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Amphipathic polyethylene glycol stabilized emulsions (o/w): physical characterization and in vivo distribution

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Abstract

Injectable submicron oil-in-water emulsions have important potential as a carrier for hydrophobic drugs. Conventional formulations, however, using phosphatidylcholine (PC) either with or without co-emulsifiers cannot generally be used because of their fast elimination from the blood by the reticuloendothelial system. To overcome this obstacle, we have included the amphipathic molecules of polyethylene glycol derivatives of phosphatidylethanolamine (PEG-PE) as a co-emulsifier into emulsions containing castor oil and phosphatidylcholine. Compared to the emulsions with phosphatidylcholine as the sole emulsifier or those with Tween 80 as the co-emulsifier, emulsions containing PEG-PE as a co-emulsifier of PC show a decreased liver uptake and increased blood circulation time in mice. Furthermore, they are much more stable than the conventional formulations as tested under various conditions. With superior stability and a prolonged circulation time in blood, emulsions containing amphipathic molecules of PEG may be useful for sustained release and targeted delivery of lipophilic drugs.

Keywords: Oil-in-water emulsion; Drug delivery system; Polyethylene glycol

1. Introduction

Oil in water emulsions (o/w) have been gaining more attention in the last few years due to their great potential as carriers for delivering lipophilic drugs to the human body, especially into the blood stream via parenteral administration (for recent review, see Benita and Levy, 1993). Lipid emulsion systems have many appealing properties as drug carriers; being biodegradable, biocompatible and physically stable. Compared to other commonly studied drug delivery systems, such as liposomes (Gregoriadis, 1988), lipid emulsions can be prepared in a large industrial scale and have been clinically well accepted. More importantly, these emulsions can solubilize a considerable amount of lipophilic drugs in the hydrophobic core of the emulsion particles.

Lipid emulsions (o/w) are commonly made by mixing an oil phase, often a long chain triglyceride, with an excess of aqueous solution in the presence of emulsifying agents. The properties of the emulsions, including particle size, stability and in vivo distribution, are primarily determined by the properties of emulsifier and the ratio of oil

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to emulsifying agents. Theoretically, any amphipathic or detergent-like molecules can be used as an emulsifier under the appropriate conditions. In reality, however, phospholipids with or without additional co-emulsifier are more commonly used than others for parenteral emulsions (Benita and Levy, 1993) because of their biocompatibility and low toxicity (as they are the major constituents of the cell membranes). Emulsions using phosphatidylcholine as the major emulsifier are stable under appropriate conditions (Davis et al., 1987), and have been widely used as a source of energy in parenteral nutrition (fat emulsion) (Rotenberg et al., 1991). However, the application of such formulations as drug delivery systems has been limited due to their short circulation time in blood. Upon intravenous administration, the emulsions, like many other types of particulate drug delivery systems, are rapidly removed from blood by mononucleophagocytes (more commonly called reticuloendothelial system (RES)). This appears to be advantageous in the delivery of drugs to the RES, but is problematic in the delivery of drugs to cells other than those in the RES. Great efforts in the past have been made to overcome the problem of RES uptake and to develop a delivery system that is able to circulate for a long time in blood, such that it will have sufficient time to find its target. Many of these attempts have been made using liposomes as a model system. It has been reported that inclusion of amphipathic molecules such as polyethylene glycol into the liposome bilayer has a profound effect on decreasing liposome affinity to the RES and increasing liposome blood circulation time (Klibanov et al., 1990; Allen et al., 1991). More importantly, long circulating, PEG-containing liposomes exhibit a high accumulation in the tumor site in tumor bearing mice, showing great potential as a carrier for tumor therapy (Papahadjopoulos et al., 1991).

