Fabrication, Characterization and Pharmacokinetic Evaluation of Doxorubicin-Loaded Water-in-Oil-in-Water Microemulsions Using a Membrane Emulsification Technique

Roshan Pradhan,^a Yong-Il Kim,^a Jee-Heon Jeong,^a Han-Gon Choi,^b Chul Soon Yong*,^a and Jong Oh Kim*,^a

^aCollege of Pharmacy, Yeungnam University; 214-1 Dae-Dong, Gyongsan 712-749, South Korea; and ^bCollege of Pharmacy, Hanyang University; 55 Hanyangdaehak-ro, Sangnok-gu, Ansan 426-791, South Korea.

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Doxorubicin (DOX)-loaded water-in-oil-in-water (W/O/W) microemulsions were produced using a shirasu-porous-glass (SPG) membrane emulsification technique. Soybean oil was used as the oil phase; polyglycerol polyricinoleate (PGPR) or tetraglycerol polyricinoleate (TGPR) was used as the surfactant to stabilize the feed W/O emulsions, while Tween 20 was used in the external water phase to stabilize oil droplets containing water droplets. Increasing the feed pressure from 50 to 90 kPa increased the particle size of W/O/W emulsions, whereas it was decreased by increasing the agitator speed. The smallest particle sizes of multiple emulsions were obtained at the feed pressure of 50 kPa and agitator speed of 350 rpm. Under this set of conditions, the increase in the concentration of PGPR or TGPR showed a decrease in the particle size of DOX-loaded W/O/W emulsions. The optimized formulation comprising of 5% w/v PGPR and 3% w/v Tween 20 in the oil phase and external water phase, respectively, with 0.5% w/v of DOX had a particle size of $0.440 \pm 0.007 \mu\text{m}$ and polydispersity index of 0.220 ± 0.087 , which was supported by the transmission electron microscopy image. The formulations showed a sustained release profile in phosphate buffer solution (pH 7.4). The plasma concentrations of DOX after intravenous administration to rats were prolonged and gave approximately 17-fold higher area under the drug concentration–time curve (AUC) compared to free DOX solution. Thus, these results demonstrated that the SPG membrane emulsification technique could be used as a promising technique to prepare W/O/W microemulsions for delivering DOX with sustained release characteristics and better bioavailability.

Key words microemulsion; shirasu-porous-glass; doxorubicin; sustained release; pharmacokinetics

Over the past two decades, an increasing interest has been devoted to multiple emulsions due to their multiple compartment structure and an immense capability of encapsulating hydrophilic drug substances for sustained released characteristics.^{1–4)} The potential application of water-in-oil-in-water (W/O/W) multiple emulsion is not only limited to pharmaceuticals but also in the field of cosmetics and food industry. In cosmetics and pharmaceuticals, W/O/W emulsions have been used for controlled release and targeted delivery of drugs.^{5–8)} Food applications include the encapsulation of vitamin/minerals,^{9,10)} and the production of low-calorie foods.¹¹⁾

W/O/W emulsions are usually created using conventional homogenization technology in a two-step procedure.¹²⁾ First, a primary water-in-oil (W/O) emulsion is prepared by homogenizing an oil phase and an aqueous phase together in the presence of a suitable oil-soluble emulsifier (low hydrophilic to lipophilic balance (HLB) < 10). Second, W/O/W emulsion is prepared by homogenizing W/O emulsion with another aqueous phase in the presence of a suitable water-soluble emulsifier (high HLB > 10).¹⁾ However, the second step often results in high polydispersity or low encapsulation efficiency. Therefore, the conventional homogenization techniques for preparing emulsions have been gradually replaced because of their high energy consumption, intricate technology and polydispersity of emulsions,¹³⁾ and their intrinsic thermodynamic instability derived from the relatively larger droplets size.¹⁴⁾

