Drug Development and Industrial Pharmacy, 25(10), 1107–1113 (1999)

RESEARCH PAPER

Effect of Divalent Cations on the Membrane Properties of the Lipid A Analog E5531

Y. Asai and S. Watanabe

Formulation Research Laboratory, Kawashima, Eisai Company, Limited, Takehaya-machi, Kawashima-cho, Hashima-gun, Gifu 501-6195, Japan

ABSTRACT

To obtain information on the effects of Mg^{2+} on the membrane properties of the lipid A analog E5531, we determined the size, structure, zeta potential, membrane fluidity, and micropolarity of the aggregates and the permeability of the E5531 membrane after the addition of Mg^{2+}. E5531 forms a vesicle structure and within the molar ratio of [E5531]:[Mg^{2+}] = 1:3, Mg^{2+} increased the zeta potential of the E5531 membrane, but did not change the size of the aggregates (approximately 20 nm). Within that molar ratio, Mg^{2+} decreased the membrane fluidity and micropolarity of E5531 and increased the phase transition temperature. Above the molar ratio of [E5531]:[Mg^{2+}] = 1:5, the size of the aggregates was increased, but at [E5531]:[Mg^{2+}] = 1:3, the size of the aggregates was similar to that in the absence of Mg^{2+} (approximately 20 nm), and we could stabilize the aggregates in rat plasma.

INTRODUCTION

Lipopolysaccharide (LPS) is a characteristic component of the cell envelope of gram-negative bacteria. It is anchored to the outer leaflet of the outer membrane of the bacteria through its lipid A component (1). The elucidation of the architecture and conformation of this highly complex lipid is of great scientific interest not only because of its crucial role in the barrier function of the outer membrane (e.g., to certain antibiotics) (2,3), but also because of its various biological activities, such as the induction of fever and the Schwartzmann bleeding reaction (4).

Recent research has focused on the description of the phase structure and conformation of isolated LPS and lipid A (5,6). From these studies, it can be concluded that LPS, and also lipid A, as with other amphiphilic lipids, seem to express remarkable polymorphism, including lamellar and nonlamellar phases, depending such experimental conditions as hydration, temperature, pH, and the like. However, especially in the case of lipid A, it is still uncertain whether the lipid exists in a lamellar (7,8) or

nonlamellar (9,10) state under conditions of full hydration and at different temperatures. These environmental changes may affect the hydrophilic head group region. A decrease in pH or an increase in the concentration of divalent cations, for instance, leads to tighter packing (11). It is, therefore, very important to guarantee the physiological conditions if such measurements are to be used to interpret biological effects.

Recent research has also focused on the synthesis of low-toxicity lipid A analogs. Christ and coworkers have indicated that E5531 (12), a synthetic disaccharide analog of lipid A (Fig. 1), has low toxicity but retains some of the biological activities of lipid A, such as reduction of tumor necrosis factor (TNF) production. This compound has been found to be a specific agent in the LPS-binding assay and is an inhibitor of LPS-induced TNF production in monocytes/macrophages. It is therefore expected that E5531 will be useful for the treatment of septic shock.

An injectable E5531 formulation would be extremely useful; however, the dispersion of E5531 in aqueous solution was a major problem. In the present study, a new ''pH-jump method'' for dispersing E5531 has been developed (13). This method involves the dispersion of E5531 in 0.003 N NaOH solution (pH 11.0) at 50°C and subsequent mixing with phosphate-NaOH buffer to neutralize the pH to 7.3. The advantages of this method include its suitability for large-scale production (without mechanical input such as sonication), a neutral pH, and the small size of the aggregates.

