxy groups as an initiation group for pseudoanionic dispersion polymerization. The particle diameter and the coefficient of variation concerning the diameter distribution of the obtained PDLLA microspheres are 3.9 μm and 4.3%, respectively. In addition, from the results of dynamic light scattering measurements, it is found that P(DMA-*co*-HEMA) and the PDLLA-grafted copolymer form a micellar structure in solution.



Introduction

Biodegradable polymeric microspheres have been developed for the chemical industry, e.g., for coatings, inks, adhesives, and controlled drug delivery systems.[1–4] Polylactide (PLA), a typical biodegradable polymer, shows good mechanical properties, biodegradability, and biocompatibility to use in those areas.[5–7] PLA microspheres are mainly prepared by solvent evaporation and spray drying techniques.[8–11] However, these techniques have drawbacks such as the requirement of a multi-step preparation process and polydispersity of particle diameter. Alternatively, dispersion polymerization is well known to provide monodisperse polymeric microspheres that range from submicrometer to 15 μm. The dispersion polymerization is carried out in a reaction medium that dissolves monomer, but does not dissolve the polymer. The polymerization proceeds in the reaction medium until a critical molecular weight for the solubility of the polymer chain, and then the precipitated primary particles are stabilized by a polymeric stabilizer. Slomkowski et al. reported that poly(D,L-lactide) (PDLLA) (or poly(L,L-lactide, PLLA) microspheres with a narrow size distribution, and a coefficient of variation (CV) of 11.5%, were prepared by dispersion polymerization of D,L (or L,L)-lactide in 1,4-dioxane/heptane (1:4, v/v) using poly(dodecyl acrylate)-*graft*-poly(ε-caprolactone) as a polymeric stabilizer.[12–14] We also reported that PDLLA microspheres with a narrow

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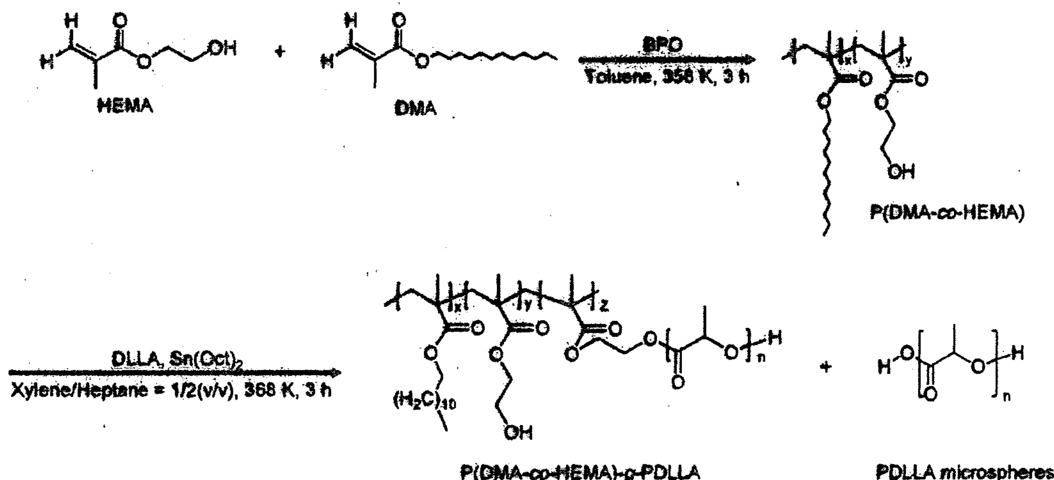

Scheme 1. Synthesis of P(DMA-*co*-HEMA) and preparation of the PDLLA microspheres.

diameter distribution, and a CV of 14.1%, were prepared by dispersion polymerization of D,L-lactide in xylene/heptane (1:2, v/v) using the copolymer grafted with PLLA, poly(dodecyl methacrylate)-*graft*-poly(L,L-lactide), as a polymeric stabilizer.[15] However, monodisperse PDLLA microspheres (less than 10% of CV) have not been prepared.

In this study, we designed a new polymeric stabilizer to prepare monodisperse PDLLA microspheres by dispersion polymerization of D,L-lactide. The stabilizer plays a critical role in the primary particle formation stage. A block or graft copolymer that contains both soluble and insoluble polymer segments for a solvent has been used as a stabilizer.[16,17] Winnik et al. showed the existence of regular micelles comprised of several hundred diblock copolymer molecules and the micelle clusters corresponded to the aggregation of tens of micelles in the aqueous solution by dynamic light scattering measurements.[18] The equilibrium state between polymeric micelles and unimers affects the adsorption rate of copolymeric stabilizer on the surface of primary particles. Thus, the mechanism of particle formation in the dispersion polymerization is complex and poorly understood when using a diblock copolymer as a dispersion stabilizer, and it is difficult to prepare monodisperse PDLLA microspheres. On the other hand, a graft copolymer is produced in situ when a precursor polymer that contains active sites for the chain transfer of radicals, such as hydroxypropyl cellulose, poly(acrylic acid), and poly(vinyl pyrrolidone) (PVP), is employed as a stabilizer in the dispersion polymerization of styrene.[19–21] Since these polymeric stabilizers are able to ignore the formation of micelles in the solution in an initial stage, it is promising to prepare monodisperse polystyrene microspheres.

Our idea is motivated by the case of the graft copolymeric stabilizer produced in situ, and thus we synthesized poly[(dodecyl methacrylate)-*co*-(2-hydroxyethyl methacrylate)] (P(DMA-*co*-HEMA)) to prepare monodisperse PDLLA microspheres (Scheme 1). P(DMA-*co*-HEMA) has hydroxy groups as an initiation group for pseudoanionic dispersion polymerization, which leads to the formation of the copolymer grafted with PDLLA, P(DMA-*co*-HEMA)-*g*-PDLLA. This paper describes the preparation of monodisperse PDLLA microspheres by dispersion polymerization of D,L-lactide using P(DMA-*co*-HEMA) as a polymeric stabilizer.

Experimental Part

Materials

D,L-Lactide (Purac) was purified by recrystallization from toluene. 2-Hydroxyethyl methacrylate (HEMA), dodecyl methacrylate (DMA) (Wako Pure Chemical Industries, Ltd.), and tin(II) 2-ethylhexanoate ($Sn(Oct)_2$, Aldrich)[22] were purified by distillation under reduced pressure. Xylene and heptane (dehydrated grade, Wako Pure Chemical Industries, Ltd.) were stored in a glove box filled with argon gas. Other reagents (Wako Pure Chemical Industries Ltd.) were used as received.