Membrane emulsification is a relatively new method for the production of emulsions. The technique is attractive due

to the low energy consumption, the better control of droplet size and droplet size distribution, and especially the scale-up possibility of the process. In particular, a special type of glass membrane, called shirasu-porous-glass (SPG) membranes, is potentially suitable for membrane emulsification due to their uniformly sized pores and wide range of available mean pore diameters.¹⁵⁾ The multiple emulsions are produced by forcing a primary emulsion (W/O) through a microporous membrane into a continuous aqueous phase.^{16–19)} This results in much less shear than in conventional emulsification processes so that the droplets are intact and both a high entrapment efficiency and monodispersity could be achieved.^{20,21)}

In the present work, we prepared the doxorubicin (DOX)-loaded W/O/W microemulsions using membrane emulsification by pressurizing the premixed W/O emulsions into the external water phase through SPG membrane. Soybean oil was used as the oil phase, polyglycerol polyricinoleate (PGPR) or tetraglycerol polyricinoleate (TGPR) as the hydrophobic emulsifiers (HLB value of 4), and Tween 20 (HLB value of 16.7) was chosen as the hydrophilic surfactant in the external water phase. Doxorubicin hydrochloride (DOX) was used as a hydrophilic model drug. DOX is an antineoplastic agent widely used in the treatment of acute leukemia, endometrial, ovarian and breast cancers.²²⁾ Despite good efficacy, its clinical utility may be hampered by cumulative, dose-limiting cardiotoxicity, myelosuppression, and drug resistance.^{23,24)} Thus, W/O/W emulsion systems can be suitable drug carriers because of the encapsulation of the drug in the internal water phase and the low viscosity due to the external water phase. Moreover, in the form of an emulsion, it is possible to control release rates

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* To whom correspondence should be addressed. e-mail: csyong@yu.ac.kr; jongohkim@yu.ac.kr

of medicine and suppress strong side effects of the drug.

Experimental

Materials DOX was supplied by Dong-A chemical (Suwon, South Korea). Soybean oil was purchased from Sigma Chemical Co., Ltd. (Germany). Polyglycerol polyricinoleate (SY-GLYSTER CRS-75) and tetraglycerol polyricinoleate (SY-GLYSTER CR-310) were supplied from Sakamoto Yakuhin Kogyo (Osaka, Japan). Tween 20 was purchased from DC Chemical Co., Ltd. (China). Semi-permeable membrane tubing (MEMBRA-CEL[®] Dialysis Tubing) was purchased from SERVA Electrophoresis (Heidelberg, Germany). A miniature kit for emulsification with an MPG module (microporous glass, a brand name of SPG) was purchased from Kiyomoto Works Co. (Miyazaki, Japan). All other chemical reagents were of analytical grade and were used without further purification.

Preparation of W/O/W Emulsions The W/O/W emulsions were prepared by a two step process. The first-step emulsification consisted of preparing premixed W/O emulsions. For preparation of premixed W/O emulsions, various concentrations of the hydrophobic emulsifier PGPR or TGPR were initially mixed with soybean oil. The oil phase containing PGPR or TGPR was mixed with the water phase (pure water or drug solution). The volume fraction of the water to oil phase was 0.25 and the total volume was 25 mL. The premixed W/O emulsions were homogenized using a homogenizer (Ultra-Turrax[®] Model T25, IKA Works, Inc., Germany) at 9000 rpm for 5 min.

Membrane emulsification was used for the second step. A schematic diagram of the SPG membrane emulsification technique is shown in Fig. 1. The SPG membrane system consisted of a tube-shaped hydrophilic SPG membrane with an outer diameter of 10 mm, a thickness of 0.75 mm and a pore size of 1.4 μm , pressurized N₂ gas, a dispersed phase container, a collecting vessel, and a stirrer. The premixed W/O emulsion as the dispersed phase was poured in a pressure-tight vessel that was connected to a nitrogen gas inlet attached to a PG-200-163GP-S pressure gauge (COPAL Electronics, Japan). The continuous phase, 100 mL of water containing 3% w/v Tween 20 was continuously stirred to prevent creaming of the droplets. Before starting the emulsification process, SPG membrane was dipped into the continuous phase and subjected to ultrasonic treatment for 30 min in order to wet the membrane thoroughly with the continuous phase.¹⁷⁾ The dispersed phase was pumped under gas pressure through the pores of the membrane into the continuous phase, which circulated through the membrane module to form the W/O/W micro-emulsion droplets. The experiments were carried out over a wide range of feed pressures (50–90 kPa) and agitator speeds (150–450 rpm), at 25°C to optimize the process parameters.