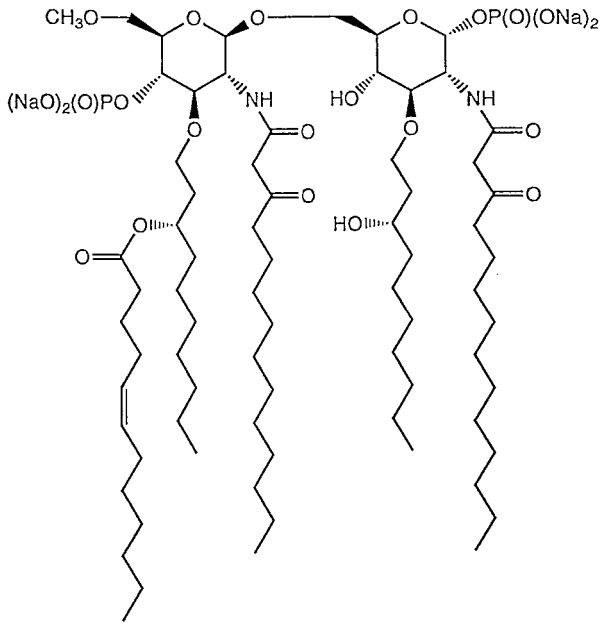

Figure 1. Chemical structure of the synthetic lipid A analog E5531.

The purpose of this study was to investigate the effects of Mg^{2+} on the E5531 membrane properties, such as the zeta potential, membrane fluidity, micropolarity, and stability in rat plasma.

EXPERIMENTAL

Materials

E5531 was obtained from Eisai Chemical Company, Limited (Ibaraki, Japan). Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Wako Pure Chemical Industrial Limited (Osaka, Japan). 3,3′-Bis[*N*,*N*-bis(carboxymethyl) aminomethyl]-fluorescein (calcein) was purchased from Dojin Company, Limited (Kumamoto, Japan). *N*-Dansylhexadecylamine (DSHA) was from Lambda Company, Limited (Graz, Austria).

Methods

Preparation of E5531 Dispersions

Samples were prepared by the pH-jump method (13) using 1.3 g of E5531 dispersed in 650 ml of 0.003 N NaOH solution by stirring at 50°C for 60 min. This solution was mixed with phosphate-NaOH buffer containing lactose, and the volume was adjusted to 13 L by adding water and formulation (100 μg/ml of E5531, 4.25 mM phosphate-NaOH, and 10% lactose solution, pH 7.3). This solution was passed through a 0.22-μm filter and lyophilized. After reconstitution with water to an E5531 concentration of 100 μg/ml, 10 mM of $MgCl_2 \cdot 6H_2O$ solution was added to achieve [E5531]:[Mg^{2+}] molar ratios of 1:0, 1:1, 1:3, 1:5, 1:7, and 1:10.

Determination of the Structure of E5531 Aggregates

To obtain information on the structure of the E5531 aggregates, the trapped volume inside the aggregates was determined by dispersing 5 mg of E5531 in 2.5 ml of 70 mM calcein solution (pH 11.0), stirring at 50°C for 60 min. After cooling to 25°C, the pH of the solution was adjusted to 7.3 by the addition of 1 N HCl solution. The

untrapped calcein was removed by gel filtration (Sephadex G-50) at 25°C. The volume of the calcein solution trapped in the dispersed aggregates was determined fluorometrically (14) after solubilization of the lipid aggregates by the addition of 10% Triton X-100, and the aqueous volume trapped per mole of E5531 was evaluated. E5531 in the dispersion was assayed by high-performance liquid chromatography (HPLC) (detection wavelength 254 nm).

Effect of Mg^{2+} on E5531 Aggregate Size

The size of the E5531 aggregates was determined by the dynamic light scattering (DLS) techniques using a laser particle analyzer (model DLS-7000DL, Ohtsuka Electronics Co., Ltd., Osaka, Japan) after the addition of Mg^{2+} to the reconstituted solutions at 25°C. The data were analyzed by the histogram method (15), and the weight-average size was evaluated.

Effect of Mg^{2+} on Zeta Potentials

The zeta potentials of the E5531 aggregates after the addition of Mg^{2+} to the reconstituted solutions were measured at 25°C using a zeta potential analyzer (model ELS-800, Ohtsuka Electronics), and these data are represented as mean values of duplicate measurements.

Effect of Mg^{2+} on Membrane Fluidity of E5531 Aggregates

The membrane fluidity of E5531 aggregates after the addition of Mg^{2+} to the reconstituted solutions was determined using a fluorescence polarization technique (DPH probe) as reported by Iwamoto et al. (16). DPH was added at 1 mol% of total lipids. The excitation and emission wavelengths used were 360 nm and 428 nm, respectively. All fluorescence measurements were carried out using a model F-4500 fluorescence spectrophotometer (Hitachi Co., Ltd., Tokyo) equipped with a thermoregulated cell compartment, Atago Coolnics model REX-C10 (Atago Co., Ltd., Tokyo). The degree of polarization P was defined by the following equation:

$$P = (I_{VV} - C_f \cdot I_{VH})/(I_{VV} + C_f \cdot I_{VH})$$

where I is the fluorescence intensity and subscripts V and H indicate the vertical and horizontal orientations of excitation (first) and analysis (second) polarizers, respectively. $C_f(=I_{HV}/I_{HH})$ is the grating correction factor.