Measurements

Gel permeation chromatography (HLC 8120, Tosoh, GPC) was performed on the basis of polystyrene standards with tetrahydrofuran (THF) as an eluent to determine the weight-averaged molecular weight ($\overline{M}_w$) and polydispersity index ($\overline{M}_w/\overline{M}_n$) of the synthesized polymer. ^{1}H NMR (AL300 SC-NMR, JEOL) spectroscopy was conducted using $CDCl_3$ that contained tetramethylsilane (TMS, 1%, v/v) as an internal standard to determine the molecular structure of the synthesized polymer. Scanning electron micro-

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scopy (S-4700, Hitachi, SEM) was performed to determine the particle diameter (d_p) and the diameter distribution (CV) of the prepared PDLLA microspheres. Dynamic light scattering measurement (FPAR-1000, Otsuka Electronics Co., DLS) was carried out at 293 K to determine the hydrodynamic diameter (R_h) of the micelles composed of the copolymeric stabilizers in xylene/heptane (1:2, v/v).

P(DMA-*co*-HEMA) Synthesis

P(DMA-*co*-HEMA) was synthesized according to Scheme 1. DMA (31.18 mmol), HEMA (0.62 mmol), and 24 mL of dehydrated toluene as a solvent were placed into a round-bottom reactor. After nitrogen was admitted to remove oxygen, the reactor was immersed in an oil bath at 358 K. Benzoyl peroxide (BPO, 1.25 mmol) dissolved in dehydrated toluene was added to initiate the polymerization. The polymerization was conducted for 3 h. After cooling, the reaction mixture was poured into excess methanol to remove the remaining DMA. After the purification, the obtained polymer was dried under reduced pressure at 313 K.

PDLLA Microspheres Preparation

PDLLA microspheres were prepared according to Scheme 1. D,L-Lactide (3,47 mmol) was added to 17 mL of a solution of P(DMA-*co*-HEMA) dissolved in dehydrated xylene/heptane (1:2, v/v). The solution was stirred at 120 rpm with a magnetic stirrer. $Sn(Oct)_2$ (0.12 mmol) dissolved in a solution of dehydrated xylene and heptane (3 mL, 1:2, v/v) as a catalyst was prepared. The solution was added by a syringe and the polymerization was conducted at 368 K for 3 h. After the polymerization, the reaction solution was poured into excess cold heptane. The solution was centrifuged for 3 min at 6 000 rpm, and the microspheres were redispersed into excess heptane. The solution was filtered to collect the obtained microspheres.

Results and Discussion

P(DMA-*co*-HEMA) Synthesis

P(DMA-*co*-HEMA) ($\overline{M}_w = 40\,000$, $\overline{M}_w/\overline{M}_n = 2.48$) was successfully obtained by free radical copolymerization of DMA and HEMA using BPO as an initiator. The ^{1}H NMR spectrum of P(DMA-*co*-HEMA) is shown in Figure 1a. The spectrum shows peaks at 3.9 ppm ($COOCH_2$ for DMA unit) and 4.1 ppm ($COOCH_2$ for HEMA unit). Furthermore, the peaks at around 5.6 and 6.1 ppm (double bond for DMA and HEMA) were not detected in the spectrum. Therefore, P(DMA-*co*-HEMA) was identified. The number of HEMA units in P(DMA-*co*-HEMA) ($N_{HEMA} = 2.0$) was calculated from the ^{1}H NMR spectrum using the integration ratios that correspond to around 3.9 ppm ($COOCH_2$ for DMA unit) and 4.1 ppm ($COOCH_2$ for HEMA unit).[23]

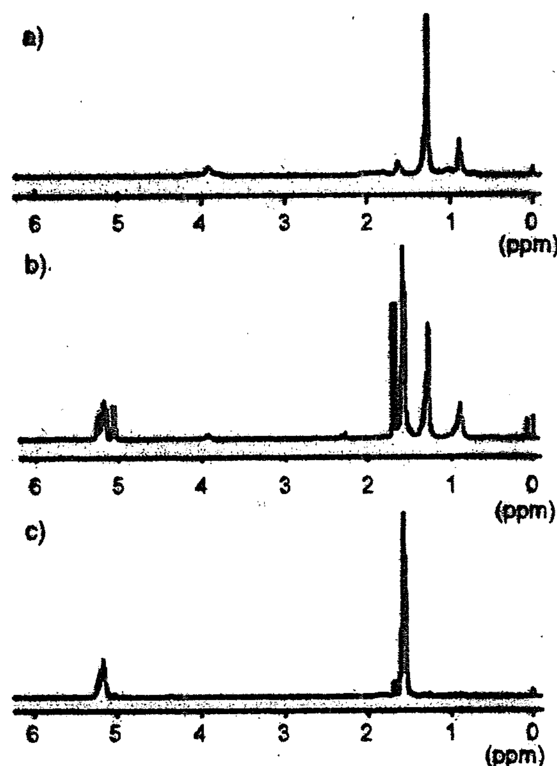

Figure 1. ^{1}H NMR spectra of a) P(DMA-*co*-HEMA), b) PDMA-*g*-PDLLA, and c) PDLLA microspheres.

PDLLA Microspheres Preparation

Figure 2 shows SEM images of PDLLA microspheres prepared by dispersion polymerization with different concentrations of P(DMA-*co*-HEMA). By observing the spherical particles, it was clear that P(DMA-*co*-HEMA) played a critical role as a dispersion stabilizer. In addition, from Figure 1b, the ^{1}H NMR spectrum of the polymeric

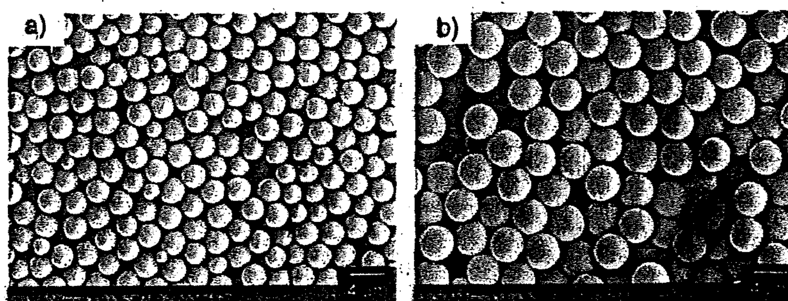

Figure 2. SEM images of monodisperse PDLLA microspheres prepared with different concentrations of P(DMA-*co*-HEMA): a) [P(DMA-*co*-HEMA)] = 250 mmol · L^{-1} (d_p = 2.7 μm, CV = 8.3%), b) [P(DMA-*co*-HEMA)] = 63 mmol · L^{-1} (d_p = 3.9 μm, CV = 4.3%).