The activity of amphipathic PEG in prolonging the circulation time of liposomes has prompted our attempt to explore the possibility of using these molecules as an emulsifier for emulsion preparations. In this report, we have systematically examined the physical properties of emulsions (o/w) stabilized by the amphipathic polyethylene glycols. The activity of PEG derivatives as a co-emulsifier in prolonging the blood circulation time of emulsions composed of castor oil and phosphatidylcholine (PC) was also examined. In comparison to the activity of Tween 80, one of the most commonly used co-emulsifiers for emulsion preparations, PEG derivatives have much better activity in stabilizing the emulsion structure and prolonging emulsion circulation time in blood. Our results suggest that amphipathic PEG derivatives may serve as a better emulsifier for emulsion preparations.

2. Materials and methods

2.1. Materials

Castor oil was purchased from Sigma. Tween 80 was from Fisher Scientific. Phosphatidylcholine (PC) from egg yolk was from Avanti Polar Lipids. $^{111}InCl_3$ (carrier-free) was from New England Nuclear. Diethylenetriaminepentaacetic acid stearylamide (DTPA-SA) and dioleoyl *N*-(monomethoxypolyethylene glycol succinyl)phosphatidylethanolamine (PEG-PE) were kindly provided by Dr Leaf Huang (Department of Pharmacology, University of Pittsburgh). The syntheses of these compounds have been described in previous reports (Kabalka et al., 1987; Klibanov et al., 1990). Mice were purchased from Harlan Sprague Dawley Inc.

2.2. Preparation of emulsions

Castor oil (1.0 mg) diluted in chloroform was mixed with PC and PEG derivatives or Tween 80 in different weight ratios. The organic solvent was evaporated under a stream of nitrogen gas. The lipid film was then vacuum desiccated at 4°C overnight to remove the residual organic solvent. 1 ml of phosphate-buffered saline (PBS, pH7.4) was then added and the mixture was allowed to hydrate for 1 h at room temperature. Lipid suspension was then vortexed and subsequently homogenized using a tissue tearer (Model 985-370, BioSpec Products, Inc.) at a speed of 22000 rpm. The average diameter of emulsion particles was

determined by using quasi elastic laser light scattering with a Coulter N4SD submicron particle analyzer and presented as a mean plus standard variation of unimodal analysis. For animal studies, a trace amount of ^{111}In-DTPA-SA was added into the lipid mixture as part of the emulsions. ^{111}In-DTPA-SA is one of the most commonly used radioactive markers for liposome biodistribution studies (Klibanov et al., 1991; Liu et al., 1992).

2.3. Stability of emulsions

The stability of emulsions was tested using two methods. The first is by measurement of the size change of emulsion particles using accelerated tests and long-term storage. For accelerated tests, emulsions were either incubated at high temperatures (30 min in water bath), repeat of freeze-thaw cycles (−20 to 25°C), or incubated for 3 h at 37°C at pH 1 (directly adjusted using 1 M HCl). The average diameter of emulsion particles for different preparations was measured before and after each treatment. For long-term storage tests, samples were kept at room temperature or 4°C. The mean diameter of emulsions at different storage time was recorded. The second method for stability testing was by centrifugation (Lundberg, 1994). Emulsions (200 μl, ^{111}In DTPA-SA labeled) were diluted to 700 μl with PBS (pH7.4) and the mixed solution was then centrifuged in small test tubes (6 × 50 mm, Fisher Scientific) for the designated time at 1000 × g. 20 μl of the centrifuged solution taken from the top was counted using a Beckman gamma-counter. The amount of radioactivity in this fraction was presented as the function of centrifugation time.

2.4. Biodistribution studies

Biodistribution studies were performed in mice (male, NIH Swiss, 20–25 g) as described previously (Liu et al., 1992). Emulsions (100 μl, containing total lipids of 0.152 mg for oil/PC/Tween 80 and 0.16 mg for oil/PC and oil/PC/PEG2000-PE) labeled with ^{111}In-DTPA-SA were injected into mice via the tail vein. 1 h later, the animals were killed and ^{111}In radioactivity in different organs were analyzed using a Beckman gamma-counter. The percent injected dose in blood was determined by assuming that the total volume of blood is 7.3% of the body weight (Wu et al., 1981). Blood contamination in other organs was corrected using correction factors obtained via ^{51}Cr-labeled red blood cells (Liu et al., 1992).

