Evaluation of Liposomal Erythropoietin Prepared with Reverse-Phase Evaporation Vesicle Method by Subcutaneous Administration in Rats

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We encapsulated erythropoietin (Epo) in dipalmitoylphosphatidylcholine (DPPC) liposomes with soybean-derived sterols (SS-liposomes) and its glucoside (SG-liposomes) by reverse-phase evaporation vesicle method, and evaluated them by subcutaneous administration in rats. With 4 min of sonication, the damage to Epo activity was observed mainly in the non-encapsulated Epo in the liposomes. This study indicated that the bilayer of liposomes had the ability to protect the Epo activity, by reducing the aggregation that was caused by interaction between Epo molecules. The SG-liposomes had a higher retention of the Epo activity than SS-liposomes. 25.3% or 33.6% of activity was retained by SS-liposomes under the conditions of 4 min or 1 min of sonication, while 53.3% or 58.3% of the activity was retained by SG-liposomes under the same conditions. Shorter sonication was available to minimize the loss of the Epo activity. Epo in SG-liposomes appeared to increase the activity.

Key words erythropoietin; dipalmitoylphosphatidylcholine liposome; activity retention; preparation, evaluation; soybean-derived sterols; sterylglucoside

Erythropoietin (Epo) is a glycoprotein produced primarily in the kidneys and to a lesser extent in the liver. Epo is a single-chain polypeptide with a molecular weight of 30.4 kDa, 40% of which is ascribed to carbohydrates. The physiological function of Epo is to regulate the proliferation and differentiation of erythroid progenitor cells. The application of Epo is currently limited to intravenous injection. The use of delivery systems for liposomes could extend the therapeutic possibilities for Epo, for example, parenteral and nonparenteral administration. The use of liposomes as carriers of Epo has not been studied in the past however, since the glycoprotein is not stable in a solution when subjected to mechanical stress, especially shaking and sonication.[1]

Recently we demonstrated that dipalmitoylphosphatidylcholine (DPPC) liposomes with soybean-derived sterols (SS) are very stable compared with liposomes composed of DPPC alone,[2,3] and its monoglucoside (SG) was used as an enhancer for nasal absorption.[4] SG is a mixture of monoglucosides of β-sitosterol (49.9%), campesterol (29.1%), stigmasterol (13.8%), and brassicasterol (7.2%) as shown in Fig. 1. SS is obtained by the hydrolysis of the glucoside bond of SG, *i.e.*, SS is the aglycon of SG. The average molecular weight of SG and SS are 570.5 and 409.2, respectively. It has therefore been anticipated that the stabilizing effect of SS on liposomes would lead to the stabilization of Epo encapsulated in liposomes, and that DPPC liposomes with SG might be effective for intestinal absorption of Epo.

The purpose of this work was to develop the conditions for preparation of liposomal Epo with high encapsulation efficiency and preservation of activity. We selected the reverse-phase evaporation vesicle (REV) method to encapsulate Epo into liposomes using DPPC with SS and SG (SS-liposomes, SG-liposomes, respectively). The activity of Epo encapsulated in SS and SG-liposomes was evaluated by measuring the circulating reticulocyte counts in blood after subcutaneous administration in rats, and

was compared with measurement of the Epo concentration by high performance liquid chromatography (HPLC).

Materials and Methods

Materials DPPC was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). SS and SG were generously supplied by Ryukakusan Co., Ltd. (Tokyo, Japan). Epo was a gift from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). Calcein was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals used were of reagent grade. Male Wistar rats were purchased from Saitama Experimental Animal Supply (Saitama, Japan).