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stabilizer after polymerization showed peaks for both DMA and PLA (CH_3; 1.7 ppm, CH; 5.2 ppm). Furthermore, the peak at 4.1 ppm ($COOCH_2$ for HEMA unit) was not detected. Therefore, the graft copolymer, PDMA-*g*-PDLLA, was produced in situ by polymerization of D,L-lactide from all the hydroxy groups in P(DMA-*co*-HEMA). According to the ^1H NMR spectrum of the purified PDLLA microspheres, only two peaks are observed at 1.7 and 5.2 ppm (CH_3 and CH for PLA), and the peaks of PDMA-*g*-PDLLA were not detected (Figure 1c). In the dispersion polymerization of styrene or methyl methacrylate using a conventional stabilizer, PVP, the existence of a graft copolymer on the resultant particle surface has been confirmed by FT-IR and X-ray photoelectron spectroscopy.[24,25] Therefore, it was suggested that the adsorption of the graft copolymeric stabilizer on the particle surface was low in the case of the dispersion polymerization of D,L-lactide using P(DMA-*co*-HEMA) as a dispersion stabilizer. The particle diameter of the PDLLA microspheres decreased with increasing P(DMA-*co*-HEMA) concentration. This tendency of particle size control has been reported by other researchers.[24,25] In addition, monodisperse PDLLA microspheres ($d_p = 3.9$ μm, CV = 4.3%) were obtained when using 63 mmol · L^{-1} P(DMA-*co*-HEMA).

The hydrodynamic diameter (R_h) of P(DMA-*co*-HEMA) was estimated by DLS measurement to investigate the presence of P(DMA-*co*-HEMA) micelles and the micelles comprised of the graft copolymers derived from P(DMA-*co*-HEMA) in xylene/heptane (1:2, v/v). The results of the DLS measurements are shown in Figure 3. P(DMA-*co*-HEMA) exhibited a bimodal size distribution that consists of the unimers and the polymeric micelles (formed by self-aggregation) (Figure 3a). The average diameters of the unimers and the polymeric micelles were about 1.3 and 8.8 nm, respectively. In addition, it was found that most of P(DMA-*co*-HEMA) were in a unimer state in the solution. Thus, the presence of P(DMA-*co*-HEMA) unimers during the initial stage would be a contributing factor to prepare monodisperse PDLLA microspheres. By producing graft copolymers in situ, the single size distribution based on the polymeric micelles appeared (Figure 3b). The average diameter was about 43.8 nm, which was almost same as

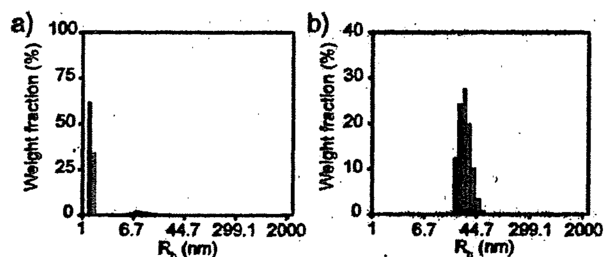

Figure 3. Size distributions of aggregates of the polymeric stabilizer in xylene/heptane (1:2, v/v): a) before the polymerization, b) after the polymerization, [P(DMA-*co*-HEMA)] = 250 mmol · L^{-1}.

that obtained with dextran grafted with PLLA.[26] This result indicates that the equilibrium state between the polymeric micelles and unimers in the solution influences the particle diameter and the distribution of PDLLA microspheres. Moreover, it was proposed that the molecular structure of P(DMA-*co*-HEMA) strongly affected the equilibrium state, which was important to prepare monodisperse PDLLA microspheres.

Conclusion

We synthesized P(DMA-*co*-HEMA) that contained hydroxy groups and used it as a stabilizer in the dispersion polymerization of D,L-lactide. From the results, PDLLA microspheres in the size range of 2.7–3.9 μm were obtained using P(DMA-*co*-HEMA). In particular, when using 63 mmol · L^{-1} P(DMA-*co*-HEMA), monodisperse PDLLA microspheres ($d_p = 3.9$ μm, CV = 4.3%) were obtained. In addition, most of P(DMA-*co*-HEMA) showed a unimer state in the solution. It produced a graft copolymer in situ during the polymerization and formed polymeric micelles (by self-aggregation). Therefore, it is supposed that the molecular structure of P(DMA-*co*-HEMA) affects the equilibrium state between the polymeric micelles and unimers in the solution, which would be a contributing factor to prepare monodisperse PDLLA microspheres.

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Release Behavior from Hydrogen-Bonded Polymer Gels Prepared by Pressurization

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ABSTRACT: Our previous research showed that a simple ultra-high-pressure process made poly(vinyl alcohol) (PVA) solution into a macrogel and nanoparticles. To investigate the release properties of PVA hydrogels prepared by the ultra-high-pressure treatment, we prepared hydrogels containing model drugs by pressurizing a PVA solution with Alfa-G Hesperidin or Oil Blue N as a water-soluble or an oil-soluble model drug, respectively. In the case of the oil-soluble drug, an oil-in-water emulsion, Oil Blue N containing dodecane in a PVA solution, was used by homogenization before pressurization. The average diameter and the diameter distribution of oil droplets before and after the ultra-high-pressure treatment were almost the same. However, the PVA hydrogel prepared at 10,000 atm for 10 min exhibited the slowest release rate of model drugs. Thus, we found that the release rates of the model drugs from the PVA hydrogels were controlled by the degree of crosslinking in the resulting gels, which was determined from the operation parameters of the ultra-high-pressure treatment, such as the pressure, time, and concentration of the PVA solution. Therefore, an ultra-high-pressure process is promising for drug-carrier development because of the nonharmful simple preparation process. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 000: 000–000, 2009