Determination of Particle Size and Size Distribution NanoZS light-scattering particle size analyser (Malvern, U.K.) with non-invasive back scatter (NIBS[®]) technology was used to measure the droplet size and size distribution of the samples. It allowed detection of droplets in the range of 0.6–6000 nm. The data on particle size distribution were collected using Dispersion Technology Software (DTS) (nano) software (version 5.0) which was provided with the instrument. All the experiments were repeated three times and the values of the *z*-average diameters were used. The *z*-average

Fig. 1. Schematic Diagram of the Experimental Apparatus Used for Preparation of W/O/W Emulsion Using SPG Membrane Emulsification

(i) SPG membrane module; (ii) compressed nitrogen gas; (iii) dispersed phase container; (iv) continuous phase and emulsion container; (v) magnetic stirrer; (vi) dispersed phase; (vii) continuous phase.

diameter of the emulsion was derived from cumulative analysis by the Automeasure software (Malvern Instruments, Malvern, U.K.). The data were expressed as the mean $\pm$ standard deviation (S.D.). A two-tailed unpaired Student's *t*-test was performed with a significance level of $p < 0.05$.

Determination of the Encapsulation Efficiency The encapsulation efficiency (EE) of W/O/W emulsion was assessed by the fraction of DOX retained within the inner aqueous phase. Briefly, 100 μL of the W/O/W emulsions and 900 μL of distilled water were poured into a sampling tube and mixed. The tube was centrifuged at 7500 rpm for 10 min. Six hundreds microliter of the clear part of the solution in the tube was taken up by a syringe equipped with a needle (Greenject-1, Korea). The needle was replaced with a polyvinylidene difluoride (PVDF) syringe filter (Whatman, 0.45 μm pore diameter), and then the solution was filtered to eliminate the oil droplets. The filtrate would contain DOX derived from the outer aqueous phase of the original W/O/W emulsion, so its concentration was determined by HPLC as described below. The encapsulation efficiency of DOX in the inner-water phase of a W/O/W emulsion was calculated by the following equation:

$$\text{EE (\%)} = \frac{D_o - D_{\text{out}}}{D_o} \times 100\%$$

where D_o is the initial amount of DOX loaded into the inner-water phase and was calculated from the composition of the W/O/W emulsion; D_{out} is the amount of DOX that leaked into the outer-phase solution and was calculated using the initial volume of the outer aqueous phase.

In Vitro Release Study Each 2 mL of W/O/W emulsion and free drug solution (as a control) containing 0.5% DOX was put into the MEMBRA-CEL[®] dialysis membrane bag. Both the ends of the membrane bag were sealed with a Mediscell clip to prevent leakage. Each bag was immersed in placed in 200 mL phosphate buffer solution (PBS, pH 7.4) contained in the jars of the dissolution tester (DST-600; Fine Chemical, Hwasung, South Korea). The temperature of the water-bath was maintained at 36.5°C and the stirring was done using the paddle at 50 rpm. At pre-determined intervals, 1 mL of the medium was sampled and filtered.^{25,26)} The resulting solution was then analyzed by HPLC as described below.