3. Results

In order to test whether PEG-PE has advantages over the existent emulsifiers, we have used oil/PC and oil/PC/Tween 80 as control groups. Oil/PC was chosen because it serves as a background for PEG-PE effect. Oil/PC/Tween 80 with a weight ratio of 1:0.4:0.12 has been believed to be one of the optimal ratios for the preparation of emulsions for drug delivery (Lundberg, 1994). In a series of experiments, hydrated oil/lipids mixture was homogenized using a tissue tearer. The average diameter of emulsion particles as the function of homogenization time is shown in Fig. 1. The longer the emulsions were homogenized, the smaller the average diameter of the emulsion particles. This diameter reached

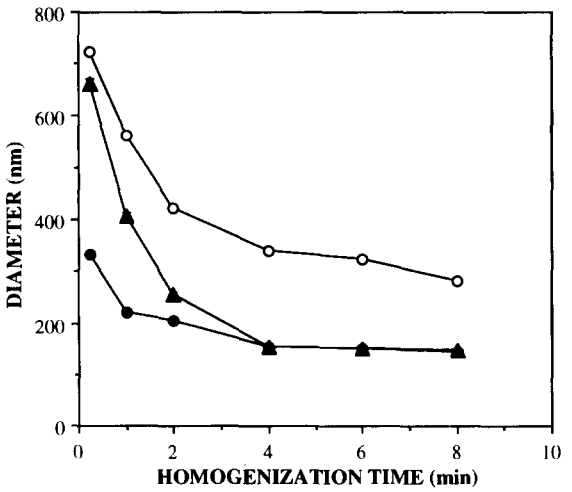

Fig. 1. Size change of emulsion particles as a function of homogenization time. (○) Oil/PC (1:0.6, weight ratio), (▲) oil/PC/Tween 80 (1:0.4:0.12, weight ratio) and (●) oil/PC/PEG2000-PE (1:0.2:0.4, weight ratio).

a plateau after homogenization for 4 min or longer. The final particle size after homogenization depends on the emulsion composition. For example, the average diameter of emulsion particles composed of oil and PC (1:0.6) was about 300 nm after 8 min of homogenization in comparison to 150 nm for emulsions where 2/3 of the PC was replaced by PEG (MW = 2000)-PE (oil/PC/PEG2000-PE = 1:0.2:0.4). Emulsions composed of oil/PC/Tween 80 (1:0.4:0.12, weight ratio) exhibit the same average diameter as that of emulsions containing PEG2000-PE. A marked effect of temperature was observed in emulsion preparations with PC as sole emulsifier (data not shown). The average diameter of emulsion particles of oil/PC (1:0.6) reached 190 nm after 4 min homogenization at 50°C. From these experiments, the standard homogenization conditions were established at 4 min (22,000 rpm) at room temperature for emulsions with dual emulsifiers, and 50°C for emulsions with PC as sole emulsifier.

The size of emulsion particles is generally a function of the oil to emulsifier ratio. To evaluate the effect of PEG 2000-PE concentration on particle size of emulsions, a series of samples with a constant weight ratio of 1:0.2 (oil/PC) were prepared with increasing concentration of PEG2000-PE. The results (Fig. 2) show a rapid decrease in particle size with increasing concentration of PEG 2000-PE, reaching a minimum mean diameter of about 150 nm at the ratio of 1:0.2:0.4