Key words: diffusion; gels; hydrogels; hydrophilic polymers

Author Proof

INTRODUCTION

A considerable number of studies have been devoted to the study of the molecular assembly technology in the material processing field, such as in molecular machines,[1] carbon nanotubes for biological systems[2] and circuit wires,[3,4] and drug carriers.[5,6] Molecular assembly is organized by noncovalent bonding, such as electrostatic interaction, van der Waals forces, and hydrogen bonding. It is important for the formation of molecular assembles to control these interactions by changing intensive variables.[7–9] Therefore, we showed that pressure must also be available to control the intermolecular forces to generate molecular assembles. We found that a poly(vinyl alcohol) (PVA) solution turned into a macrogel or nanoparticles through a simple ultra-high-pressure process (10,000 atm for 10 min).[10] Our results demonstrate that ultra-high pressure induces hydrogen bonding in water that is strong enough to maintain microassemblies, such as gels and particles. In addition to the formation of gels and nanoparticles, we found that the swelling ratio of the gels and the size of nanoparticles were easily controlled by the operative parameters in an ultra-high-pressure process. Furthermore, the macrogel prepared by this process indicated discriminating elasticity, which was never seen in the conventional PVA hydrogels.

Hydrogels are used in a wide variety of applications as a soft material, including in soft contact lenses,[11] shock absorption materials,[12] and drug carriers.[13,14] So far, several articles have been written on the drug-release behavior from PVA hydrogels prepared by physical crosslinking[15,16] and chemical crosslinking.[17,18] In these reports, the authors have concluded that PVA hydrogels are suitable for drug delivery because of their excellent drug-release characteristics and biocompatibility. As the ultra-high-pressure process does not require any harmful compound for crosslinking, we remarked on the hydrogel prepared by an ultra-high-pressure process as a

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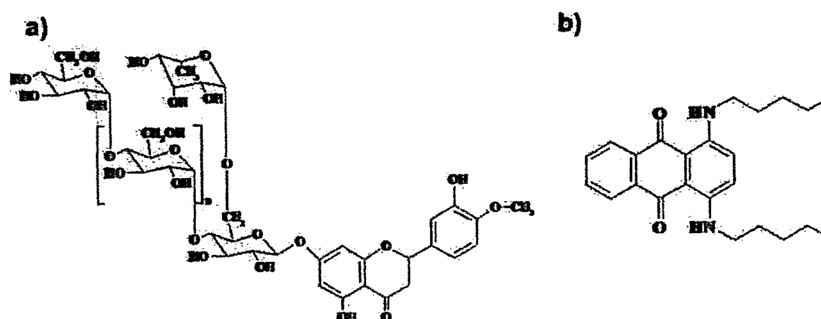

Figure 1 Chemical structures of the model drugs: (a) Alfa-G Hesperidin and (b) Oil Blue N.

drug carrier. That is why it is important to clarify the capability of controlled release from PVA hydrogels produced by an ultra-high-pressure process.

In this study, we prepared PVA hydrogels with a water-soluble or oil-soluble drug through an ultra-high-pressure process, and the drug-release behavior from the gels was investigated.

EXPERIMENTAL

Materials

PVA (99.85 mol % hydrolyzed) was used without further purification. This was kindly supplied by AQ2 Kuraray Co., Ltd. We used Alfa-G Hesperidin and Oil Blue N as the water-soluble and oil-soluble model drugs, respectively. Alfa-G Hesperidin was kindly supplied by Hayashibara Biochemical AQ2 Research Laboratory Co., Ltd. Oil Blue N was pur-AQ2 chased from Sigma-Aldrich Japan Co. The chemical structures of Alfa-G Hesperidin and Oil Blue N are F1 shown in Figure 1.

Preparation of the cylindrical PVA hydrogels

PVA (10 or 20% w/v) aqueous solutions were prepared with an autoclave. A PVA aqueous solution mixed with a 10% w/v Alfa-G Hesperidin aqueous solution was treated under ultra-high pressure. The ultra-high-pressure apparatus was Dr. CHEF, which AQ2 was made by Kobe Steel, Ltd. The treatment conditions were as follows: the ultra-high-pressure treatment condition was 6000–10,000 atm, the treatment time was 1–60 min, and the treatment temperature was kept at 313 K, at which no ice crystals were formed.[19] After the treatment, the prepared gels were cut into the desired shape. A representative photograph of a prepared gel is shown in Figure F2 2(a). The shape of gel was cylindrical. The length was 10 mm, and the diameter was 5 mm.

A dodecane solution dissolving 0.1% w/v Oil Blue N was mixed with a 10% w/v PVA aqueous solution. The solution was homogenized for 10 min at 6000 rpm to prepare oil-in-water (o/w) emulsions with a homogenizer. After that, the emulsion solution was treated under ultra-high pressure. The ultra-high-pressure treatments were carried out at 6000–10,000 atm for 1–60 min at 313 K. A typical prepared gel is shown in Figure 2(b). The diameter of the dodecane droplets was measured under a microscope before and after ultra-high-pressure treatment.

Evaluation of the model drug-release behavior from the gels

The release behavior of the water-soluble model drug from the PVA hydrogel was evaluated in ultrapure water. The hydrogel in the vial with ultrapure water was placed in a thermostated water bath at 303 K. The solution was shaken at 100 rpm to keep the concentration constant. Samples were withdrawn AQ3 at predetermined intervals to measure the amount of

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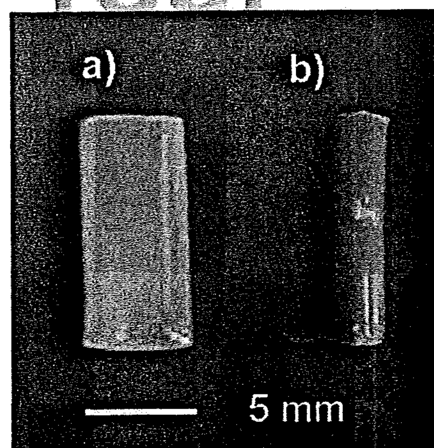

