VOLUME 71, NO. 3
(Completing vol. 71)
28 APRIL 2001
71(3) 221–372
ISSN: 0168-3659

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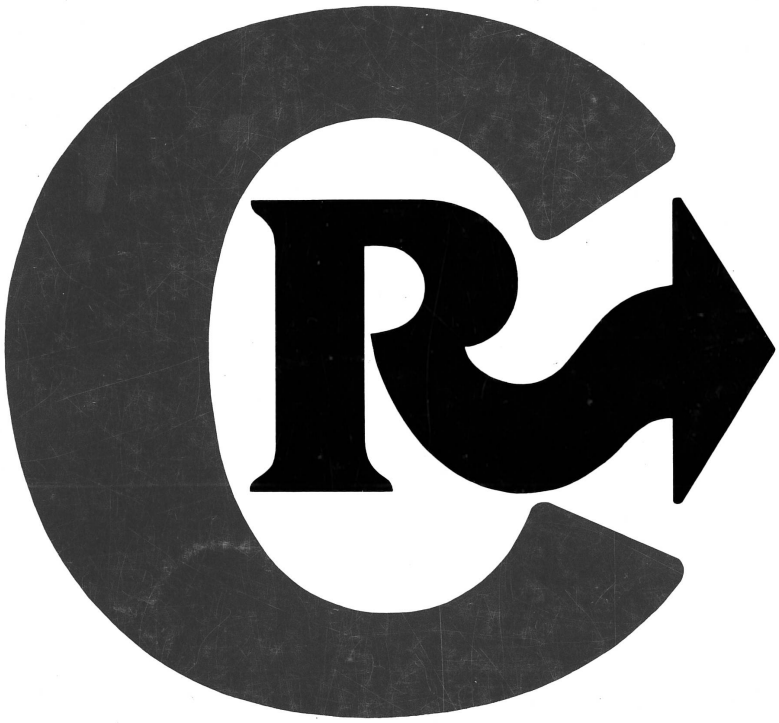
journal of controlled release

OFFICIAL JOURNAL OF THE CONTROLLED RELEASE SOCIETY
AND THE JAPANESE SOCIETY OF DRUG DELIVERY SYSTEM



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journal of controlled release

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GENE DELIVERY

Oil components modulate physical characteristics and function of the natural oil emulsions as drug or gene delivery system

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Received 14 September 2000; accepted 24 November 2000

Abstract

Oil-in-water (o/w) type lipid emulsions were formulated by using 18 different natural oils and egg phosphatidylcholine (egg PC) to investigate how emulsion particle size and stability change with different oils. Cottonseed, linseed and evening primrose oils formed emulsions with very large and unstable particles. Squalene, light mineral oil and jojoba bean oil formed stable emulsions with small particles. The remaining natural oils formed moderately stable emulsions. Emulsions with smaller initial particle size were more stable than those with larger particles. The correlation between emulsion size made with different oils and two physical properties of the oils was also investigated. The o/w interfacial tension and particle size of the emulsion were inversely proportional. The effect of viscosity was less pronounced. To study how the oil component in the emulsion modulates the in vitro release characteristics of lipophilic drugs, three different emulsions loaded with two different drugs were prepared. Squalene, soybean oil and linseed oil emulsions represented the most, medium and the least stable systems, respectively. For the lipophilic drugs, release was the slowest from the most stable squalene emulsion, followed by soybean oil and then by linseed oil emulsions. Cationic emulsions were also prepared with the above three different oils as gene carriers. In vitro transfection activity was the highest for the most stable squalene emulsion followed by soybean oil and then by linseed oil emulsions. Even though the in vitro transfection activity of emulsions were lower than the liposome in the absence of serum, the activity of squalene emulsion, for instance, was ca. 30 times higher than that of liposome in the presence of 80% (v/v) serum. In conclusion, the choice of oil component in o/w emulsion is important in formulating emulsion-based drug or gene delivery systems.

Keywords: Cationic lipid; Egg phosphatidylcholine; Interfacial tension; Squalene; Stability; Transfection; Vegetable and animal oils

1. Introduction

Lipid emulsions have been widely used in pharmaceutical and medical fields as drug carriers. To be applied as parenteral, oral or topical formulations, emulsions must be physically stable and non-toxic [1–4]. Since o/w emulsion is a thermodynamically unstable system, it is bound to phase-separate with time. One of the most important pre-requisites in formulating an emulsion, therefore, is to maintain its physical stability. Emulsion size stability is defined as the ability to maintain initial particle size distribution without undergoing phase separation. Many factors are known to change the stability of emul-

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0168-3659/01/$ – see front matter
PII: S0168-3659(00)00363-1

sions. To mention a few, components, composition, preparation method and formulation conditions are important factors ([1,2] and Refs. cited therein). It has been a long-standing aim to formulate stable emulsions with small particles since the stabilization of emulsion could be achieved by particle size reduction [5]. Our aim is to produce a stable emulsion that has small particles using natural oils. We also tried to elucidate what physical or chemical factors influence the emulsion size stability. Specifically, we investigated how different oils affect the emulsion particle size and stability. We also investigated whether there is a correlation between emulsion stability and initial emulsion particle size and its distribution. To this end, 18 different natural oils, including vegetable and animal oils, were chosen to formulate o/w type emulsions. Many oils that are frequently used in producing parenteral emulsions have been included in this study.