Preparation of SS and SG-Liposomes Encapsulated Epo Liposomes were prepared according to the REV method.[5] DPPC (105 μmol) and SS or SG (30 μmol) (DPPC: SS or SG = 7:2, molar ratio) in chloroform solution were deposited in a 50 ml pear-shaped flask, and the organic solvent was removed under reduced pressure by rotary evaporation at room temperature. The lipid film was redissolved in 3 ml of chloroform and 3 ml of isopropyl ether. To the resulting organic phase, the aqueous phase including 1 ml of Epo solution (180000 IU/ml) and 1 ml of phosphate-buffered saline (1/10 of dilution of the phosphate-buffered saline in distilled water: 137 mM NaCl/2.6 mM KCl/6.4 mM Na$_2$HPO$_4$·12-H$_2$O/1.4 mM KH$_2$PO$_4$; pH 7.31; PBS) was added. The mixture was sonicated in a bath-type sonicator (Honda Electronics, W220R, 200W, 40 kHz, Tokyo, Japan) at 50 °C for 1 min or 4 min until it had become a homogeneous w/o emulsion. The emulsion was then placed on a rotary evaporator, the organic solvent was removed under reduced pressure (400 mmHg) using a water aspirator, and flushed with a gentle stream of nitrogen (500 ml/min) at 50—55 °C for 30 min. The preparation was diluted two times with the PBS and extruded successively through polycarbonate membrane (Nuclepore, U.S.A.) with pore size of 0.4 and 0.2 μm at about 60 °C. The size distribution of liposomes was measured
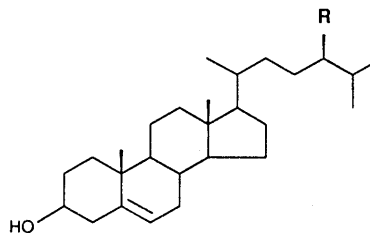

Fig. 1. Chemical Structure of Soybean-Derived Sterols (SS)

R = CH$_3$, campesterol; R = C$_2$H$_5$, sitosterol; R = C$_2$H$_5$ and Δ^{22}, stigmasterol; R = CH$_3$ and Δ^{22}, brassicasterol.

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by a Nicomp 370 submicron particle analyzer (Pacific Scientific, CA, U.S.A.). The sizes estimated for SS and SG-liposomes were quite homogeneously distributed and the mean diameters were 134—166 nm, respectively. After extrusion, 0.5 ml of the preparation was passed through a Sephadex G-50 column (1.8 × 35 cm, Pharmacia, Sweden) with PBS to remove non-encapsulated Epo. Each fraction contained 4.5 ml. The diluted factor of liposome suspension after gel filtration was 9. Epo encapsulated in SS-liposomes and SG-liposomes is expressed as Epo/SS-liposomes and Epo/SG-liposomes, respectively.

Determination of Encapsulated Volume of Liposomes Encapsulated volume is defined as the volume enclosed by a given amount of a lipid and units of liters entrapped per mol of total lipid (l/mol). Calcein was used as a marker to determine the encapsulated volume. In preparation of the liposomes (105 μmol of DPPC and 30 μmol of SS or SG), the dried lipid film was dissolved in 3 ml of chloroform, 3 ml of isopropyl ether and 2 ml of PBS containing 20 mM calcein. The same REV and extrusion methods were applied to prepare Epo liposomes. The preparation that calcein was encapsulated after passing through gel filtration was diluted 1000-fold with PBS and the fluorescence intensity (F_b) was measured with a fluorescence spectrometer (excitation at 490 nm, emission at 520 nm; Hitachi F-4010, Tokyo, Japan). The liposomes were then completely disrupted by adding 30 μl of 10% Triton X-100 solution to 1 ml of sample and then the fluorescence intensity (F_a) was measured. The fluorescence intensity of calcein encapsulated in the liposomes (F_{lipo}) was calculated according to a equation ($F_{lipo} = F_a - F_b$). The amount of calcein encapsulated in the liposomes (C_{lipo}) was calculated from F_{lipo} and the standard curve prepared by a known concentration of calcein. The encapsulated volume of SS or SG-liposomes was 7.08 or 4.74 l/mol lipid, respectively, when calculated from C_{lipo} and the concentration of DPPC in the liposomes determined using a Phospholipid B-test Wako (Wako Pure Chemical Ind., Ltd., Osaka, Japan).

Animal Experiments Male Wistar rats 9 weeks old (about 300 g) were used in all experiments. Rats (3 per group) were lightly anesthetized with diethyl ether and then free Epo solution or Epo encapsulated in liposome suspension was administered subcutaneously in the dorsal neck. Twenty μl of blood sample was collected from the dorsal metatarsal vein before administration and on days 2, 4 and 7 after administration, and the sample was immediately diluted with 10 ml of Cellent (Sysmex, Toa Medical Electronics, Japan) to make a diluted blood solution. This solution was treated with about 100 μl of hemolyzing reagent, Quicklyser (Sysmex, Toa), about 15 min before counting. The hemolyzed blood cells were counted using a microcell counter (Sysmex F-500) and a cell monitor (Sysmex CM-5). Sensitivity of the cell monitor was set at 4 and the discriminator at 1 or 5. The difference in numbers counted at discriminator levels 1 and 5 was viewed as the circulating reticulocyte counts. The technical and operating principles of the automatic microcell counter have been described elsewhere.[6,7] Epo/SS and Epo/SG-liposome suspensions after gel filtration were administered to 0.3, 0.1 and 0.03 ml/300 g rats, whereas those before gel filtration to 0.033, 0.011 and 0.003 ml/300 g rats, respectively. The difference of dose between before and after gel filtration is equal to the dilution factor (9) of liposome suspension after gel filtration.