Figure 2 Photograph of the PVA gel prepared by ultra-high-pressure treatment (10,000 atm for 10 min at 313 K): (a) PVA gel with 5% w/v Alfa-G Hesperidin, the water-soluble model drug, with a PVA concentration of 10% w/v and (b) PVA gel with 0.1% w/v Oil Blue N, the oil-soluble model drug, with a PVA concentration of 10% w/v. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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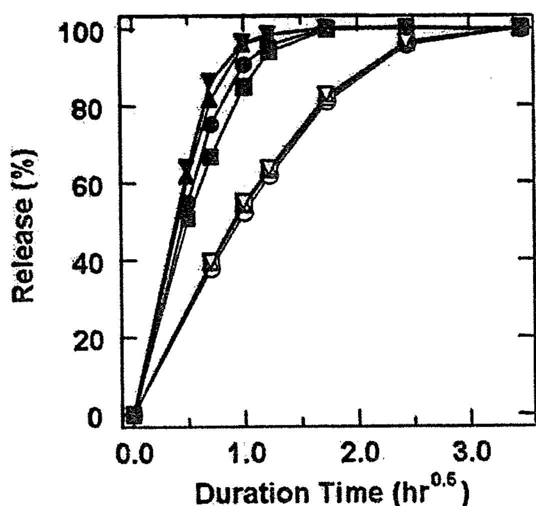

Figure 3 Release behavior of Alfa-G Hesperidin from the PVA hydrogels. A 5% w/v PVA solution with a 5% w/v Alfa-G Hesperidin solution was treated under the following conditions: (●) 10,000 atm for 10 min, (■) 9000 atm for 10 min, (▲) 8000 atm for 10 min, and (▼) 8000 atm for 20 min. A 10% w/v PVA solution with a 5% w/v Alfa-G Hesperidin solution was treated under the following conditions: (○) 10,000 atm for 10 min, (□) 9000 atm for 10 min, (△) 8000 atm for 10 min, and (▽) 8000 atm for 20 min.

released Alfa-G Hesperidin. The concentration of Alfa-G Hesperidin in the samples was determined by measurement of the absorption at a wavelength of 282.5 nm with an ultraviolet–visible spectrophotometer (U-2000A, Hitachi, Ltd.).

The release behavior of the oil-soluble model drug from the PVA hydrogel was evaluated in an ethanol solution.[20] The hydrogel in a vial with the ethanol solution was placed in a thermostated water bath at 303 K. The solution was shaken at 100 rpm to keep the concentration constant. The released Oil Blue N was determined by measurement of the concentration of Oil Blue N in the collected samples at predetermined intervals at 642.5 nm with the ultraviolet–visible spectrophotometer.

The release data were shown with a Higuchi plot, with the cumulative amount of released drug versus the square root of the duration time ($h^{0.5}$).[21] It was available with the estimation of an initial drug release rate because the release data in the early stages were proportional to the root of the duration time.

RESULTS AND DISCUSSION

Release behavior of Alfa-G Hesperidin from the gels

The PVA hydrogels, as shown in Figure 2(a), were obtained in the case that the PVA aqueous solution dissolving Alfa-G Hesperidin was treated under ultra-high pressure. The prepared PVA hydrogels were uniformly yellowish, which color was based on Alfa-G Hesperidin, so Alfa-G Hesperidin was homogeneously distributed in hydrogels. The PVA hydrogels with Alfa-G Hesperidin prepared in this study were as elastic and flexible as the gel without any solutes.

Figure 3 shows the release behavior of Alfa-G Hesperidin from the PVA hydrogels. We found that Alfa-G Hesperidin, the water-soluble model drug, was completely released from the 5% w/v PVA gel within about 3 h. However, the complete release of Alfa-G Hesperidin from the 10% w/v PVA gel took about 12 h. This delay in the release behavior of the water-soluble compound was caused by the dense polymer network in the 10% w/v PVA gel.

The release rate was estimated from the initial slopes in Figure 3 and is shown in Figure 4. The release rates from the hydrogels treated at higher pressure (9000–10,000 atm) were lower than that from the hydrogel treated under 8000 atm because the treatment at a higher pressure enhanced crosslinking in the polymer network and induced a more elastic and stronger hydrogel. However, the treatment time in the ultra-high-pressure process at 8000 atm hardly affected the release rates of Alfa-G Hesperidin. Generally, release behaviors are affected by the crosslinking density in the gels, which is derived from the swelling ratio of the gels. In our previous study, we found that the swelling ratio of PVA hydrogels prepared by ultrahigh pressurization decreased with increasing pressure and was constant when a fixed pressure (≥6000 atm) was applied for more than 10 min.[10] Because these drug-release

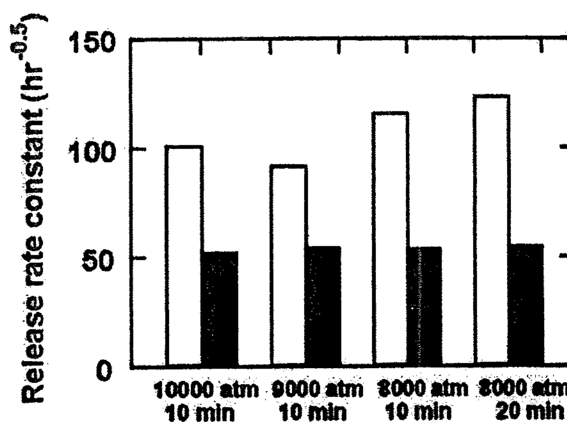

Figure 4 Release rate constants of Alfa-G Hesperidin from the PVA hydrogels derived from the initial slopes of the release curve of Alfa-G Hesperidin from the PVA hydrogels. The compositions of the gels were as follow: (white bar) 5% w/v PVA and 5% w/v Alfa-G Hesperidin and (black bar) 10% w/v PVA and 5% w/v Alfa-G Hesperidin.