There are indications in the literature that the size stability of the emulsion changes greatly by changing oils [6,7]. The o/w interfacial tension [6,7] or the intrinsic viscosity [6] of the oils have a correlation with the particle size of the emulsions when binary and more complicated oil systems were used. The main stabilizing factor, however, is not certain. In our dissertation, we also attempted to correlate these two physical properties of oils with the stability of emulsions.

We also investigated how the emulsions made with different oils could influence the in vitro release properties of lipophilic drugs. To this end, squalene, soybean oil and linseed oil were chosen to form emulsions wherein a lipophilic drug is loaded in the discontinuous oil phase. The results show that the in vitro release rates of lipophilic drugs are significantly different for three emulsion systems.

Recently, we have reported that cationic o/w lipid emulsions can become efficient in vitro and in vivo gene carriers [8,9]. We have also demonstrated that the transfection activity depends greatly on the composition of cationic emulsifiers and co-emulsifiers (Submitted for publication). As an on-going research project to develop efficient cationic emulsion-based gene carriers, we have evaluated whether the transfection efficiency can be enhanced by altering oil components in the cationic emulsion in this paper. Our findings demonstrate that emulsion stability as well as the transfection activity is greatly dependent on the choice of the oil component in the emulsion.

2. Materials and methods

2.1. Materials

Borage, castor, coconut, corn, cottonseed, evening primrose, fish, jojoba bean, lard, linseed, mineral, olive, peanut, safflower seed, sesame, soybean, sunflower and wheat germ oils and squalene were purchased from Sigma Chemical Company (St Louis, MO) and used without further purification. L-α-phosphatidylcholine from dried egg-yolk (egg PC, 60% pure by TLC) and 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid sodium salt (diclofenac) were also from Sigma. Rifampicin was kindly supplied by Yuhan Pharmaceutical Company, Ltd. (Korea). 1,2-Dioleoyl-*sn*-glycero-3-trimethylammonium-propane (DOTAP) was from Avanti Polar Lipids, (Alabaster, AL). Water was purified by using a water purification system (Milli-Q Plus; Millipore Corp., Bedford, MA).

2.2. Preparation of emulsions

The o/w emulsions contained 100 μl/ml of oils and various concentrations (1–48 mg/ml) of emulsifiers. In most of the studies, egg PC was used as an emulsifier. For in vitro gene delivery experiments, DOTAP at 24 mg/ml was used as a cationic emulsifier. The emulsifier was weighed and mixed with deionized distilled water. The mixture was sonicated until clear by using a probe type sonicator (High Intensity Ultrasonic Processor, 600 W model, Sonics and Materials, Danbury, CT) to form a liposome solution in an ice/water bath. The aqueous phase was added to oil and sonicated in an ice/water bath for ca. 4 min to form emulsions. The emulsions were kept at room temperature for further experiments, unless otherwise specified.

2.3. Determination of the particle size of emulsion and emulsion/DNA complex

The average particle size of the emulsions as well

as emulsion/DNA complex was determined by quasielastic laser light scattering with a Malvern Zetasizer® (Malvern Instruments Limited, UK). Emulsion or emulsion/DNA complex was diluted by 300 times before the measurement. The size measurements were performed 1 day after the emulsions were prepared and at preset intervals to monitor the emulsion size stability. The size determination was repeated three times/sample for at least three samples comprising an identical composition. It is conventional to show the size distribution function of the emulsion as shown in Fig. 1A or B. In this paper, however, it would be space consuming to show the distribution function of all the emulsions especially since we intend to show the size distribution function change with time. An alternative way to display data would be to show the average size values only sacrificing the information on size heterogeneity in a single system. To display the data efficiently, we adopted a new method to display size distribution function data clearly by using the following procedure in this paper. The size distribution function followed a so-called log-normal distribution function

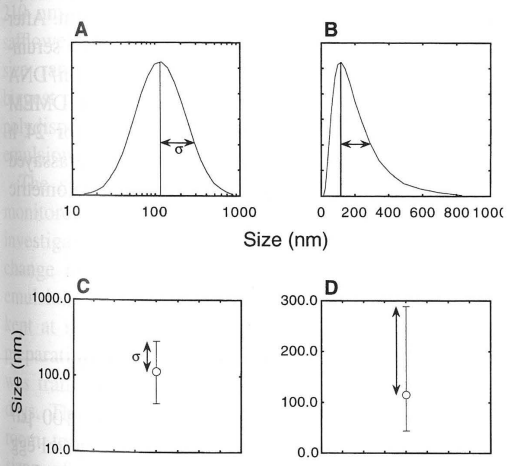

Fig. 1. Typical size distribution of the emulsion function in (A) logarithmic axis mode. When the horizontal axis is converted to the normal axis mode, the distribution looks positively-skewed as shown in (B). The polydispersity is the variance of the log-normal distribution function. From the polydispersity, standard deviation can be calculated and is shown as the bars in (C). Therefore, the bars in the size distribution function in normal scale (D) are calculated from polydispersity in the log-normal size distribution function.

for our emulsion systems as shown in Fig. 1 [10]. The fact that the size distribution of our emulsion systems displays a unimodal log-normal distribution has also been confirmed by using a dynamic laser light scattering device (Model BI-9000 AT Digital Correlator, Brookhaven Instruments Corp., Holtsville, NY). Log-normal distribution function shows a Gaussian distribution in logarithmic axis mode. The variance of the function is called polydispersity. The bars in Fig. 1C in logarithmic axis mode indicate the standard deviation that corresponds to (polydispersity)$^{1/2}$. In the normal axis mode, the distribution function is positively-skewed (Fig. 1B). The bars in normal axis mode, also positively-skewed, represent the standard deviation (σ) of the log-normal size distribution function (Fig. 1D). Therefore, in this paper, the average particle size and standard deviation will represent the size distribution function of an emulsion system.