Determination of Epo by HPLC The Epo concentration after preparation of liposomes was determined by HPLC. A 0.3 ml of liposome suspension containing Epo was shaken with 0.09 ml of chloroform in order to disrupt the liposomes. After centrifugation at 3000 rpm for 10 min, 0.2 ml of the aqueous phase containing Epo or a standard solution of Epo was injected into HPLC. The HPLC system for analysis of Epo consisted of a butylsilil silicagel column (Vydac C4, 250 × 4 mm, 5 μm), a Waters 600 multisolvent delivery system, and a Waters 486 tunable absorbance detector (Nihon Waters, Ltd., Tokyo, Japan). Mobile phase A consisted of water–acetonitrile–trifluoroacetic acid (400 : 100 : 1), and B consisted of water–acetonitrile–trifluoroacetic acid (100 : 400 : 1). The column was equilibrated with a mobile phase consisting of 35% B and 65% A. Percentage of B was maintained at 35% for 5 min after injection and was linearly changed to 100% over 15 min, where it was maintained for 2 min. The flow rate was 1 ml/min all the while. Epo was detected at a wavelength of 214 nm and was eluted at a retention time of about 20 min at room temperature.

Results and Discussion

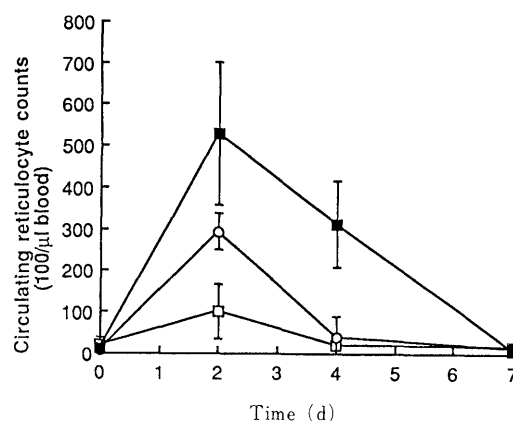**Epo/SS and Epo/SG-Liposomes** The liposomes of the molar composition ratio of DPPC and SS at 7 : 4 may

Fig. 2. Effect of a Single Subcutaneous Administration of Free Epo Solution on the Circulating Reticulocyte Counts

Administration doses are 100 (□), 300 (○) and 1000 (■) IU/kg of rats. Values are means ± S.D. ($n=3$).

be limited loading since the rigidity of DPPC liposomes with SS shows the highest, as measured by fluorescence anisotropy.[8] On the other hand, SG distributes homogeneously in the DPPC liposomes up to a 7 : 3 molar ratio of DPPC and SG, measured by differential scanning calorimetry (data not shown). SS-Liposomes and SG-liposomes consist of a molar ratio of DPPC and SS or SG of 7 : 2, therefore, SS and SG may distribute in DPPC liposomes homogeneously.

The liposomal Epo was estimated by three methods: (1) the curve of the circulating reticulocyte counts vs. dose at 2 d after subcutaneous injection of the Epo/SS and Epo/SG-liposome suspensions; (2) measurement of Epo concentration by HPLC; and (3) encapsulated volume and lipid recovery of SS and SG-liposomes.

Circulating Reticulocyte Counts after Subcutaneous Administration of Free and Liposomal Epo Many investigators have reported the administration of Epo results in an increase of circulating and macrocytic reticulocyte counts,[9,10] and the relative circulating reticulocyte counts were markedly increased on the second day.[7,11,12]

When Epo solution was administered subcutaneously, the circulating reticulocyte counts in blood significantly increased and reached a peak at 2 d, then decreased to the level of pre-administration at 7 d, as shown in Fig. 2.