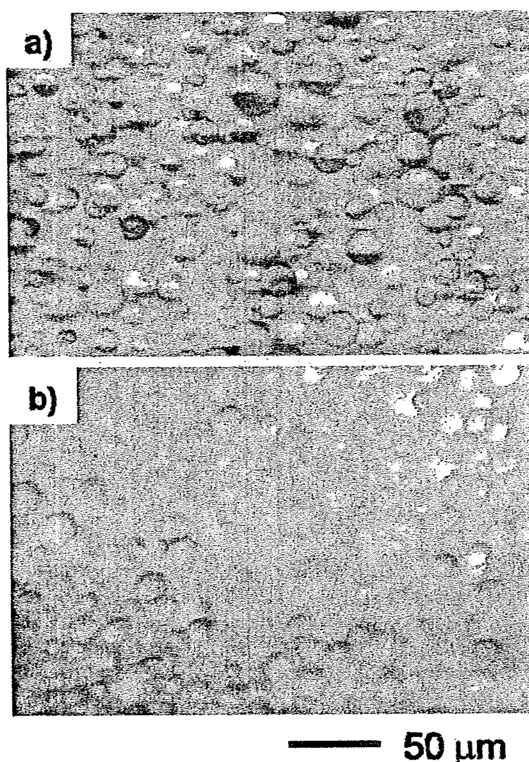

Figure 5 Photographs of o/w emulsion droplets: (a) in solution (before pressurization) and (b) in PVA hydrogel (after pressurization). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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behaviors indeed corresponded to the crosslinking state in the gels, the treatment pressure played a most important role in the operation parameters of the ultra-high-pressure treatment to control the release rate from the gel. The effect of PVA concentration on the release properties was found to be significant; the release rates from the 10% w/v PVA gels were approximately half of those from the 5% w/v PVA gels. From the results shown in Figures 3 and 4, the effects of the treatment pressure and treatment time on the release rate of Alfa-G Hesperidin were smaller than those of the PVA concentration.

Release behavior of Oil Blue N from the gels

The PVA hydrogel with dodecane droplets was obtained by ultra-high-pressure treatment [Fig. 2(b)]. Figure 5 shows the o/w emulsion droplets dissolving Oil Blue N in solution and that in the PVA hydrogel prepared by ultra-high-pressure treatment. Figure 6 shows the diameters and the coefficients of variation (CVs) of dodecane droplets before and after the ultra-high-pressure treatment. Dodecane droplets were stably dispersed in the media. According to Figure 6, the average diameter and the diameter distribution before and after the ultra-high-pressure treatment were almost the same. Under ultra-high pressure, the volumes of the oil and water phases seemed to be decreased, and the density and the concentration of the solution were enhanced. Such a condition may have involved the droplet instability through the interfacial tension change. In fact, however, we observed no droplet coalescence in the gels. This result suggests that a homogeneously

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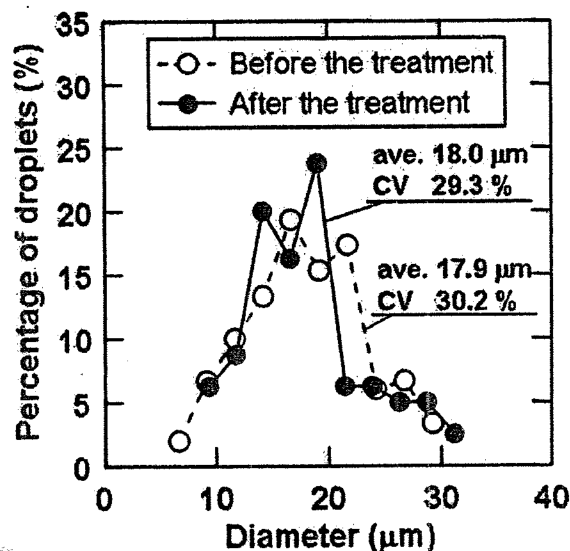

Figure 6 Diameter distributions of dodecane droplets before and after the ultra-high-pressure treatment.

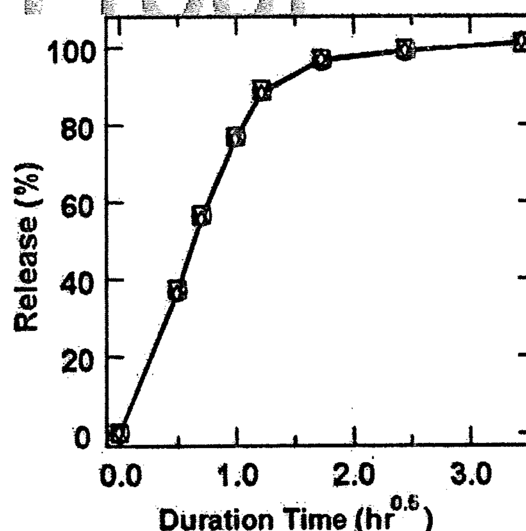

Figure 7 Release behavior of Oil Blue N from 10% w/v PVA hydrogels in ethanol. The pressurized conditions were (○) 10,000 atm for 10 min, (□) 9000 atm for 10 min, (△) 8000 atm for 10 min, (□) 8000 atm for 20 min, and (◇) 8000 atm for 60 min.

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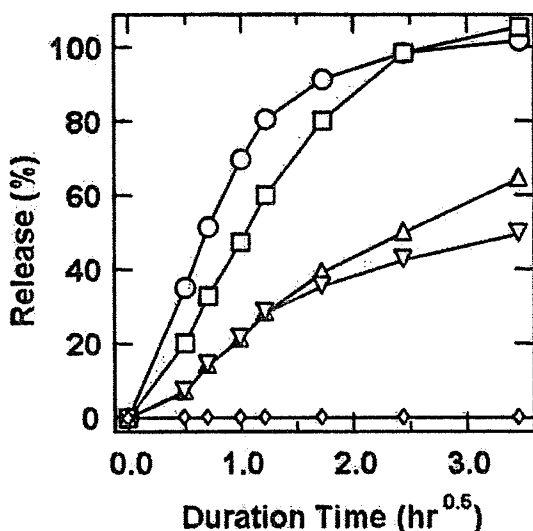

Figure 8 Effect of ethanol concentration on the release rates. All of the hydrogels were prepared by pressurization (treatment conditions: 10,000 atm for 10 min). The ethanol concentrations were as follows. (○) 100, (□) 75, (△) 50, (□) 25, and (◇) 0 wt %.

hydrophobic drug-dispersed hydrogel was prepared by an ultra-high-pressure process when a stable o/w emulsion was obtained in the PVA solution.

The release behavior of Oil Blue N is shown in Figure 7. The release behavior was almost the same over a wide range of treatment pressure and treatment time. The release rate from this PVA hydrogel was about 75 $h^{-0.5}$, which was slightly higher than that from the hydrogels. This difference in the release rate between the water-soluble and oil-soluble model drugs may suggest the diffusion resistance of a drug in PVA matrix. That is, Alfa-G Hesperidin was incorporated into the PVA hydrogel matrix by hydrogen bonding, which was formed between the hydroxyl groups of Alfa-G Hesperidin and those of PVA. Therefore, the release rate of the oil-soluble model drug from the PVA hydrogels was slightly higher than that of the water-soluble model drug.