2.4. Interfacial tension and viscosity measurements

Interfacial tension between oil and aqueous subphase was measured by using du Nouy type surface tensiometer (Fisher Surface Tensiomat, Model 21, Fisher Scientific Company, Pittsburgh, PA). The interfacial tension was measured from more dense liquid, aqueous solution to less dense liquid, oil by slowly lifting the platinum–iridium ring while increasing the scale to maintain the zero reading. The scale reading at the breaking point of the interfacial film was the apparent interfacial tension. In this paper, apparent values are reported. The measurement was performed at 22±2°C.

The lower and upper phases were deionized distilled water and pure oil, respectively, without emulsifiers. In some cases, especially for the less hydrophobic oils, some of the components diffused from the oil phase to the aqueous sub-phase with time. We measured the o/w interfacial tension within 2 min from the contact time between the two phases. Therefore, the interfacial tension in this paper does not represent the equilibrium values.

The viscosity of the oils was measured by using a kinematic viscometer at 22±2°C (Cannon-Fenske Type, Calibrated, Cat. No. 13-617E, Size 200, Fisher Scientific, Pittsburgh, PA).

2.5. In vitro release of lipophilic drugs

A lipophilic drug (3 mg) was solubilized completely in 1 ml oil and mixed with 9 ml of a liposome solution of 18 mg/ml egg PC. Linseed oil, soybean oil and squalene were chosen as the oil phase. The mixture was sonicated in an ice/water bath for ca. 4 min to form emulsions. The emulsions were kept at room temperature. For the release experiment, 2 ml of emulsion was pipetted into a dialysis bag (Spectra/Por® membranes, MWCO: 3500, Spectrum Medical Industries, Inc., Los Angeles, CA). The bag was sealed and immersed in 10 ml phosphate-buffered saline (PBS) at pH 7.4 at room temperature. For the emulsions containing rifampicin, ascorbic acid (0.5 mM) was added in PBS to prevent rifampicin oxidation during the release experiment. The tubes were incubated in a shaking water bath (Vision Co. Ltd., Korea) at 37°C at a shaking frequency of 150 rpm. The release medium was exchanged totally with fresh PBS solution of an equal volume when the concentration of the released drugs was determined. Concentration of rifampicin was determined by measuring the fluorescence emission (K2 Multifrequency Phase Fluorometer, ISS Inc., Champaign, IL) at 480 nm (λ_{ex}=370 nm). Before the fluorescence measurements, 2 ml of released medium was reacted with 0.5 ml each of 0.1 N sodium hydroxide aqueous solution and hydrogen peroxide for 2 to 3 h [11]. The concentration of released diclofenac was determined by performing high performance liquid chromatography (HPLC) as described elsewhere [12]. Briefly, HPLC system consisted of a SP8810 precision isocratic pump (Spectra-Physics Inc., San Jose, CA). Mobile phase for diclofenac consisted of 50% (w/w) each of acetonitrile and water with a trace of acetic acid to adjust pH to 3.3. The flow rate of the mobile phase was controlled to 1 ml/min. Waters μBondpack™ C18 Column (3.9 mm×300 mm, Waters Corp., Milford, MA) was used. The column effluent was monitored at 230 nm by using Spectra 100 variable wavelength detector (Spectra-Physics).

2.6. In vitro gene transfer

A derivative of simian kidney cell line, COS-1, was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL/Life Technologies, New York, NY) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37°C in a humidified 5% carbon dioxide incubator. Cells were seeded at 2×10^4 cells per well onto 96-well plates 12 h before transfection. Cells were ca. 70–80% confluent at the time of transfection.

The plasmid pCMV-beta encoding *Escherichia coli* (*E. coli*) *lac*Z (β-galactosidase) gene expression plasmid driven by the human cytomegalovirus immediate-early promoter was purchased from Clontech Laboratories (Palo Alto, CA). The plasmid was amplified in the *E. coli* DH5-α strain and purified by using a Qiagen mega-kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instruction. DNA purity was determined by agarose gel electrophoresis and by measuring optical density. DNA having $OD_{260}/OD_{280} \geq 1.8$ was used. pCMV-beta (0.5 μg) and 0.0556 μl of emulsion (corresponding to 2 μg DOTAP) each diluted with 20 μl serum-free DMEM were mixed to form a complex. After washing the COS-1 cells with serum-free DMEM, 40 μl of complex and 160 μl serum free DMEM were added to each well. To test effect of serum, 160 μl FBS was added instead of serum free medium. After 1 h of incubation, the cells were washed with serum-free media to remove the remaining emulsion/DNA complexes. The cells were fed again with DMEM containing 10% (v/v) FBS and cultured for 24 h after transfection. The transfected cells were assayed for β-galactosidase activity using a photometric assay.