Table 1. Compositions of Primary W/O Emulsion

W/O emulsion	Soybean oil (mL)	PGPR (g)	TGPR (g)	Distilled water (mL)	Phase separation
P1	20	0.20	—	5	+
P2	20	0.40	—	5	—
P3	20	0.60	—	5	—
P4	20	0.80	—	5	—
P5	20	1.00	—	5	—
T1	20	—	0.20	5	+
T2	20	—	0.40	5	+
T3	20	—	0.60	5	+
T4	20	—	0.80	5	+
T5	20	—	1.00	5	+
T6	20	—	1.20	5	—
T7	20	—	1.40	5	—
T8	20	—	1.60	5	—
T9	20	—	1.80	5	—

+: Phase separation for 24 h. —: No phase separation for 3 d.

Pharmacokinetic Study In Vivo Experiments Male Sprague-Dawley rats weighing 250 ± 20 g were fasted for 12 h prior to the experiments, but allowed free access to water. Eight rats were divided into two groups. Under light ether anaesthesia, the femoral arteries and veins of rats were cannulated with PE-50 polyethylene tubing. After complete recovery from anaesthesia, DOX-loaded W/O/W emulsion or DOX solution were administered intravenously to the femoral vein through the catheter at a dose of 5 mg/kg as doxorubicin, respectively. Blood samples (0.2 mL) were collected *via* the femoral artery at designated time intervals and put into heparinized glass tubes and centrifuged at $12000 \times g$ for 10 min using a 5415C centrifuge (Eppendorf, Hauppauge, NY, U.S.A.).²⁷⁾ All animal care and procedures were conducted according to the Guiding Principles in the Use of Animals in Toxicology, as adopted in 1989, revised in 1999, and amended in 2008 by the Society of Toxicology.²⁸⁾ Furthermore, the protocols for the animal studies were approved by the Institute of Laboratory Animal Resources of Yeungnam University.

Blood Sample Analysis Plasma (0.1 mL) was thoroughly mixed with 0.1 mL of acetonitrile and 25 μ L of acetonitrile solution containing daunomycin (100 μ g/mL) as an internal standard. It was centrifuged at $12000 \times g$ for 10 min to precipitate the proteins. The supernatant layer (0.2 mL) was evaporated under N_2 (g). The residue was reconstituted in 50 μ L of mobile phase. The resulting solution (20 μ L) was analyzed by an HPLC system (Varian ProStar 210/215; Varian, Chicago, IL, U.S.A.) equipped with an Inertsil ODS-3 C_{18} column (GL Science, 5 μ m, 15 cm $\times$ 0.46 cm i.d.; Tokyo, Japan) and a UV detector (Model L-7450). The mobile phase consisted of water, acetonitrile, and acetic acid (77:22:1, v/v/v) adjusted to pH 2.8 with 1 M hydrochloric acid. The eluent was monitored at 254 nm with a flow rate of 1.0 mL/min.^{29–31)}

Pharmacokinetic Data Analysis and Statistical Analysis The area under the drug concentration–time curve from zero to infinity (*AUC*), the mean residence time (*MRT*), the elimination constant (K_{el}), and half-life ($t_{1/2}$) alpha and beta were calculated using a two compartmental analysis (WinNonlin; professional edition, version 2.1; Pharsight Co., Mountain View, CA, U.S.A.). The maximum plasma concentrations of drug (C_{max}) were obtained directly from the plasma data.³²⁾

Levels of statistical significance ($p < 0.05$) were assessed using the Student's *t*-test between two means for unpaired data. All data are expressed as mean $\pm$ standard deviation (S.D.).

Results and Discussion

Effect of Hydrophobic Emulsifiers on Phase Separation of Primary W/O Emulsions In order to develop a stable DOX-loaded W/O/W emulsions using membrane emulsification technique, various process parameters were optimized. First, the volume fraction of water to oil was determined by adding different quantities of distilled water (10–50% v/v) to the soybean oil containing 2% (w/v) PGPR, prior to homogenization. Emulsions with 20–30% volume of water were stable without phase separation for seven days. The increase in water phase induced greater instability, which may be due to the increased incidence of coalescence and bridging at higher water volume, both of which led to reduction in total water droplet surface area.³³⁾ Similar results were demonstrated that 20–30% of dispersed phase to continuous phase was the optimum volume fraction for an emulsion to have good stability and high encapsulation efficiency.^{34,35)} Therefore, the volume fraction of water and oil in primary W/O emulsion, were set at 0.25 in all the following experiments.