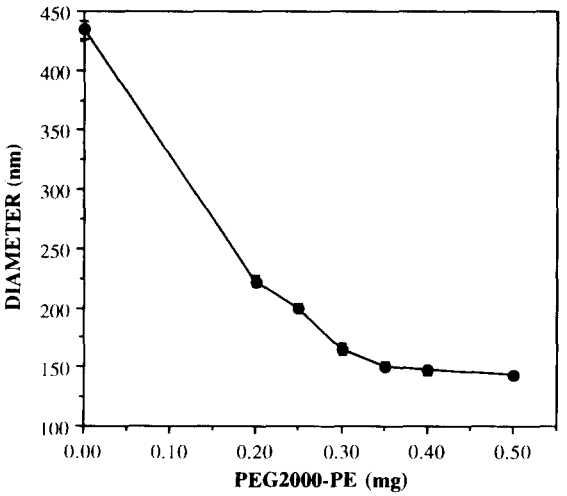

Fig. 2. Effect of PEG 2000-PE concentration on mean diameter of emulsion particles. 1 mg of caster oil was mixed with 0.2 mg of phosphatidylcholine in the presence of different amounts of PEG2000-PE and the emulsions were prepared as described in section 2. Lipid suspensions (total, 1 ml) were homogenized (22000 rpm) for 4 min at room temperature.

Table 1
Effect of different treatments on particle size of emulsions

Emulsions (weight ratio)	Diameter (nm)			
	No treatments	100°C [a] (1 h)	pH 1.0 [b] (3 h)	Freeze-thaw [c] (10 cycles)
Oil/PC (1.0:0.6)	198 ± 8	572 ± 12	199 ± 6	200 ± 9
Oil/PC/Tween 80 (1.0:0.4:0.12)	149 ± 4	1125 ± 10	150 ± 4	150 ± 1
Oil/PC/PEG1000-PE (1.0:0.2:0.4)	123 ± 6	125 ± 7	201 ± 9	124 ± 5
Oil/PC/PEG2000-PE (1.0:0.2:0.4)	144 ± 7	149 ± 3	150 ± 4	144 ± 8
Oil/PC/PEG3000-PE (1.0:0.2:0.4)	156 ± 5	158 ± 4	158 ± 4	155 ± 6
Oil/PC/PEG5000-PE (1.0:0.2:0.4)	163 ± 8	165 ± 6	162 ± 8	163 ± 7

[a] Emulsions (400 μl) were heated in boiling water for 1 h. The average diameter of emulsion particles was measured after the solution was cooled to room temperature. Data represent average ± standard variation of triplicate samples.
[b] The pH of the emulsion suspensions was directly titrated with a pH-meter to 1 by HCl (1 M). Emulsions were then incubated for 3 h at 37°C and the particle size was measured at room temperature ($n = 3$).
[c] Samples were freeze-thawed (−20 to 25°C) for 10 cycles before the particle size was measured ($n = 3$).

(oil/PC/PEG-PE, weight ratio). Further increase of PEG 2000-PE concentration did not significantly decrease the mean diameter of the emulsion particles. Therefore, the ratio of 1:0.2:0.4 (oil/PC/PEG-PE) was chosen for additional characterizations.

The effect of chain length of PEG in PEG-PE on the mean diameter of emulsion particles was also tested. PEG-PE with different chain lengths of PEG ranging from 1,000 to 5,000 was included in emulsions. The weight ratio of oil/PC/PEG-PE was kept at 1:0.2:0.4 for all emulsion preparations. Table 1 shows the mean diameter of emulsion particles containing PEG-PE with different PEG chain lengths. It is evident that the chain length of PEG under our experimental conditions does not affect the ultimate particle size. Emulsions containing either PEG1000-PE, PEG3000-PE or PEG5000-PE exhibit an average particle size of about 150 nm, indicating that they are all potent emulsifiers for emulsion preparation.