3. Results

3.1. Emulsion particle size and size stability

The o/w emulsions with different oils (100 μl/ml) were prepared by sonication with 12 mg/ml egg PC as an emulsifier in water. The average size of the emulsion and standard deviation (=(polydispersity)$^{1/2}$ in logarithmic axis mode) are summarized in Table 1. The average sizes of the emulsions were in the range 200–400 nm. The size of the emulsions in 1 day after preparation at room temperature varied depending on oils. Squalene formed the smallest emulsions (190 nm, polydispersity 0.13). Jojoba oil

Table 1
The average particle size and polydispersity of natural oil emulsions and the o/w interfacial tension (22±2°C) and viscosity of oils (20±2°C)

Oil	Size (nm)	Polydispersity	Surface tension (dyne/cm)	Viscosity (cSt/s)
Castor oil	273.8	0.306	12.8	723.0
Coconut oil	246.3	0.216	–	–
Corn oil	261.0	0.220	22.0	70.4
Cottonseed oil	263.6	0.199	23.8	62.3
Evening primrose oil	247.1	0.257	–	–
Fish oil	247.0	0.251	15.6	42.0
Jojoba oil	224.7	0.213	25.8	43.0
Lard oil	282.7	0.271	18.6	73.4
Linseed oil	354.9	0.116	1.8	51.2
Olive oil	263.2	0.195	23.2	84.0
Peanut oil	256.8	0.212	23.3	66.1
Safflower oil	283.6	0.225	22.0	54.2
Sesame oil	263.3	0.246	26.0	62.9
Soybean oil	249.7	0.279	14.0	69.3
Squalene	191.7	0.125	33.9	15.9
Sunflower oil	249.2	0.185	26.5	47.4
Wheatgerm oil	253.2	0.217	24.0	63.1
Mineral oil	209.9	0.154	–	37.8

and light mineral oil yielded the emulsions with average sizes of 225 nm (polydispersity 0.21) and 210 nm (polydispersity 0.15), respectively. Castor, safflower and lard oils formed the emulsions in the size range 270–285 nm. The emulsion with the biggest particles was formed by linseed oil (355 nm, polydispersity 0.12). The rest of the oils formed the emulsions in the size range 240–270 nm.

The size and distribution of the emulsions were monitored for 20 days at room temperature to investigate the size stability of the emulsions. Size change as a function of time for six representative emulsions is shown in Fig. 2. The emulsions were kept at room temperature for the first 10 days after preparation. After day 10, a portion of each emulsion was transferred to a 4°C refrigerator for another 10 days. The rest of the emulsion was kept further at room temperature for an additional 10 days. The sizes of the emulsions with cottonseed, evening primrose and linseed oils grew further ca. 10 days after preparation when stored at room temperature. The size of these emulsions did not vary significantly if they were stored at 4°C after the first 10-day storage period at room temperature. In the case of linseed oil emulsion, phase separation was observed by day 20. The sizes of the squalene and jojoba oil emulsions did not change with time for the first 20 days beyond the statistical error range. The castor and lard oil emulsions were stable at 4°C, but the polydispersity became slightly bigger (0.3–0.4) with time at room temperature (data not shown). The coconut, corn, fish, olive, peanut, safflower, sesame, sunflower, wheatgerm and light mineral oil emulsions stayed stable for 20 days at both temperatures. In general, the emulsion whose initial size was bigger became unstable more rapidly whereas those with smaller initial size remained stable for the duration of the experiments. Also, the size variation of the emulsions was less at 4°C than at room temperature. We also have performed preliminary experiments on the stability of the squalene, soybean oil and linseed oil emulsions at 37, 100 and 120°C. The results show that the linseed oil emulsion was unstable at these three temperatures. Phase separation of the linseed oil emulsion was observed in ca. 5 days at 37°C and in 5 min at 100 and 120°C. The soybean oil emulsion was stable for 20 days at 37°C without any size change. At 100 and 120°C, however, the particle size became bigger (400 nm) in 10 min, but phase separation was not observed. The squalene emulsion stayed stable for 20 days at 37°C and for 20 min at 100 and 120°C. We also confirmed

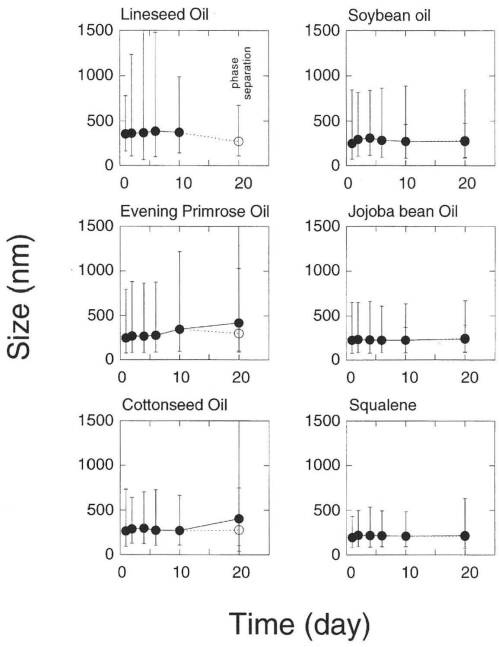

Fig. 2. Time-dependent changes in average particle size and its distribution of emulsions prepared with different oils. Egg PC (12 mg/ml) was used as an emulsifier. The emulsions were kept at room temperature for the first 10 days. At day 10, a portion of each emulsion was transferred to and kept in a 4°C refrigerator for the rest of the experiment (open circles). The rest of the emulsion was stored further at room temperature (filled circles).

that squalene emulsion could be autoclaved without sacrificing the emulsion stability.