In addition, the effects of ethanol concentration on the release rate of the model drug are shown in Figure 8. As shown in this figure, the release rate of Oil Blue N from the PVA hydrogel increased with increasing ethanol concentration in the solution. In the case when water (0 wt % ethanol) was used as the medium, no release of Oil Blue N was observed. According to the results shown in Figures 7 and 8, the diffusion of ethanol into the hydrogel was a predominant step for the release of the oil-soluble drug.

CONCLUSIONS

As the PVA solutions dissolving the water-soluble model drug or dispersing the oil-soluble model drug were treated under the ultra-high pressure, hydrogels were obtained. The release rate of each model drug from the PVA hydrogel was controlled by the operation parameters of the ultra-high-pressure treatment, such as the pressure, time, and concentration of the PVA solution. The average diameter and diameter distribution of dodecane droplets before and after the ultra-high-pressure treatment were almost the same. Thus, we expect this technique to be useful as a preparation technique for innocuous sustained-release materials. AQ3

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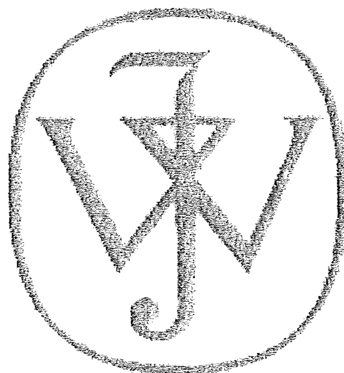

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In vivo anti-tumor effect of PEG liposomal doxorubicin (DOX) in DOX-resistant tumor-bearing mice: Involvement of cytotoxic effect on vascular endothelial cells

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ABSTRACT

We evaluated the *in vivo* anti-tumor effect of polyethylene glycol-modified liposomal doxorubicin (PEG liposomal DOX) in the DOX-resistant Colon-26 cancer cells (C26/DOX)-bearing mice model. IC_{50} value of DOX to C26/DOX in vitro (40.0 μM) was about 250 times higher than that to control C26 (C26/control) (0.15 μM). However, *in vivo* anti-tumor effect of PEG liposomal DOX was similar in both C26/control- and C26/DOX-bearing mice, suggesting that the *in vivo* anti-tumor effect of PEG liposomal DOX was not directly reflecting the sensitivity of these tumor cells to DOX. IC_{50} value (0.10 μM) of DOX to HUVEC, a model vascular endothelial cell, was similar to that of C26/control. Double immunohistochemical staining of vascular endothelial cells and apoptotic cells within the tumor tissue after intravenous administration of PEG liposomal DOX showed that the extent of co-localization of apoptotic cells with endothelial cells was significantly higher for C26/DOX tumors (60%) than C26/control ones (20%), suggesting that the apoptosis is caused preferentially for vascular endothelial cells in C26/DOX tumor. From these results, it was suggested that the cytotoxic effect of DOX on vascular endothelial cells in the tumor would be involved in the *in vivo* anti-tumor effect of PEG liposomal DOX in C26/DOX-bearing mice.

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1. Introduction

Generally, chemotherapeutic drugs are administered to cancer patients for a long term with low dosage to prevent severe side effects, which often causes the cancer cells to acquire the resistance against the chemotherapeutic drug and the effectiveness of the drug gradually decreases. Resistance acquisition of the cancer cells by long-term exposure of the chemotherapeutic drugs, called "multidrug resistance", has been considered as a major obstacle in the current clinical cancer chemotherapy [1].

In the past years, various mechanisms of multidrug resistance have been proposed, including the induced expression of multidrug efflux transporters (P-glycoprotein (P-gp) or multidrug resistance-associated proteins), excessive expression of metabolic enzymes (cytochrome P450 and glutathione/glutathione S-transferase), and various changes of target proteins within tumor cells (topoisomerase or β-tubulin) for chemotherapeutic drugs [2–6]. Accumulating knowledge on the mechanism behind multidrug resistance shows that two or more mechanisms are simultaneously contributing to the resistance acquisition, but the over-expression of P-gp on the tumor cells has been considered to be the main factor associating with the resistant acquisition [7–9].

A number of approaches have been made to circumvent P-gp-based multidrug resistance of cancer cells, including the use of P-gp inhibitor to block P-gp function [10–12]. Among them, nanoparticles encapsulating chemotherapeutic drugs are considered to be one of the effective approaches [13]. This is because the receptor mediated-endocytosis of nanoparticulate formulations of drugs has been considered as a primary method for bypassing P-gp. For instance, liposomes decorated with ligands such as folic acid, transferrin and lipoprotein, which are expected to bind to cancer cells *via* corresponding receptors that are over-expressed on the cell surface and followed by endocytotic uptake, were examined to bypass the efflux transporters [14]. On the other hand, another mechanism behind the circumvention of P-gp-based multidrug resistance without entering the cells by an endocytotic process was also reported in the case of doxorubicin-loaded polyalkylcyanoacrylate nanoparticles [15]. However, except for the limited number of successful approaches that have launched into the clinical trial [16], the outcomes from most of the approaches especially in the *in vivo* studies have been found to be still unsatisfactory to overcome the multidrug resistance of cancer cells.

Most solid tumors possess unique pathophysiological characteristics that are not observed in normal tissues/organs, such as extensive angiogenesis, defective vascular architecture and impaired lymphatic drainage/recovery system. Due to the leakiness of the microvasculature in the solid tumors, the nanoparticles that exhibit the long blood-

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circulating property have been shown to preferentially accumulate in the tumors [17–19]. This phenomenon known as the enhanced permeability and retention (EPR) effect has been generally observed in many types of solid tumors and provides a great opportunity for passive targeting of anti-cancer agents into the tumor tissue [20–23]. For example, the polyethylene glycol (PEG)-modified liposomal formulation encapsulating doxorubicin (DOX), known as "DOXIL" in the United States, has been used for the cancer patients. PEG liposomal DOX has revealed an increased therapeutic efficacy and reduced cardiotoxicity compared to free DOX [24,25]. However, there is little information on the therapeutic efficacy of PEG liposomal DOX in multidrug-resistant tumor-bearing animal model.

In the present study, therefore, we established the DOX-resistant Colon-26 cancer cells (C26/DOX) with P-gp over-expressed, and evaluated the *in vivo* anti-tumor effect of PEG liposomal DOX in the C26/DOX-bearing mice model.