Effect of Mg^{2+} on the Micropolarity Around *N*-Dansylhexadecylamine

The effect of Mg^{2+} on the micropolarity of E5531 aggregates in the reconstituted solutions was determined using a fluorescence technique (DSHA probe). DSHA has been reported to give information on the phase transition by giving a large increase in fluorescence intensity, mainly as a result of higher partitioning of the dye in the head group phase of the fluid bilayers (17,18). DSHA was added at 1 mol% of total lipids. The fluorescence spectra were measured on excitation at 330 nm. The micropolarity of DSHA incorporated into the lipid bilayer was evaluated using the wavelength of maximum intensity of emission. DSHA (3 mg) was dissolved in 10 mg of methanol, ethanol, propanol, butanol, acetone, tetrahydrofuran, benzene, and hexane, and 5 μl of each solution were then diluted with 5 ml of the same solvent. The wavelengths at the maximum fluorescence intensity of each solution were plotted against the polarity of each solvent (19).

Effect of Mg^{2+} on the Stability of E5531 Aggregates in Rat Plasma

To evaluate the stability of E5531 aggregates ([E5531]:[Mg^{2+}] molar ratios of 1:0, 1:1, and 1:3) after intravenous injection into rats, the leakage of calcein from the aggregates in the plasma was determined according to a previously described method (20,21). In 25 ml of 70 mM calcein solution (pH 11.0), 50 mg of E5531 were dispersed with stirring at 50°C. After stirring for 60 min, the solution was cooled to 25°C, and the pH was adjusted to 7.3 by the addition of 1N HCl solution. The solution of $MgCl_2 \cdot 6H_2O$ was added to the E5531 solution to achieve [E5531]:[Mg^{2+}] molar ratios of 1:0, 1:1, and 1:3.

The untrapped calcein was eluted from Sephadex G-50 gel in the void fraction with 4.25 mM phosphate-NaOH, 10% lactose buffer solution (pH 7.3), and 0.5 ml of the fraction was added to 2.5 ml of rat plasma. The permeability of the E5531 aggregates was evaluated fluorometrically by monitoring the leakage of calcein during incubation with rat plasma at 37°C. The percentage leakage of calcein was calculated according to the following equation:

$$\text{Leakage (\%)} = [(F - F_0)/(F_\infty - F_0)] \times 100$$

where F_0 represents the initial fluorescence intensity at time zero, F is the fluorescence intensity monitored during the incubation at 37°C, and F_∞ denotes the maximum

fluorescence intensity after lysis of the aggregates by the addition of 0.1 ml of 10% Triton X-100.

RESULTS AND DISCUSSION

Determination of the Structure and Size of E5531 Aggregates

The size and the volumes of trapped inner space in the aggregates per mole of E5531 were determined. The trapped volumes of small unilamellar vesicles (diameter 20–50 nm), large unilamellar vesicles (200–1000 nm), and multilamellar vesicles (diameter 400–3500 nm) of phosphatidylcholine have been estimated to be 0.2–0.5, 3–4, and 7–10 L/mol, respectively (22). E5531 aggregates (diameter 20.4 nm) had a trapped volume of 0.27 L/mol. These data indicate that E5531 molecules form a liposomelike structure (small unilamellar vesicle).