To evaluate the physical stability of the emulsions containing PEG-PE derivative as the co-emulsifier, we have systematically studied the stability of PEG2000-PE stabilized emulsions in comparison to that of oil/PC and oil/PC/Tween 80 under different conditions. In these experiments, change of the particle size was used as a stability indicator. It is clear from Fig. 3 that emulsions with all the compositions were very stable at temperature ranging from 20 to 70°C. No size change was observed after 30 min incubation at these temperatures either with or without co-emulsifier. However, size increase was observed when emulsions were incubated at higher temperatures. Depending on the emulsion composition, the average diameter of emulsion particles increased by approx. 50% for PC-stabilized emulsions when the temperature increased from 70 to 100°C. An approx. 8-fold increase in average diameter of emulsion particles was obtained when the optimal composition according to Lundberg (1994) was used (oil/PC/Tween 80 = 1:0.4:0.12). In contrast, no change of particle size was observed for emulsions stabilized by PEG2000-PE and PC (oil/PC/PEG2000-PE = 1:0.2:0.4), suggesting a stronger stabilization activity of PEG-PE than that of the traditional emulsifier such as Tween 80.

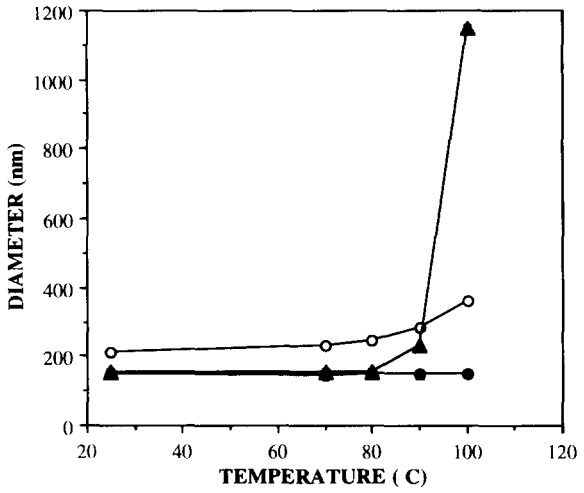

Fig. 3. Stability of emulsions at different temperature. Emulsions were incubated for 30 min at designated temperature (in water bath). Particle size was measured after the solutions were cooled to room temperature. (○) Oil/PC (10:6, prepared at 50°C), (▲) oil/PC/Tween 80 (1:0.4:0.12) and (●) oil/PC/PEG2000-PE (1:0.2:0.4). All ratios are presented as weight ratios ($n = 3$).

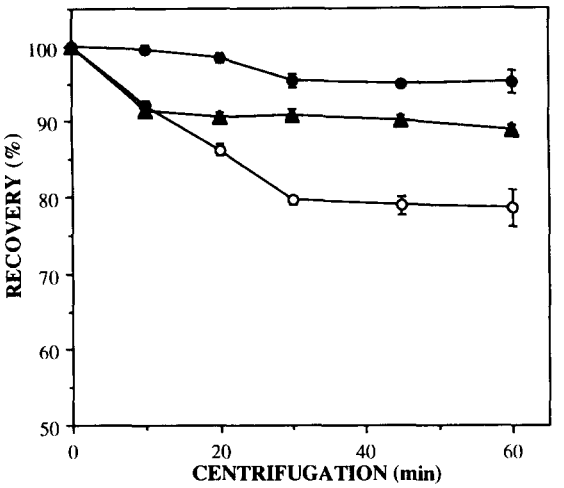

Fig. 4. Stability of emulsions assayed by centrifugation. Percent remaining of emulsions at the top of the supernatant after repeated centrifugation was plotted as the function of centrifugation time. (○) Oil/PC (1:0.6), (▲) oil/PC/Tween 80 (1:0.4:0.12) and (●) oil/PC/PEG2000-PE (1:0.2:0.4). Data represent the average of triplicate measurements with standard variation.

To confirm that the PEG-PE is a better co-emulsifier than Tween 80 in stabilizing the emulsion particles, emulsions were centrifuged at 1000 $\times g$ at room temperature using the conditions as described by Lundberg (1994). The results from the centrifugation experiments are shown in Fig. 4. There was about 20% loss of materials for emulsions without co-emulsifier, compared to 10% of loss for emulsions with Tween 80 as a co-emulsifier, after centrifugation for 30 min. Repeated centrifugation of emulsions containing PEG2000-PE showed minimal loss. Less than 5% of the emulsions were lost after centrifugation for 60 min. The particle size of PEG2000-PE stabilized emulsions were constant upon storage either at room temperature or 4°C at least for 3 months (ongoing test, data not shown).