The circulating reticulocyte counts in blood at 2 d show a linear relationship with the logarithmic dose of Epo after subcutaneous administration of Epo solution. The linear equation of the circulating reticulocyte counts (y, × 100/μl) in blood at 2 d vs. the dose of Epo (x, IU/kg) after subcutaneous administration of free Epo solution was

$$y = -761 + 429 \times \log x \quad (r = 0.999) \quad (1)$$

This equation corresponds with that after intravenous administration in an earlier paper.[12,13]

When, prep. 1-a and prep. 2-a were administered subcutaneously, the circulating reticulocyte counts increased as Epo solution as shown in Fig. 3.

Therefore, Epo/SS and Epo/SG-liposome suspension was administered with one dose, 0.10 or 0.011 ml/300 g weight of rats after or before gel filtration, respectively,
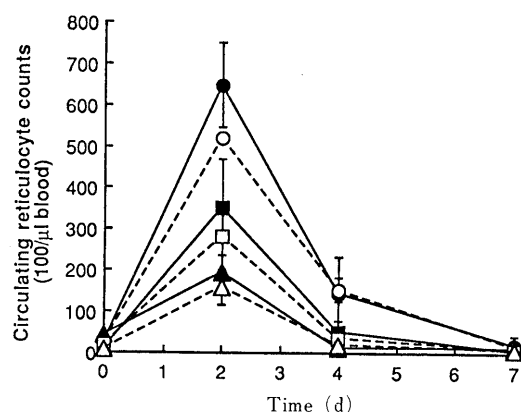
Fig. 3. Effect of a Single Subcutaneous Administration of Epo/SS and Epo/SG-Liposome Suspensions after Gel Filtration on the Circulating Reticulocyte Counts

The dotted line and empty symbol represent Epo/SS-liposomes (prep. 1-a). The solid line and solid symbol represent Epo/SG-liposomes (prep. 2-a). Administration volumes are 0.3 (○), 0.1 (□) and 0.03 (△) ml/300 g. Values are means ± S.D. ($n = 3$).
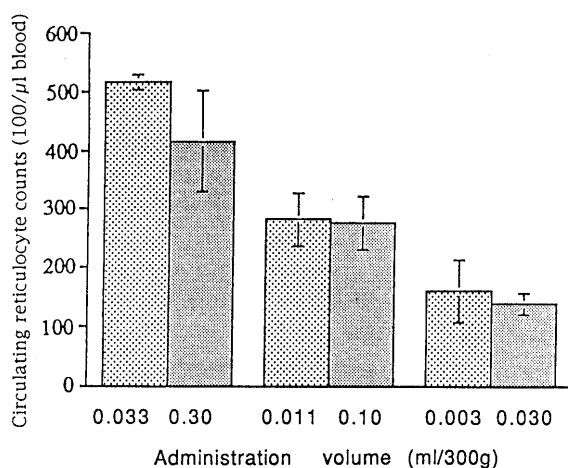

Fig. 4. Comparison of the Epo/SS-Liposomes before (Prep. 1-b, ▨) with after (Prep. 1-a, ▨), Gel Filtration on the Circulating Reticulocyte Counts at 2 d after a Single Subcutaneous Administration

Administration volume of liposomes before gel filtration is 1/9 of that after gel filtration since the concentration of the liposomes suspension is diluted to 1/9 after gel filtration. Values are means ± S.D. ($n = 3$).

TABLE I. Circulating Reticulocyte Counts at 2 d after Subcutaneous Administration of Epo/SS and Epo/SG-Liposomes Prepared with 1 min and 4 min Sonication

Prep. No.	Type of liposomes	Gel filtration	Sonication duration (min)	Administration volume (ml/300 g)	Reticulocyte counts[a] (100/μl)
1-b	SS	Before	4	0.011	281 ± 45
1-a	SS	After	4	0.10	275 ± 45
2-b	SG	Before	4	0.011	421 ± 102
2-a	SG	After	4	0.10	353 ± 116
3-b	SS	Before	1	0.011	569 ± 31
3-a	SS	After	1	0.10	263 ± 38
4-b	SG	Before	1	0.011	515 ± 27
4-a	SG	After	1	0.10	204 ± 69

a) Mean ± S.D.

TABLE II. Comparison of Epo Concentration Estimated by HPLC Method with the Activity of Epo Estimated by the Curve of Circulating Reticulocyte Counts at 2 d after Subcutaneous Administration of Epo/SS and Epo/SG-Liposomes vs. Dose

Prep. No.	Epo conc. (IU/ml)[a]	Activity of Epo (IU/ml)[b]
1-b	9406[c]	7293
1-a	756[c]	785
2-b	10917[d]	15494
2-a	511[c]	1193
3-b	37435[c]	34234
3-a	814[c]	734
4-b	17446[d]	25629
4-a	312[d]	537

a) Epo concentration estimated by HPLC method. b) Calculated using the circulating reticulocyte counts (Table I), administration volume and Eq. 1. c) Mean value for double measurements. d) Value for single measurement.

instead of three doses as described in the Animal Experiments.