The composition of the primary W/O emulsions (10 mL) and the effects of various PGPR concentrations (1–5% w/v) or TGPR concentrations (1–9%) on the phase separation with respect to the volume of soybean oil are shown in Table 1. The PGPR and TGPR were used as the hydrophobic emulsifiers due to their excellent water-binding capacity.³⁶⁾ For all the concentrations of PGPR tested, no phase separation into discrete oil and water phases was observed except formulation P1. In case of TGPR, lower concentrations showed phase separation (formulations T1–T5), while at higher concentrations (formulations T6–T9), no phase separation was observed. It suggested that the inadequate emulsifier would result in coalescence between water droplets which eventually would lead to phase separation. Moreover, due to the presence of the long hydrophilic polyglycerol chain in PGPR as compared to TGPR, the lower concentrations of PGPR were sufficient to stabilize the water droplets in primary W/O emulsions. The formulations which showed no phase separation were consid-

Fig. 2. Effect of the Feed Pressure on (A) *z*-Average Particle Size and (B) Droplet Size Distribution of W/O/W Microemulsion. Each value represents the mean $\pm$ S.D. ($n=3$).

ered for the SPG membrane emulsification for the preparation of W/O/W multiple emulsions.

Determination of Concentration of Hydrophilic Emulsifier Tween 20, a non-ionic surfactant with HLB of 16.9 was selected as hydrophilic emulsifier because it is regarded as having a high level of stability and a low level of toxicity and is widely used in the preparation of emulsions. Preliminary W/O/W emulsions with 2% (w/v) PGPR in the oil phase and different concentrations of Tween 20 (1–3% w/v) in the external water phase were prepared by high speed homogenizer, considering 0.25 as the volume fraction of the dispersed phase to continuous phase. Although the droplet size was large (more than 50–300 μm), the decrease in the droplet size was clearly observed when the concentration of Tween 20 was increased from 1 to 3% (w/v). The results were in good agreement with those showing that the droplet size decreased when the concentration of hydrophilic emulsifier in the external water phase was increased.³⁵⁾ Therefore, the concentration of Tween 20 was set at 3% w/v for the preparation of narrowly sized W/O/W microemulsions by SPG membrane emulsification technique.

Effect of Process Parameters on Mean Droplet Size and Distribution **Effect of Feed Pressure on the Dispersion Phase** Feed pressure is known to be one of the key factors that influence the formation of emulsion and particle size distribution. By controlling feed pressure in the emulsification process, the flux rate of the dispersed phase across the membrane channel, as well as the detachment of the droplets, can be modulated for the preparation of uniform emulsion droplets.³⁷⁾ The hydrophilic SPG membrane, with an average pore size of 1.4 μm was fitted in the SPG membrane equipment. The dispersed phase tank was filled with primary W/O emulsion and the continuous phase tank was filled with distilled water containing 3% w/v Tween 20. The system was run with constant agitator speed, but the applied feed pressure was varied. The results showed that increasing the feed pressure from 50 to 90 kPa increased the *z*-average particle size from *ca.* 0.678 to 0.854 μm, respectively, as shown in Fig. 2A. This

was in general agreement with the literature.³⁸⁾ The increase in feed pressure causes the growth of droplets at the pores to occur faster, and the higher pressure of the fluid inside the growing droplets allows their shape to be held better, and so they become larger before detachment. In addition, as the rate of droplet formation increases with increasing pressure, the rate of interface formation increases. However, the rate of adsorption of emulsifier remains the same and interfacial tension is reduced more slowly relative to the formation rate. The droplet size distribution was increased at high feed pressure of 90 kPa than 50 kPa, as shown in Fig. 2B. These phenomena may be either due to the collision of the dispersed phase particles with the continuous phase components thereby breaking the emulsion or the delicate force balance against the interfacial tension was broken, thereby resulting in a nonuniform size distribution of the emulsion droplets. In this case, feed pressure of 50 kPa was chosen as an optimal condition for the preparation of DOX-loaded W/O/W emulsions in further studies.