3.2. Dependence of emulsion stability on emulsifier concentration and choice of oil

In forming an emulsion, it is essential to add an appropriate amount of emulsifier into oil and water to stabilize the system. The concentration of the emulsifier is one of the important factors in determining the emulsion size stability. Among the emulsions prepared with different oils, squalene emulsion had the smallest average size while the linseed oil emulsion had the biggest (Table 1 and Fig. 2). Therefore, we chose these two systems to investigate the correlation between emulsifier concentration and particle size in the emulsion. We also selected soybean oil, which forms medium-sized particles, for comparison. For these three different systems, we prepared emulsions at three different egg PC concentrations, 3, 12 and 30 mg/ml, to observe the emulsion stability at different emulsifier concentrations by monitoring the average particle size change for 20 days (Fig. 3). When the egg PC concentration was 3 mg/ml, the average particle sizes of the linseed, soybean, squalene emulsions were 647, 440 and 324.6 nm, respectively, immediately after preparation. Particle size of the linseed oil emulsion was the biggest among the three different emulsion systems for 10 days, and phase separation was observed in 20 days. The soybean oil emulsion was medium in size among the three and the size distribution became broad in a few days after preparation. In 20 days, phase separation was also observed in soybean oil emulsion. Squalene emulsion had the smallest particle size and narrow size distribution for 20 days. At 12 mg/ml egg PC, the average particle size of the three emulsions was much smaller with improved size stability than at 3

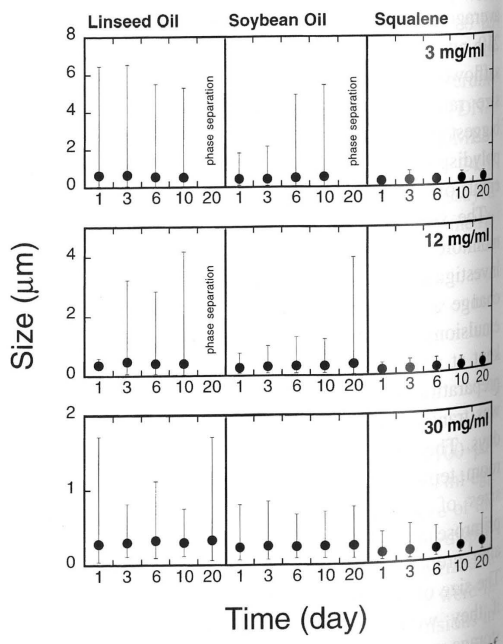

Fig. 3. Changes in the average particle size and its distribution of the linseed oil, soybean oil and squalene emulsions at 3 mg/ml, 12 mg/ml and 30 mg/ml of egg PC concentrations.

mg/ml egg PC. Linseed oil emulsion was unstable whereas squalene emulsion was stable for 20 days. Size distribution of soybean oil emulsion became broad in 20 days. Unlike linseed oil emulsion, however, phase separation was not observed in soybean oil emulsion for the duration of the experiment. Squalene emulsion stayed stable with no apparent size changes for 20 days. At the emulsifier concentration of 30 mg/ml, size stability of the soybean oil and squalene emulsions was greatly enhanced. Even though phase separation was not observed for linseed oil emulsion for 20 days, the size distribution became broader with time. For any given oil systems, emulsion has smaller size and higher stability at higher emulsifier concentrations. Among the three emulsions, squalene emulsion was the most stable with small particles at any given emulsifier concentrations.

3.3. Oil/water interfacial tension and viscosity of the oil

There are many physical and chemical properties of the oils that could regulate the size and stability of the emulsions. Recently, Jumaa and co-workers have addressed that the o/w interfacial tension [6,7] and the intrinsic viscosity [7] of the oils are considered two of the most important physical properties that control emulsion particle size. To test if their observation could be reproduced in our oil systems, we have determined the oil/water interfacial tension and viscosity of the oils and the relationship between these physical properties and the particle size of the emulsions (Table 1).

Linseed oil had the lowest interfacial tension whereas squalene had the highest interfacial tension against water among the studied oils. The o/w interfacial tension was plotted against the size of the emulsion made of that particular oil by using egg PC as an emulsifier (12 mg/ml) in Fig. 4A. We note that o/w interfacial tension represents the values without emulsifiers. The interfacial tension and the particle size of the emulsion, immediately after preparation, were inversely proportional. When the o/w interfacial tension was the biggest, as in squalene, the size of the emulsion was the smallest. The interfacial tension was the smallest for linseed oil that produced the emulsion with the biggest particle size. Since

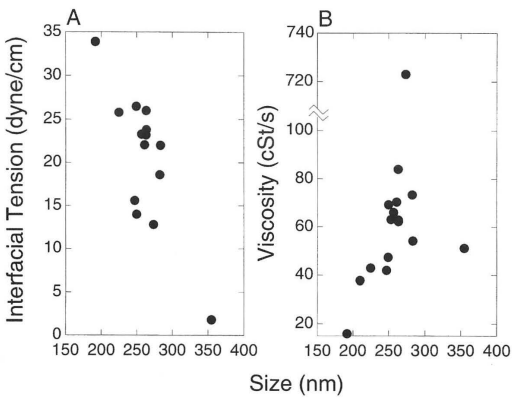

Fig. 4. Correlation between the emulsion size and (A) the o/w interfacial tension and (B) viscosity. The emulsions were composed of natural oils (100 μl/ml) and egg PC (12 mg/ml). The interfacial tension was measured between water and pure natural oil that was used to form the corresponding emulsion at 22±2°C. Viscosity of pure oils were measured at 20±2°C.

more hydrophobic oil has a larger oil/water interfacial tension, it is possible to infer that more hydrophobic oil forms emulsion with smaller particles when egg PC is used as an emulsifier.