Effect of Mg^{2+} on E5531 Aggregate Size and Zeta Potential

Figure 2 shows the weight-average size of E5531 aggregates evaluated by DLS measurements. At the molar ratios of [E5531]:[Mg^{2+}] = 1:0, 1:1, and 1:3, mean diameters were almost 20 nm, and no differences were observed. At the ratio of [E5531]:[Mg^{2+}] = 1:5 or greater, the aggregate size increased with the increase in the ratio of Mg^{2+}. In this study, to evaluate the effect of Mg^{2+} on the physicochemical properties in the similar size of the aggregates (approximately 20 nm), we decided to determine the zeta potentials, fluidity, and micropolarity of the membrane and stability in the plasma using the samples at the molar ratios of [E5531]:[Mg^{2+}] = 1:0, 1:1, and 1:3. Figure 3 represents the zeta potentials as a function of [Mg^{2+}]/[E5531]. Zeta potentials were negative and increased slightly as the concentration of Mg^{2+} increased. Since E5531 is negatively charged in neutral pH, the phosphate group at the head sugar moiety will give a net negative charge, and zeta potentials will have negative values. By addition of Mg^{2+}, the negative charge of the head phosphate group will be neutralized, and the zeta potential will be increased.

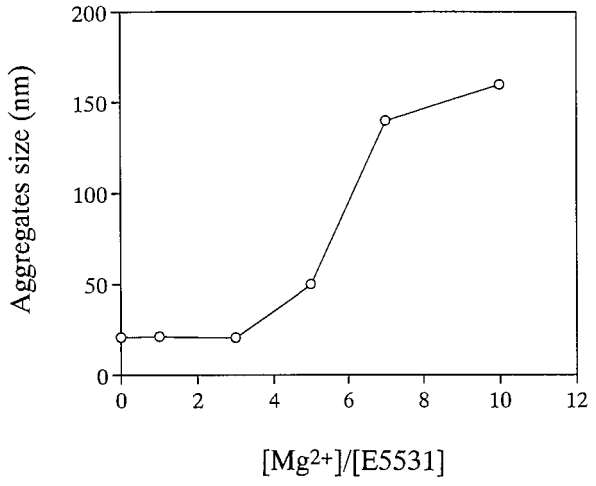

Figure 2. Relationship between the molar ratio [E5531]:[Mg^{2+}] and the weight-average size of aggregates of E5531.

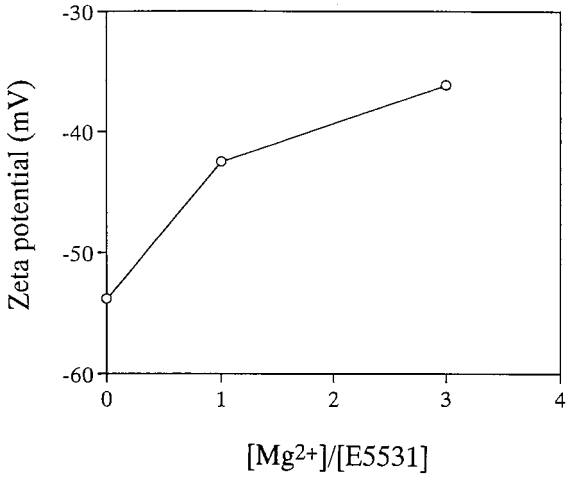

Figure 3. Relationship between the molar ratio [E5531]:[Mg^{2+}] and the zeta potential of aggregates of E5531.

Effect of Mg^{2+} on E5531 Membrane Fluidity

The influence of Mg^{2+} on the membrane fluidity of E5531 aggregates was evaluated by fluorescence polarization (DPH probe). Figure 4 illustrates the relationship between the temperature and fluorescence polarization as a function of [Mg^{2+}]/[E5531]. A dramatic change in fluorescence polarization and an increase in the phase transition temperature were seen with increases in [Mg^{2+}]/[E5531]. In the absence of the Mg^{2+}, the phase transition took place at 29°C, and as the [Mg^{2+}]/[E5531] increased, the phase transition shifted to a slightly higher temperature. When [E5531]:[Mg^{2+}] = 1:1, the phase transition temperatures increased to 34°C, and when [E5531]:[Mg^{2+}] = 1:3, the phase transition temperature increased and was not detected in the temperature range 20°C to 50°C. It was probable that the phase transition