Effect of Agitator Speed The effect of the agitator speed was investigated using the same experimental setup. The feed pressure was set at 50 kPa, and the system was run with various agitator speeds. As shown in Figs. 3A and B, the increase in agitator speed from 150 to 450 rpm decreased the *z*-average particle size from *ca.* 0.856 to 0.648 μm, respectively, and also decreased the droplet size distribution. Although many mechanisms have been postulated for emulsion formation, the effects of agitator speed on the properties of an emulsion have not yet been clarified.^{39,40)} However, in one of the study, it was reported that the particle size of emulsions decreased as the agitator speed increased, which was in close agreement with the present results.⁴¹⁾ Thus, 350 rpm was selected as the optimal agitator speed for preparation of DOX-loaded W/O/W microemulsion, because it gave relatively uniform emulsion droplets with a narrow particle size distribution.

Based on these findings, the optimized conditions to produce an optimal uniform DOX-loaded W/O/W microemulsion using an SPG membrane were the feed pressure of 50 kPa and

Fig. 3. Effect of Agitator Speed on (A) z-Average Particle Size and (B) Droplet Size Distribution of W/O/W Microemulsion

Each value represents the mean $\pm$ S.D. ($n=3$).

Fig. 4. Effect of PGPR and TGPR on z-Average Particle Size of W/O/W Microemulsion

Each value represents the mean $\pm$ S.D. ($n=3$).

the agitator speed of 350 rpm.

Effect of PGPR and TGPR on DOX-Loaded W/O/W Emulsion The effect of various concentrations of PGPR and TGPR on droplet size and size distribution of DOX-loaded W/O/W emulsions were investigated. Initially, 625 mg of DOX was dissolved in 5 mL of internal water phase and various concentrations of PGPR (2–5% w/v) or TGPR (6–9% w/v) were added to 20 mL of the oil phase. Primary emulsification was carried out by using homogenization technique in order to obtain primary W/O emulsion. It was then filled into the dispersed phase tank, while the continuous phase tank was filled with 100 mL of distilled water containing 3% w/v Tween 20. Secondary emulsification was carried out under the optimized conditions using the SPG membrane emulsification technique at the feed pressure of 50 kPa and the agitator speed of 350 rpm. Figure 4 exhibited that the z-average particle size were decreased from *ca.* 0.690 to 0.440 μm as concentration of PGPR was increased from 2 to 5% w/v, and from *ca.* 0.765 to

0.610 μm as concentration of TGPR was increased from 6 to 9% w/v, respectively. Less emulsifier allows more coalescence of the droplets and this may explain the increase in droplet size.⁴²⁾ The lowest z-average particle size of 0.440 ± 0.007 μm with polydispersity index of 0.220 ± 0.087 were produced with the PGPR 5% w/v in the oil phase and 3% w/v Tween 20 in the external water phase. Moreover, the size and the spherical morphology of W/O/W microemulsion produced by SPG membrane emulsification technique were further supported by the TEM images as shown in Fig. 5. Thus, DOX-loaded W/O/W microemulsions had a narrowly sized, uniformly distributed spherical morphology.