Among the other stability tests, including low pH (pH 1.0) and freeze-thaw (10 cycles), no difference with or without coemulsifier was observed (Table 1). PEG1000-PE was as an efficient stabilizer as those with longer PEG chain length (Table 1).

The superior property of PEG-PE in decreasing the liver uptake of emulsions and prolonging the circulation time is demonstrated in Fig. 5. 1 h after intravenous injection of emulsions into mice, about 60% of the injected dose was already in the liver, while only about 25% left in the blood for PC-stabilized emulsions. Conversely, approx. 20% of the injected dose was recovered from the liver and over 60% was still remaining in blood at the same time period for emulsions containing PEG2000-PE. Tween 80 exhibited low activities in prolonging emulsion circulation time, with 45% in the liver and 25% in the blood. Other organs including lung, spleen, kidney and heart had a minimal uptake.

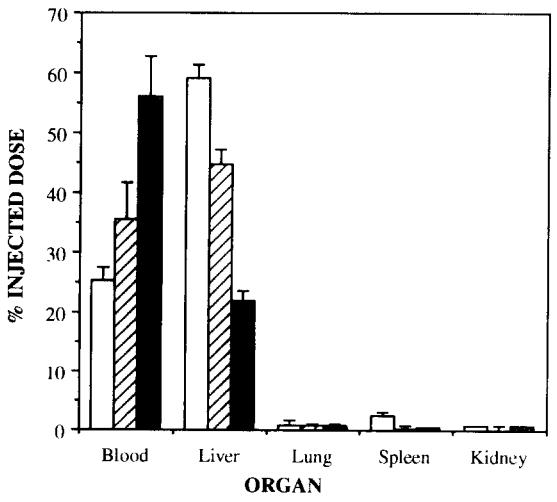

Fig. 5. Biodistribution of emulsions. (□) Oil/PC (1:0.6), (▨) oil/PC/Tween 80 and (▩) oil/PC/PEG2000-PE (1:0.2:0.4). Data represent average with standard variation of three animals.

4. Discussion

The goal of the studies presented in this report was to study the effect of amphipathic molecules of PEG-PE on stabilizing and prolonging the circulation time of oil-in-water emulsions that are suitable for drug delivery. As summarized in Table 1, PEG-PE stabilized emulsions exhibit a superior activity to those with PC as a sole emulsifier, or with Tween 80 as a co-emulsifier in resisting harsh conditions such as high temperature and centrifugation. The function of PEG2000-PE in decreasing the affinity of emulsions to the RES and prolonging the blood circulation time (Fig. 5) was also demonstrated.

It has been known for many years that PEG can prolong the circulation time of proteins once they are covalently conjugated to the protein molecules (Beauchamp et al., 1983; Knauf et al., 1988). It is only recently, however, that PEG has been used to prolong the circulation time of particulate drug carriers such as liposomes (Klibanov et al., 1990; Allen et al., 1991), nanoparticles (Gref et al., 1994) and oil-in-water emulsions (Wheeler et al., 1994; Liu and Liu, 1995). The universal function of PEG in these cases is the prevention of PEG-containing substances from recognition by the immune system, especially by the macrophages in the RES, thus, resulting in prolonged blood circulation time. The mechanism for this function, even though still lacking direct evidence, is believed to involve the activity of PEG in decreasing the affinity of those substances to the RES by preventing the op-

sonization of the serum components (opsonins) or decreasing the hydrophobic interactions of those substances with the RES (Torchilin et al., 1994). It is likely that the function of PEG in prolonging the circulation time of emulsions as demonstrated in Fig. 5 is through the same mechanism.