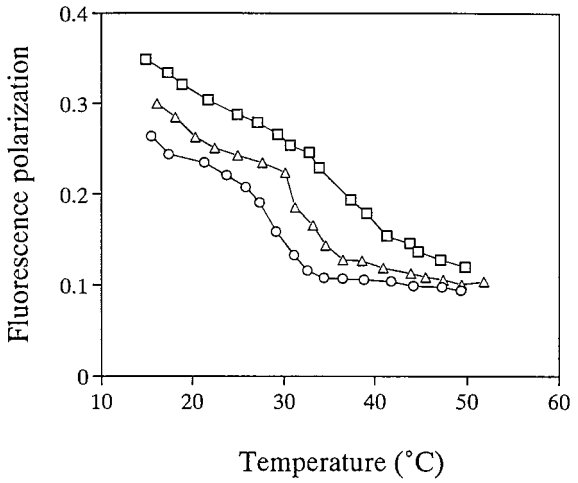

Figure 4. Relationship between temperature and fluorescence polarization of E5531 membranes (DPH probe) as a function of the molar ratio [E5531]:[Mg^{2+}]: ○, 1:0; △, 1:1; □, 1:3.

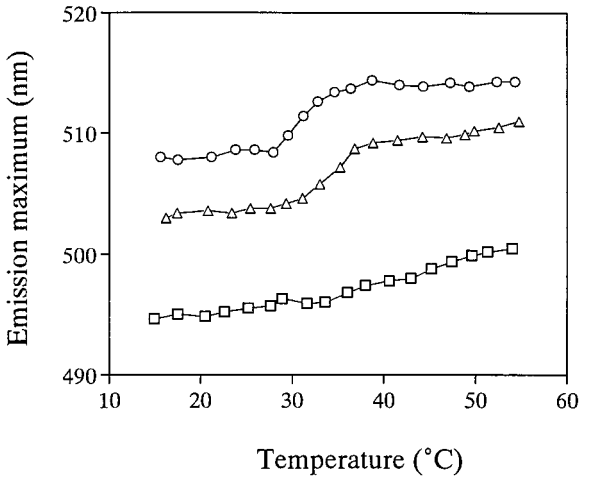

Figure 5. Relationship between temperature and fluorescence emission maxima of E5531 membranes (DSHA probe) as a function of the molar ratio [E5531]:[Mg^{2+}]: ○, 1:0; △, 1:1; □, 1:3.

temperature was shifted to above 50°C and therefore could not be detected in this study.

It has been reported that there is a strong correlation between the molar ratio of these divalent cations and the lipid A and LPS. The phase transition temperature (measured by Fourier transform infrared [FTIR] spectroscopy) of aggregates of LPS from *Salmonella minnesota* strain Rz have been reported by Seydel et al. (23). The LPS had a phase transition at 37°C after the addition of Mg^{2+}, and the transition temperature increased to 39°C ([LPS]:[Mg^{2+}] = 1:0.3) and 44°C ([LPS]:[Mg^{2+}] = 1:1). Brandenburg et al. reported that the lipid A from *S. minnesota* (strain R595) and *Escherichia coli* (strain F515) had a polymorphism (lamellar to hexagonal H_{II}) initiated in a highly cooperative fashion by increasing the molar ratio of Mg^{2+} to the lipids (11).

Within the molar ratios of [E5531]:[Mg^{2+}] = 1:0, 1:1, and 1:3, the aggregate size did not change, but the membrane fluidity decreased, indicating that, within that range of the molar ratio, Mg^{2+} had an intramolecular, not intermolecular, effect on the E5531 aggregates.

Effect of Mg^{2+} on the Micropolarity Around *N*-Dansylhexadecylamine in E5531 Aggregates

The influence of Mg^{2+} on the micropolarity around DSHA in E5531 aggregates was evaluated by the fluorescence intensity taken from the shift in the emission maximum wavelength. The emission maximum wavelength as a function of temperature is illustrated in Fig. 5 for E5531 aggregates at three different Mg^{2+} concentrations. It has been reported that the fluorescence characteristics of DSHA depend on the micropolarity around the probe, and that the dansyl fluorophore is located in the glycerol backbone of liposomal bilayers (18). It has also been reported that hydration increased greatly in phospholipid liposomes above the phase transition temperature (19). Therefore, it is expected that the emission maxima of DSHA in E5531 aggregates will provide information on the micropolarity around the surface. The emission maxima for E5531 aggregates with the molar ratio [E5531]:[Mg^{2+}] = 1:0, 1:1, and 1:3 at 25°C were approximately 508 nm, 504 nm, and 494 nm, respectively, indicating that the micropolarity around the probe in E5531 aggregates is comparable to that of butanol, acetone, and tetrahydrofuran, respectively.