Encapsulation Efficiency The encapsulation efficiencies (EEs) of DOX-loaded W/O/W emulsions prepared with different concentrations of PGPR (between 2 and 5% w/v) or TGPR (between 6 and 9% w/v) in oil phase and Tween 20 in the external water phase are presented in Fig. 6. Increasing PGPR or TGPR concentrations resulted in higher EEs. In particular, at PGPR concentrations of 3 to 5% w/v and TGPR concentrations of 7 to 9% w/v, EEs of almost 100% were achieved. However, at PGPR concentration of 2% w/v and TGPR concentration of 6% w/v, the EEs of 76.2% and 69.0%, respectively was obtained indicating that almost 30% of the aqueous phase of the original primary emulsion was exposed to, and mixed with the external water phase during the SPG emulsification process. In addition, these multiple emulsions were unstable at low PGPR and TGPR concentrations, and resulted in a significant decrease in EE values compared to the initial EE values (data not shown). The decrease in the EE values was probably due to the rupture or expelling of the internal water droplets.⁴³⁾ In contrast, The EEs of W/O/W emulsions prepared with higher concentrations of PGPR (4 and 5% w/v) or TGPR (8 and 9% w/v) showed no significant change for at least three months at room temperature, indicating that DOX remained entrapped within the internal aqueous phase during the storage period.

In Vitro Drug Release The nature of the surfactants in the W/O/W emulsions can affect the DOX release profiles. As depicted in Figs. 7A and B, for both types of surfactants, the releases of DOX from W/O/W emulsions were decreased

Fig. 5. TEM Images of Doxorubicin Hydrochloride-Loaded W/O/W Emulsion

W/O/W emulsion was prepared by the SPG membrane emulsification. (A) Scale bar: 500 nm; (B) Scale bar: 100 nm; (C) Size distribution.

Fig. 6. Effect of Different Concentrations of PGPR (% w/v) and TGPR (% w/v) on the Encapsulation Efficiencies of DOX-Loaded W/O/W Emulsion

Each value represents the mean $\pm$ S.D. ($n=3$).

as surfactant concentrations increased. In addition, PGPR showed more release retarding capacity than TGPR at the lower concentration. The lower concentrations of surfactants resulted in a higher initial loss of DOX, indicating that it was released immediately after formation of the W/O/W emulsions. The internal water droplets in these emulsions appear to have an insufficient amount of PGPR or TGPR and would be more susceptible to damage by shear forces during the SPG membrane emulsification process. More elaborately, the release of DOX in W/O/W emulsions with lower concentration of PGPR (2% w/v), was very rapid and reached between 50–60% within first 6 h, and up to 70% within 48 h. Similarly, TGPR (6% w/v) showed the fast release profile in the first 6 h and the release was up to 80% within 48 h. At higher concentrations of PGPR and TGPR, the release of DOX was slower and in a sustained manner. The increased hydrophobic emulsifier concentration increase the viscosity in the oil phase, which might form the strong membrane, and slow down the water permeation coefficients, and the internal water droplets are more resistant to rupture or coalescence.⁴⁴ On the contrary, the free DOX solution under similar experimental conditions released around 70% and 95% in 1 and 4 h, respectively, which indicated immediate release of DOX as compared to the W/O/W emulsion. Since the dialysis membrane itself acted as the barrier for the drug release, the DOX released from the solution was extended upto 4 h.

In order to assess the exact mechanism of drug release from the W/O/W emulsions, the rate of dissolution was calculated using Korsmeyer–Peppas equations, given below as follows:

$$M_t / M = kt^n$$

$$\log[M_t / M] = \log k + n \log[t]$$

where M_t/M is the fraction of drug dissolved at time t , k is a kinetic constant and n is the release exponent which is an indication of the dissolution mechanism. The release exponent n value indicated that the mechanism of drug release was zero-order, non-Fickian, and Fickian (Higuchi model) whenever $n=1$, $0.5 < n < 1$ and $n=0.5$, respectively.⁴⁵ Our results showed that most n values were between 0.5 and 1, suggesting that the drug was released from the microemulsion system by non-Fickian, “anomalous” mechanism.