Epo existed as non-encapsulated and encapsulated in the liposome suspension before gel filtration. The circulating reticulocyte counts incresed with administration of non-encapsulated and encapsulated Epo, and at 2 d after subcutaneous administration of prep. 1-b they were not significantly higher than those of prep. 1-a at any administration volume by Student's t-test ($p < 0.05$) (Fig. 4).

Table I shows the circulating reticulocyte counts at 2 d after subcutaneous administration of a series of liposome suspension preparations.

The circulating reticulocyte counts of prep. 2-b and prep. 2-a also did not show significant difference by Student's t-test ($p < 0.05$). But for prep. 3-b and prep. 3-a, as well as prep. 4-b and prep. 4-a, these counts did show significant difference by this test ($p < 0.05$). These results appear to

indicate that the Epo non-encapsulated in Epo/SS and Epo/SG-liposome suspensions prepared by 4 min of sonication did not demonstrate notable action, while those prepared by 1 min of sonication demonstrated notable action in the circulating reticulocyte counts.

Epo Concentration Estimated by Comparison of Epo Activity Using HPLC Method   The Epo concentration of Epo/SS and Epo/SG-liposomes before and after gel filtration estimated by HPLC method is summarized in Table II.

The acivity of Epo is calculated from the circulating reticulocyte counts 2 d after subcutaneous administration of the liposome suspensions using Eq. 1 and the administration volume (Table I).

SS-Liposomes (prep. 1-a, 3-a) showed the Epo concentration estimated by HPLC to be comparable with the activity of Epo estimated by the circulating reticulocyte counts, while SG-liposomes (prep. 2-a, 4-a) showed the Epo concentration to be incomparable. The reasons SG had a different effect on Epo were not clear in the present study. It is speculated that SG might interact with the Epo molecule specially so that Epo in SG-liposomes would increase the activity.

Epo Concentration Estimated by Encapsulated Volume   The encapsulated volume naturally depends on the radius of the liposomes produced by a given technique and is affected by the lipid composition of each vesicle and the ionic composition of the medium. We estimated the

Table III. Activity of Total Epo Estimated by Encapsulated Volume (7.08 or 4.74 l/mol Lipid, Respectively) and Retention of Activity of Epo/SS and Epo/SG-Liposomes

Prep. No.	Lipid recovery	Yield of Epo (%)	Activity of total Epo (IU/ml)	Retention of activity[e] (IU/ml)
1-b	100[a]	100[c]	45000[c]	16.2
1-a	83.6	62.1[b]	3105[d]	25.3
2-b	100[a]	100[c]	45000[c]	34.4
2-a	90.0	44.8[b]	2240[d]	53.3
3-b	100[a]	100[c]	45000[c]	76.1
3-a	58.8	43.7[b]	2185[d]	33.6
4-b	100[a]	100[c]	45000[c]	57.0
4-a	37.0	18.4[b]	920[d]	58.3

a) The lipid recovery (%) was estimated as 100% before gel filtration. b) The yield of Epo was calculated according to encapsulated volume × 105 μmol × lipid recovery (%). c) Before gel filtration, the yield of Epo was assumed to be 100%, the activity of total Epo was calculated according to the Epo solution 180000 (IU/ml)/4 = 45000 IU/ml. d) The activity of total Epo after gel filtration was calculated using 45000 IU/ml × yield of Epo/9 (diluted factor).

e) % retention of activity = $\dfrac{\text{activity of Epo}}{\text{activity of total Epo}} \times 100$.

encapsulated volumes of Epo/SS and Epo/SG-liposomes as 7.08 and 4.74 l/mol lipid, respectively.

Encapsulation efficiency was defined as the fraction of the aqueous compartment sequestered by bilayers. The encapsulation efficiency of SS or SG-liposomes (74.3% or 49.8%, respectively) was calculated as the encapsulated volume multiplied by the amount of total lipid (105 μmol) and divided by the original volume of Epo solution (1 ml).