Above the phase transition temperature, the maximum wavelengths increased and exhibited a red shift, indicating that the micropolarity around the surface of E5531 aggregates increased. In the absence of Mg^{2+}, the phase transition takes place at 30°C, and it is shifted to slightly higher temperatures with increasing Mg^{2+} concentrations. When [E5531]:[Mg^{2+}] = 1:1, the phase transition temperatures increased to 35°C, and when [E5531]:[Mg^{2+}] = 1:3, the phase transition temperature increased and was not detected at the temperature range 20°C to 50°C. It was probable that the phase transition temperature shifted above 50°C and therefore could not

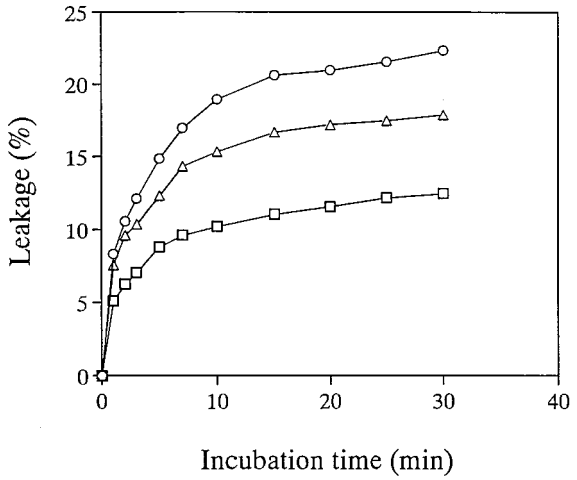

Figure 6. Leakage profile of calcein from E5531 aggregates at 37°C as a function of the molar ratio [E5531]:[Mg^{2+}]: ○, 1:0; △, 1:1; □, 1:3.

be detected in this study. Within the molar ratios of [E5531]:[Mg^{2+}] = 1:0, 1:1, and 1:3, the aggregate size did not change, but the membrane micropolarity decreased, indicating that, within that range of the molar ratio, Mg^{2+} had an intramolecular, not intermolecular, effect on the E5531 aggregates.

Stability of E5531 Aggregates in Rat Plasma

To investigate the stability of E5531 aggregates after intravenous injection into rats, the permeability of E5531 aggregates with different membrane fluidity ([E5531]:[Mg^{2+}] = 1:0, 1:1, and 1:3) in rat plasma was evaluated on the basis of the leakage profile of calcein at 37°C. Figure 6 represents the time course of the leakage of calcein from E5531 aggregates, indicating that the permeability of E5531 aggregates for calcein decreased with an increase in the concentration of Mg^{2+}. Based on the results from membrane fluidity (Fig. 4 and Table 1), the fluorescence polarization of E5531 aggregates at 37°C decreased with the increase in the concentration of Mg^{2+}, indicating that the decrease of the fluidity by addition of Mg^{2+} caused the decreased permeability of the aggregates after intravenous injection in rats.

It has been reported that the size of colloidal particles is correlated with their hepatic uptake, and a smaller aggregate size (less than 100 nm) may result in reduced hepatic uptake (24). Above the molar ratio of [E5531]:[Mg^{2+}] = 1:5, the size of the aggregates was drastically increased. At the molar ratio of [E5531]:[Mg^{2+}] = 1:3, the size of the aggregates was similar to that in the absence of Mg^{2+} (approximately 20 nm), and the stability in rat plasma was improved.

CONCLUSIONS

The effect of Mg^{2+} on the aggregate size, zeta potential, membrane fluidity, and micropolarity and permeability of the E5531 aggregates was investigated using several physicochemical techniques. E5531 formed a vesicle structure, and within the molar ratios of [E5531]:[Mg^{2+}] = 1:1 and 1:3, Mg^{2+} increased the zeta potentials of the E5531 membrane, but did not change the size of the aggregates (approximately 20 nm). Within that molar ratio, Mg^{2+} decreased the membrane fluidity and mi-

Table 1

Effect of Mg^{2+} on the Physicochemical Properties of E5531 Aggregates and the Stability in Rat Plasma