Similarly, correlation between viscosity of oils and the particle size of the emulsion was evaluated. Castor oil is one of the most viscous oils (Table 1). Squalene was the least viscous among the studied oils. The viscosity of the oils was plotted against the size of the emulsion containing egg PC as an emulsifier (12 mg/ml) in Fig. 4B. In general, no correlation between viscosity of the oils and size of the emulsions was apparent. In the case of the linseed oil emulsion, the emulsion particle size was the biggest among the investigated oil systems although the viscosity of linseed oil was smaller than corn or olive oil. The viscosity of castor oil was more than 10 times bigger than other oils studied. The average particle size of the castor oil emulsion was ca. 280 nm, which was much smaller than linseed oil emulsion (355 nm).

3.4. In vitro release of lipophilic drugs

A lipophilic drug, rifampicin or diclofenac (0.3 mg/ml) was loaded in linseed oil, soybean oil or squalene emulsion to investigate the effect of oils on

the in vitro release characteristics. Egg PC at 18 mg/ml was used as an emulsifier. Release of each drug dissolved in water (0.3 mg/ml) was also investigated as a control experiment. The release rate of rifampicin from the emulsions depended on the identity of the oils (Fig. 5A). The rate was the fastest from the linseed oil emulsion, followed by soybean oil and then by squalene emulsions. Cumulative rifampicin release from each emulsion increased smoothly as a function of time without showing initial burst. The release rates of rifampicin from the three emulsions were slower than that from the aqueous solution.

The release rate of diclofenac depended on the identity of the oils in a lesser degree (Fig. 5B). The initial release rate of diclofenac from the emulsions was faster than that of rifampicin. Whereas release of rifampicin from the emulsions did not show initial burst, initial burst of diclofenac release was followed by a slow drug release. Among the emulsions, the rate was the fastest for the linseed oil emulsion. The rate was virtually identical for soybean oil and squalene emulsions. Release of diclofenac from the aqueous solution was faster (50% release in 1 h) than from all three emulsions.

To eliminate the possibility that the release rate is governed by the difference in the o/w partition coefficient of drugs, the partition coefficient of rifampicin was measured between oil and water containing 1 mg/ml egg PC for linseed oil, soybean oil and squalene. The o/w partition coefficients of rifampicin were 2, 1.3 and 1, respectively. Since the o/w partition coefficient of rifampicin was the biggest from the fastest releasing linseed oil emulsion, the difference in the release rates from three different emulsions does not originate from the difference in o/w partition coefficient in case the loaded drug was rifampicin [2].

The emulsion particle size and stability of the emulsions containing lipophilic drugs were also studied (Fig. 6). The particle size of the linseed oil emulsion containing rifampicin was ca. 500 nm immediately after preparation. The phase separation was observed in ca. 10 days. The average particle sizes of the soybean oil and squalene emulsion containing rifampicin were ca. 250 nm and 170 nm, respectively, and did not change for 25 days. The emulsions containing diclofenac were more stable

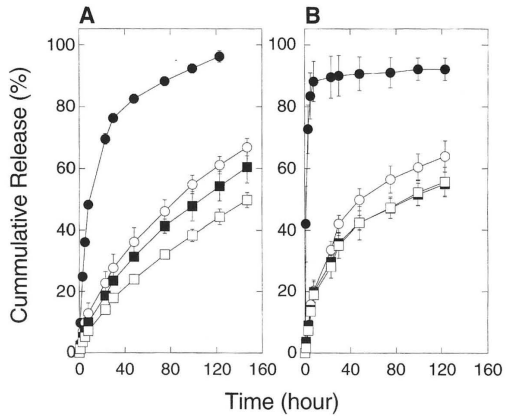

Fig. 5. In vitro release of (A) rifampicin and (B) diclofenac from linseed oil (○), soybean oil (■) and squalene (□) emulsions at 37°C. Release of drugs dissolved in water (●) was also presented as a control. The drug concentration in the emulsions was 0.3 mg/ml. Egg PC at 18 mg/ml was used as an emulsifier.

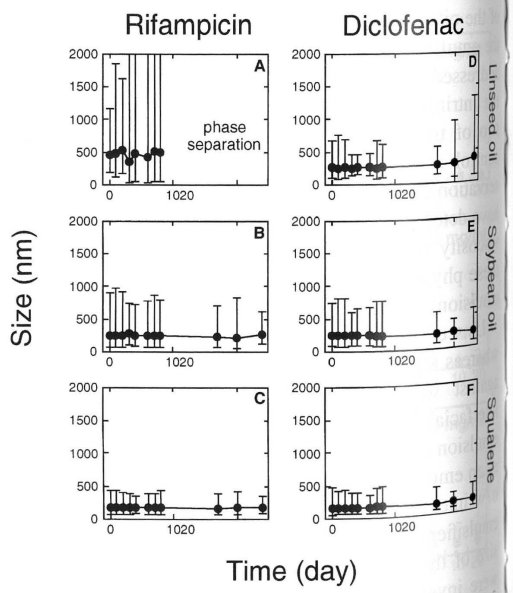

Fig. 6. Changes in the average particle size and its distribution of (A and D) linseed oil, (B and E) soybean oil and (C and F) squalene emulsions containing 0.3 mg/ml of rifampicin (A, B and C) and diclofenac (D, E and F). The emulsions were stored at room temperature. Egg PC at 18 mg/ml was used as an emulsifier.

than those containing rifampicin. Average particle sizes of the linseed oil, soybean oil and squalene emulsions containing diclofenac were ca. 260 nm, 250 nm and 160 nm, respectively. Even though the particle size of the linseed oil emulsion containing diclofenac became ca. 370 nm in 25 days, phase separation was not observed for the duration of experiment. The average particle sizes of the soybean oil and squalene emulsion containing diclofenac did not change for 25 days. The drug loaded squalene and soybean oil emulsions were stable for 25 days at 37°C whereas large aggregations were observed in the linseed oil emulsion.