Pharmacokinetic Evaluation Figure 8 shows the mean plasma concentration–time profiles of DOX after intravenous administration of DOX solution and DOX-loaded W/O/W emulsions to rats at the dose of 5 mg/kg DOX. It has been mentioned that the particle sizes of the W/O/W emulsions for the intravenous (i.v.) administration were recommended to be kept under $7\mu\text{m}$ in diameter in order to allow free flow of the particles in the blood vessels.⁴⁵ Our formulation had the particle size of *ca.* $0.440\mu\text{m}$ which was considered suitable for the i.v. route of administration. The total plasma concentrations of DOX after administration of W/O/W emulsions were

Fig. 7. Effect of Different Concentrations of (A) PGPR or (B) TGPR on the Drug Release

The W/O/W emulsion composed of soybean oil, DOX, PGPR or TGPR in the primary W/O emulsion and Tween 20 in the external water phase. Each value represents the mean $\pm$ S.D. ($n=3$).

significantly higher than free DOX solution at $p<0.05$, and maintained about $7\mu\text{g/mL}$ up to 12h. The corresponding non-compartmental pharmacokinetic parameters are listed in Table 2. The pharmacokinetic parameters of DOX-loaded W/O/W emulsions were significantly different from that in the DOX solution.⁴⁶⁾ The W/O/W emulsions gave a significantly higher *AUC* and hence *ca.* 17-fold better bioavailability compared to the DOX solution. The enhanced bioavailability of DOX might be due to the sustained release of DOX from the W/O/W microemulsion. Moreover, the *MRT*, $t_{1/2}$, and K_{el} values of DOX from the W/O/W emulsions were significantly different from those of the DOX solution ($p<0.05$). Microemulsion formulations have distinct advantages when delivered parenterally because of the fine particle microemulsion is cleared more slowly and, therefore have a longer residence time in the body. Other advantages are that they exhibit a higher physical sta-

Fig. 8. Plasma Concentration–Time Curves of DOX-Loaded W/O/W Emulsion and DOX Solution after Intravenous Administration to Rats at the Dose of 5 mg/kg

Each value represents the mean $\pm$ S.D. ($n=4$).

Table 2. Pharmacokinetic Parameters of DOX after Intravenous Administration of Drug Solution and Drug Entrapped in W/O/W Emulsion

Parameters	DOX solution	DOX-loaded W/O/W emulsion
<i>AUC</i> ($\text{h} \cdot \mu\text{g/mL}$)	16.29 ± 1.07	$276.32 \pm 30.79^*$
$C_{\max}$ ($\mu\text{g/mL}$)	15.84 ± 0.43	13.91 ± 0.16
$t_{1/2\alpha}$ (h)	0.11 ± 0.02	$0.34 \pm 0.09^*$
$t_{1/2\beta}$ (h)	1.15 ± 0.07	$21.37 \pm 2.23^*$
K_{el} (h^{-1})	0.60 ± 0.04	$0.03 \pm 0.01^*$
<i>MRT</i> (h)	1.54 ± 0.05	$30.58 \pm 3.21^*$

* $p<0.05$ compared with DOX solution. Each value represents the mean $\pm$ S.D. ($n=4$).

bility in plasma than liposomes and the internal oil phase is more resistant against drug leaching.^{48–50)} Our results suggest that DOX-loaded W/O/W multiple emulsions would be useful for delivering DOX in a pattern that allows sustained release for a long time, leading to better bioavailability.

Conclusion

DOX-loaded W/O/W microemulsions were produced by Shirasu Porous Glass (SPG) membrane using W/O emulsions prepared by homogenization as feed emulsions. The optimized formulation was composed of 0.5% w/v DOX, 5% w/v PGPR and 3% w/v Tween 20 as sole hydrophobic and hydrophilic emulsifier with respect to the volume of oil phase of the primary W/O emulsion and the outer aqueous phase of the W/O/W microemulsion, respectively. The formulation showed the sustained release characteristic, prolonged the plasma drug concentration after intravenous administration and gave about 17-fold higher *AUC* compared to DOX solution. Thus, the SPG membrane emulsification technique could be used as the promising technique to prepare W/O/W microemulsions,

which could be effective for delivering anticancer agents with sustained release characteristics and better bioavailability.

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