The amount of lipid remaining after extrusion and gel filtration was divided by the amount in the initial preparation before extrusion to yield the lipid recovery. The lipid recovery reflected the yield of Epo that remained after extrusion and gel filtration. We calculated the overall yield of Epo in the liposomes as the encapsulation efficiency multiplied by the lipid recovery. And the overall yield of Epo in the liposomes and dilution factor after gel filtration (9) gave the activity of total Epo in liposome suspension that is summarized in Table III.

Retention of activity was expressed by the activity of total Epo estimated from encapsulated volume (Table III) and the activity of Epo (Table II):

$$\% \text{ retention of activity} = \frac{\text{activity of Epo}}{\text{activity of total Epo}} \times 100 \qquad (2)$$

We examined which part of Epo, non-encapsulated or encapsulated, is inactivated from the retention of activity of Epo, comparing prep. 1-b with prep. 1-a. Prep. 1-b represented 7293 IU/ml as the activity of Epo non-encapsulated and encapsulated. Prep. 1-a with 74.3% encapsulation efficiency of 45000 IU/ml Epo (= 33435 IU/ml of Epo in liposomes) and 25.3% of retention of Epo activity represented 8459 IU/ml as the activity of Epo encapsulated. Therefore, the activity of Epo non-encapsulated in prep. 1-b in SS-liposome suspension prepared by 4 min of sonication may not exist.

Prep. 2-b represented 15494 IU/ml and prep. 2-a, 11945 IU/ml. The activity of Epo non-encapsulated in SG-liposome suspension prepared by 4 min of sonication was 7.9%.

These results indicate that nearly 100% and about 92% of the activity of Epo non-encapsulated in the SS and SG-liposomes, and about 75% and 47% of that encapsulated in these liposomes were damaged by liposome preparation with 4 min of sonication, respectively. This indicated that the bilayer of liposomes acts to protect the activity of Epo.

Comparison between Epo/SS and Epo/SG-Liposomes after Gel Filtration The retention of activity was 25.3% (prep. 1-a) and 33.6% (prep. 3-a) for Epo/SS-liposomes, and 53.3% (prep. 2-a) and 58.3% (prep. 4-a) for Epo/SG-liposomes, respectively (Table III). These results indicated that SG-liposomes have greater ability to maintain the activity of Epo than do SS-liposomes. This might be due to the steric effect of SG inhibiting the aggregation of Epo molecules, since the glucose part of SG may interact with the sugar moiety of Epo.

Effect of Conditions in the Preparation Process on Retention of Activity From Table III the retention of activity of Epo preparation with 4 min sonication was found to be relatively low and may be dependent on the duration of sonication. We changed the conditions of preparation and decreased sonication duration from 4 min to 1 min. The change in retention of activity is also shown in Table III.

The retention of activity increased from 16.2% (prep. 1-b) to 76.1% (prep. 3-b), and from 25.3% (prep. 1-a) to 33.6% (prep. 3-a) for Epo/SS-liposomes; increased from 34.4% (prep. 2-b) to 57.0% (prep. 4-b), and from 53.3% (prep. 2-a) to 58.3% (prep. 4-a) for Epo/SG-liposomes when the sonication duration was decreased from 4 min to 1 min. The retention of activity of Epo non-encapsulated in Epo/SS or Epo/SG-liposomes was calculated to be about 51% and 28%, respectively, with sonication duration of 1 min. Thus, about 49% and 72% of the activity of Epo non-encapsulated in the SS and SG-liposomes was damaged by preparation with 1 min sonication. These values are lower than those (100%, 92%) by preparation with 4 min sonication as described previously. The retention of activity of Epo increased when the condition of preparation was "gentle". This result corresponds with the fact that the activity of Epo in Epo solution is decreased by 3 min sonication (20 kHz, 200 W) to 57.7% (private communication).

Results found in this study indicate that the bilayer of liposomes has the ability to protect Epo, to reduce the interaction between Epo molecules and to suppress Epo aggregation. When the condition of preparation was severe, damage to Epo activity was observed mainly in non-encapsulated Epo.

The SG-liposomes have a higher retention of activity than SS-liposomes, 25.3% or 33.6% of activity of Epo was retained for SS-liposomes under conditions of 4 min or 1 min of sonication, while 53.3% or 58.3% of activity of Epo was retained for SG-liposomes under the same conditions. Shorter Sonication minimizes the loss of activity of Epo. SG might interact with the Epo molecule selectively since Epo in SG-liposomes shows higher activity than that estimated from Epo concentration.

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