3.5. *In vitro gene transfection activity*

Cationic emulsions were prepared with linseed oil, soybean oil and squalene at 100 μg/ml and DOTAP at 24 mg/ml. DOTAP liposome at 24 mg/ml was also prepared for comparison. The average particle size of the liposome and emulsions was determined in Fig. 7. The particle size of the liposome was slightly bigger than 100 nm. The average particle sizes of the linseed oil, soybean oil and squalene emulsions were bigger than liposome and were 220 nm, 204 nm and 155 nm, respectively. Upon forming a complex with DNA, the size of liposome grew to 269 nm. The size of the emulsion/DNA complex, however, increased only slightly. We could not observe phase separation or oil leakage in the solution containing emulsion/DNA complex indicating that the emulsion particle remains intact. In case of linseed oil and soybean oil emulsions, particle aggregation of emulsion/DNA was observed by eye. The solution containing linseed oil emulsion/DNA complex was more turbid than that with soybean oil emulsion/DNA complex. In distinct contrast, the solution containing squalene emulsion/DNA complex remained translucent.

In vitro gene transfection was performed with the liposome and the three emulsions as gene carriers (Fig. 8). In the absence of serum, DOTAP liposome exhibited higher transfection activity, expressed as β-galactosidase activity in the figure, than emulsion carriers. Among the emulsion systems, squalene emulsion showed higher transfection activity than soybean oil (P-value=0.05, Student's t-test) or linseed oil emulsions (P-value=0.0009). The situation

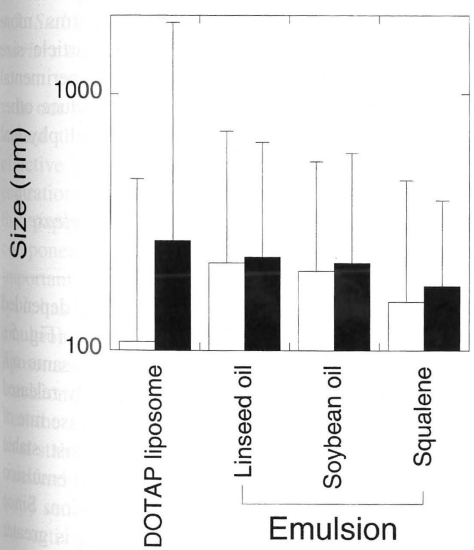

Fig. 7. Average particle size and its distribution of cationic carriers (open bars) and carrier/DNA complexes (filled bars).

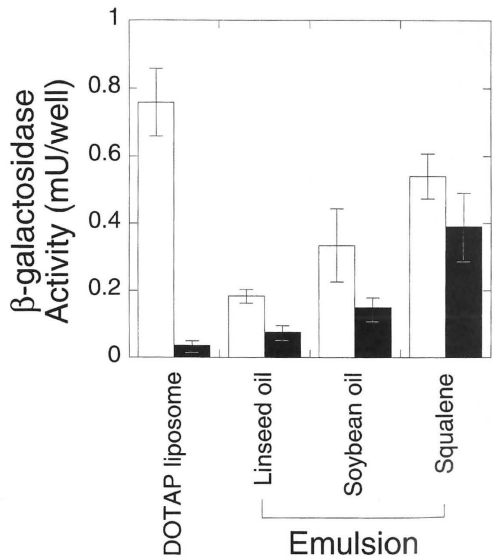

Fig. 8. In vitro gene transfection activity, expressed as β-galactoxidase activity using cationic liposome and emulsions as gene carriers. Transfection was performed in the absence (open bars) and in the presence (filled bars) of 80% (v/v) serum.

became dramatically different when 80% (v/v) serum was added during the transfection. In case of DOTAP liposome, transfection efficiency was diminished to ca. 4% of the activity without serum. On the other hand, the activity was maintained significantly for cationic emulsions. Among the emulsions, squalene emulsion was the most efficient with or without serum. Especially in the presence of 80% (v/v) serum, squalene emulsion retained more than 70% of the activity without serum and was ca. 30 times more efficient than DOTAP liposome (P-value=0.0035). On the contrary, linseed oil emulsion, though it was more efficient than the liposome with serum, was the least efficient among the cationic emulsions.

4. Discussion

4.1. Oil modulates emulsion particle size and stability

Squalene formed an emulsion with the smallest average particle size whereas size of the linseed oil emulsion was the biggest when 12 mg/ml egg PC aqueous solution was used as an emulsifier (Table 1, Figs. 2, 3). Also, particle size of the emulsion became smaller at higher emulsifier concentrations in agreement with previous observations [13]. Size stability of the emulsion depended on the initial particle size. Emulsions with smaller particles stayed stable for a longer time. For instance, squalene emulsion was stable without any changes in the size distribution for 20 days whereas the linseed oil emulsion phase-separated during the same period. Therefore, squalene would be the oil of choice in formulating a stable o/w emulsion system when egg PC is used as an emulsifier. Jojoba bean oil also formed a stable emulsion. On the other hand, linseed, mineral and borage oils formed unstable emulsions with big particles. It is apparent from the result that the oil component greatly affects the stability of the emulsions.