The size of emulsion particles is important with regard to drug loading capacity and blood circulation time. Theoretically, the larger the emulsion particles the more drug molecules can be incorporated into the hydrophobic core of the emulsion particles, and more drug molecules each particle can deliver to the target cell. However, it has been previously demonstrated that liposomes with a diameter of greater than 300 nm preferentially accumulate in the spleen regardless of the inclusion of amphipathic molecules such as ganglioside GM1 or PEG-PE which otherwise prolong the liposome circulation time in blood (Papahadjopoulos et al., 1991). The size range of PEG- containing liposomes with prolonged circulation time in blood is between 100 and 300 nm (Klibanov et al., 1991). As summarized in Table 1, the average diameter of the emulsions used in our experiments is around 150 nm, which is within the favorable size range for long circulation. The chain length of PEG does not seem to play a critical role in affecting the ultimate mean diameter of the emulsion particles under the experimental conditions. We have found that the utilization of the tissue tearer as a homogenizer for the preparation of emulsions is very convenient, especially for small quantities. As low as 100 μl of emulsion can be easily prepared if an appropriate probe head is used.

Stability is another important concern for parenteral emulsions. For example, sedimentation and creaming tendencies of the emulsions during long-term storage increase the size to a mean diameter over 5 μm, thus, promoting the formation of pulmonary emboli (Burnham et al., 1983). For this reason measurement of particle size becomes the most important parameter for emulsion stability studies, and is normally tested using accelerated testing conditions (Benita and Levy, 1993), including heating, freezing-thaw cycles and centrifugation. As summarized in Table 1 and Fig. 4, PEG-PE stabilized emulsions perform much better than those with PC as a sole emulsifier or using Tween 80 as the co-emulsifier. No size change was observed for emulsions containing PC and PEG-PE as the emulsifiers (oil/PC/PEG-PE = 1:0.2:0.4) after 10 cycles of freeze-thaw, boiling for 1 h, or treatment at low pH (pH 1.0), in comparison to those containing either PC or PC plus Tween 80 as emulsifiers. Emulsions containing Tween 80 are quite sensitive to high temperature (Table 1 and Fig. 3). Over 7-fold increase in mean diameter of emulsion particles was seen after boiling the sample for 30 min. Over 2-fold increase of mean diameter was also observed under the same conditions for emulsions with PC as a sole emulsifier. The results from these physical tests and our long-term storage test suggest that PEG-PE-containing emulsions have better stability and longer shelf-lives.

Circulation time in blood is a very important factor in delivering drugs to the target other than the RES. This is simply because the drug carrier has to circulate long enough in blood to reach the target site before eliminated from the circulation. The advantages of using a carrier with prolonged circulation time in tumor therapy have been demonstrated by many groups (for review, see Allen, 1994). The enhanced blood residence time of emulsions using PEG-PE as a co-emulsifier described in this report (Fig. 5) is advantageous to the conventional formulation for targeted delivery of lipophilic drugs. The targeting ability of such emulsions using different ligands as targeting molecules is under investigation in our laboratory.

In summary, we have shown that emulsions stabilized using PEG-PE as a co-emulsifier have better characteristics than the conventional formulations for drug delivery. Many applications of such formulations can be foreseen in the future. It is possible to use such emulsion systems to deliver lipophilic drug molecules to cells or tissues other than those in the RES. A recent report on high tumor accumulation of long circulating liposomes in tumor bearing mice suggest that PEG-PE stabilized emulsions may be used to target lipophilic drugs to tumor cells for cancer therapy. These emulsions may also be used as

carrier for sustained release. Targeting emulsions to tissues other than the RES by conjugating antibodies to the emulsion surface are also achievable with PEG-PE stabilized emulsions. Formulating certain fluorocarbon oils (Biro and Blais, 1987) for the purpose of blood substitution using PEG-PE may improve oxygen delivery efficiency. The potential of using PEG-PE as the emulsifier for other types of application is yet to be realized.

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