2. Materials and methods

2.1. Materials

Hydrogenated soybean phosphatidylcholine (HSPC) and distearoyl phosphatidyl ethanolamine-*N*-[methoxy poly (ethylene glycol)-2000] (PEG-DSPE) were purchased from NOF Inc. (Tokyo, Japan). Cholesterol (Chol) and [^{3}H]-cholesteryl hexadecyl ether ([^{3}H]-CHE) were purchased from Wako Pure Chemical Industry Inc. (Osaka, Japan) and Perkin Elmer Life Science Inc. (Boston, MA, USA), respectively. Doxorubicin (DOX), 3-amino-9-ethyl carbazole (AEC) tablets, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI-1640, fetal bovine serum (FBS), gelatin and penicillin-streptomycin solution were obtained from Sigma (St. Louis, MO, USA). Gentamycin was purchased from Gibco BRL, Co. (Grand Island, NY, USA). All other chemicals were of the finest grade available.

2.2. Cells

DOX-sensitive Colon-26 carcinoma cells (C26/control) were kindly provided from Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University. C26/control was cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 20 μg/ml gentamicin at 37 °C under 5% CO_2/95% air.

Human umbilical vein endothelial cells (HUVEC) were purchased from DS Pharma Biomedical Co. (Osaka). HUVEC was cultured in gelatin-coated dishes. They were grown in RPMI-1640 supplemented with 10% heat-inactivated FBS, 10 ng/ml epidermal growth factor (EGF), 70 ng/ml endothelial cell growth supplement (ECGS), 100 U/ml penicillin, 100 μg/ml streptomycin and 20 μg/ml gentamicin at 37 °C under 5% CO_2/95% air.

2.3. Establishment of DOX-resistant Colon-26 carcinoma cells (C26/DOX)

C26/DOX was established by periodic cultivation with increasing concentrations of DOX by following the method reported previously [7]. In brief, the escalating concentrations of DOX from 10, 20, 40, 80, 160, 320, 640 nM to 1 μM were periodically added to culture medium. One day after DOX was added to cells, they were incubated in fresh medium without DOX for 2–3 days, followed by proceeding to the next higher DOX concentration cycle. C26/DOX was cultured in the presence of 1 μM DOX to maintain the degree of resistance and was grown in medium without DOX before experiments.

2.4. Cytotoxic assay for each type of cells to DOX

Sensitivity of each type of cells to DOX was evaluated using MTT assay [26]. Briefly, cells were seeded in a 96-well plate (5000 cells/well) (Asahi Techno Glass, Chiba, Japan). After the plate was incubated at 37 °C for 12 h in a humidified 5% CO_2 atmosphere, the mediums containing a series of concentrations of DOX were added to each corresponding well in the plate. After incubation for 48 h, each well was washed and rinsed with growth medium. MTT solution (5 mg/ml) was added to each well and the cultures were further incubated for 4 h. The formazan crystals were dissolved in 0.04 M HCl–isopropanol and subjected to sonication in a bath-type sonicator (ASONE Corporation, Osaka) for 15 min. Each plate was set into a microplate reader (Bio-Rad, Hercules, CA, USA) and the absorbance values were measured at 570 nm (test wavelength) and 750 nm (reference wavelength). Results were expressed as cell viability (%), calculated for each DOX concentration by the following formula (Eq. (1)):

$$\text{Cell viability}(\%) = (OD_{570,\text{sample}} - OD_{750,\text{sample}})/(OD_{570,\text{control}} - OD_{750,\text{control}}) \times 100 \tag{1}$$

where "sample" and "control" mean the cells with and without DOX treatment, respectively.

Cell viability (%) was plotted against DOX concentration and was fitted with the Hill-type Eq. (Eq. (2)) [27,28] using the non-linear least-squares regression program MULTI [29]. The concentration at which 50% of cells survived corresponds to IC_{50}.

$$E = \frac{E_0 \times IC_{50}}{IC_{50} + C} \tag{2}$$

where E or E_0 is the % cell survival with or without DOX treatment, respectively, and C is the final concentration of DOX in each well. Each experiment was performed using four replicated wells for each DOX concentration and carried out independently five times.

2.5. Western blot analysis of P-gp expression on C26/DOX

Cells were dissolved in a lysis buffer containing 50 mM Tris–HCl (pH 7.4), 120 mM NaCl, 1 mM EDTA, 1% TritonX-100 and 1% protease inhibitor cocktail (Sigma). After centrifugation (48 °C, 30 min, 22000 ×g), the resultant supernatant was collected as a sample. Samples containing 20 μg protein, determined by the DC protein assay (Bio-Rad), were loaded to SDS-PAGE using 12.5% polyacrylamide gel (Bio-Rad). After SDS-PAGE, the resultant proteins were blotted onto nitrocellulose membranes (Advantec, Tokyo). For the detection of P-gp, rabbit anti-mouse P-gp antibody (sc-8313, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at 1:250(v/v) dilution in blocking buffer. As a second antibody, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (81-6120, Zymed Laboratory, San Francisco, CA) was used at 1:5000(v/v) dilutions in blocking buffer. The protein band was visualized with the enhanced chemiluminescence (ECL) system (GE Healthcare Ltd., Buckinghamshire, UK) and the densitometric intensities of protein bands were quantified by Scion image® (Scion Co., Frederick, MD, USA).

2.6. Preparation of liposomes

Liposomes were prepared according to the procedures described previously [26]. Briefly, HSPC, Chol and PEG-DSPE from stock solution were mixed (HSPC:Chol:PEG-DSPE = 56:38:5 by molar ratio) and dried under reduced pressure. The dried lipid film was hydrated with 250 mM ammonium sulfate (pH 5.4) under mechanical agitation. Then, the resulting multilamellar preparations were sized by repeated extrusion through polycarbonate membrane filters (Millipore, Bedford, MA, USA) with the pore size of 200 nm followed by further extrusion through the one with 100 nm. For the encapsulation of DOX into liposomes by pH remote loading method [30], the external phase of the liposomes was replaced with PBS (pH 8.0) by gel filtration with a Sephadex G-25 column (PD-10, GE Healthcare Ltd.). DOX in PBS (pH 8.0) was added to liposomes at a drug-to-lipid molar ratio of 1:10 and incubated at 60 °C for 1 h. Our preliminary experiments showed