Most of the vegetable, animal and mineral oils are not usually pure compounds. Even though the major components of these oils are saturated or unsaturated triacylglycerols in general, there exist other components. For instance, the main components of jojoba bean oil are ethyl esters of C20 and C22 straight chain monoethylene acids or alcohols in the form of ester [14]. Squalene is a pure polyunsaturated and highly branched hydrocarbon. When comparing such diverse oil systems, it is not possible to correlate emulsion characteristics and the physical property of oils by using only a single parameter. Among many factors to control emulsion stability, interfacial properties, such as the surface tension and the viscosity are known to determine the stability of the emulsions [6,7]. Our results show that there is an inversely proportional relationship between o/w interfacial tension and the emulsion size. There was no apparent correlation between viscosity of the oils and size of the natural oil emulsions.

In this paper, we have attempted to correlate the physical properties of oils and the stability of the corresponding emulsion. Many studies have been performed on the effect of the emulsifier in emulsion stability. However, it seems that there are few systematic studies in the field to elucidate the correlation between the choice of the oils and the emulsion stability. At this point in time, we cannot address which physical property of oils determines the emulsion stability. Probably, it would be too complex to explain with a few variables. In this experiment, we found that more hydrophobic oil with higher o/w interfacial tension forms more stable emulsion with a small average particle size than less hydrophobic ones under our experimental conditions. Our finding, we hope, will induce other researchers to investigate the fundamental physical basis of such behavior.

4.2. Emulsion stability controls in vitro release rate of lipophilic drugs

The release rate of lipophilic drugs depended greatly on the oil phase of the emulsions (Fig. 5). Also, from the emulsions made with the same oil, more lipophilic rifampicin was more slowly released than more hydrophilic diclofenac. The release rate of rifampicin was the slowest from the most stable squalene emulsion followed by soybean oil emulsion and then by least stable linseed oil emulsion. Since the o/w partition coefficient of rifampicin is greater for linseed oil emulsion, which had the fastest release rate, than soybean oil or squalene emulsions,

there is another factor that controls the drug release rate. Generally, the release could be faster from smaller particles since a small particle system has a large surface area where drug diffusion could occur. Again, our results demonstrate otherwise. One of the possible explanations could be given if we connect drug release rate with emulsion stability. When a lipophilic drug was loaded in the emulsion, the drug was released faster if the emulsion was unstable. Unstable emulsions could coalesce and aggregate while the contents in the emulsion particles could leak out. In case of stable emulsions, particles would remain intact, thus keeping the drugs inside. Ideally, a drug must diffuse out from the emulsion particles according to the partition coefficient between oil and water if particles remain intact without experiencing dynamic equilibrium among the particles. In the squalene emulsion, which is the most stable among the three studied emulsion systems, we could assume that the particles remain intact longer. The linseed oil emulsion, on the other hand, is a more dynamic system where the particle aggregation and coalescence occur. During this dynamic interaction, the loaded drug would leak out and diffuse to the bulk aqueous phase. As a result, drug release rate can become faster from less stable emulsion.

4.3. Stable cationic emulsions are good gene carriers especially in the presence of serum

In our previous communications, we have demonstrated that the cationic emulsion can become an effective gene carrier especially at high serum concentrations as well as for in vivo conditions [8,9]. In this paper, we have shown that the choice of oil component in the cationic emulsion can be another important factor that controls the transfection activity. It has been also shown in this paper that cationic emulsions can be more potent gene carriers than liposomes in the presence of serum. Although all emulsion formulations had lower transfection activity than the DOTAP liposome without serum, even the least stable linseed oil emulsion was more potent in transferring genes than DOTAP liposome in 80% (v/v) serum (Fig. 8). Among the emulsion carriers, the activity was the highest for the most stable squalene emulsion. Under our experimental conditions, emulsion stability and its function as a gene carrier seems to be strongly related. Our result agrees well with recent findings that the stability of cationic liposome and liposome/DNA complex is related to its ability to deliver genes [15,16].

Another advantage of using cationic emulsion as a gene carrier is the stability of emulsion itself and emulsion/DNA complex in the presence of serum. Although there are many factors that control transfection activities, proteins and other anionic materials in serum are known to cause complex inactivation and act as barriers for in vivo transfection [16,17]. Therefore, it has been a challenge to formulate a non-viral gene carrier whose transfection activity does not decrease by serum components. Cationic emulsions, especially the squalene emulsion, were stable in serum, and therefore were efficient in transferring genes in the presence of serum.

5. Conclusions

Particle size and the emulsion stability varied depending on the oil when egg PC was used as an emulsifier. Among the 18 natural oils, squalene formed the most stable emulsion with the smallest average particle size whereas linseed oil formed the least stable emulsion. Emulsions with smaller initial particle size remained more stable than those with larger ones. The o/w interfacial tension or hydrophobicity of oils was related to the emulsion stability even though the fundamental physical basis of the relationship is not known. The release rate of the lipophilic drugs was also governed by the stability of the emulsions. From the stable emulsion, drug release is more sustained than from the unstable ones. Also, in vitro gene transfection activity was strongly related to the emulsion stability. Especially in the presence of serum, stable squalene emulsion was ca. 30 times more potent than DOTAP liposome.

Acknowledgements

This work was supported by the KIST-2000 project. We thank Dr Joon Woo Park for kindly helping with the interfacial tension measurements.

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