[Mg^{2+}]/[E5531]	Aggregate Size (nm)	Zeta Potential (mV)	Fluorescence Polarization at 37°C (Probe: DPH)	Leakage in Rat Plasma (%)
0	20.4 ± 6.8	−53.8	0.107	22.3
1	21.2 ± 6.0	−42.5	0.128	17.9
3	20.1 ± 6.2	−36.1	0.194	12.5
5	50.7 ± 23.2	NP	NP	NP
7	140.7 ± 75.0	NP	NP	NP
10	160.2 ± 38.1	NP	NP	NP

NP = not performed.

cropolarity of E5531 and increased the phase transition temperature. Above the ratio of [E5531]:[Mg^{2+}] = 1:5, the size of the aggregates increased. At the ratio of [E5531]:[Mg^{2+}] = 1:3, the size of the aggregates was similar to that in the absence of Mg^{2+} (approximately 20 nm), and we could stabilize the aggregates in rat plasma.

REFERENCES

1. E. Th. Rietschel, H. W. Wollenweber, H. Brade, U. Zähringer, B. Lindner, U. Seydel, H. Bradeczek, G. Barnickel, H. Labisinski, and P. Giesbrecht, in *Handbook of Endotoxins*, Vol. 1 (E. Th. Rietschel, Ed.), Elsevier, Amsterdam, 1984, p. 187.
2. H. Nikaido and M. Vaara, Microbiol. Rev., 49, 1 (1985).
3. H. Labishinski, G. Barnickel, H. Bradaczek, D. Naumann, E. Th. Rietschel, and P. Giesbrecht, J. Bacteriol., 162, 9 (1985).
4. S. N. Vogel, G. S. Madonna, L. M. Wahl, and P. D. Rick, J. Immunol., 132, 347 (1984).
5. E. Th. Rietschel, H. Brade, L. Brade, K. Brandenburg, U. Schade, U. Seydel, U. Zähringer, C. Galanos, O. Lüderits, O. Westphal, H. Labishinski, S. Kusumoto, and T. Shiba, in *Detection of Bacterial Endotoxins with the Limulus Amebocyte Lysate Test* (S. Watson, Ed.), Alan R. Liss, New York, 1987, pp. 35–39.
6. D. Naumann, Ch. Schults, J. Born, H. Labishinski, K. Brandenburg, G. Busse, H. Brade, and U. Seydel, Eur. J. Biochem., 164, 159 (1987).
7. G. Emmerling, U. Henning, and L. Gullik, Eur. J. Biochem., 78 (1977).
8. H. Labishinski, D. Naumann, Ch. Schults, S. Kusumoto, T. Shiba, E. Th. Rietschel, and P. Giebrecht, Eur. J. Biochem., 179, 659 (1989).
9. K. Brandenburg and U. Seydel, Biochim. Biophys. Acta, 755, 225 (1984).
10. U. Seydel, K. Brandenburg, M. H. J. Koch, and E. Th. Rietschel, Eur. J. Biochem., 186, 325 (1989).
11. K. Brandenburg, M. H. J. Koch, and U. Seydel, J. Struct. Biol., 105, 11 (1990).
12. W. J. Christ, O. Asano, A. L. Robidoux, M. Perez, Y. Wang, G. R. Dobuc, W. E. Gavin, L. D. Hawkins, P. D. McGuinness, M. A. Mullarkey, P. D. Lewis, T. Kishi, T. Kawata, J. R. Bristol, J. R. Rose, D. P. Rossignol, S. Kobayashi, I. Hishinura, A. Kimura, N. Asakawa, K. Katayama, and I. Yamatsu, Science, 268, 80 (1995).
13. Y. Asai and S. Watanabe, Drug Dev. Ind. Pharm. (1998).
14. T. M. Allen and L. G. Cleland, Biochim. Biophys. Acta, 597, 418 (1980).
15. E. Gulari, Y. Tsunashima, and B. Chu, J. Chem. Phys., 70, 3965 (1979).
16. K. Iwamoto, J. Sunamoto, K. Inoue, T. Endo, and S. Nojima, Biochim. Biophys. Acta, 691, 44 (1982)
17. A. S. Waggoner and L. Stryer, Proc. Natl. Acad. Sci. USA, 67, 579 (1970).
18. K. Iwamoto and J. Sunamoto, Bull. Chem. Soc. Jpn., 54, 399 (1981).
19. K. Dimorth and C. Reichardt, Liebg. Ann., 661, 1 (1963).
20. C. Kirby, J. Clarke, and G. Gregoriadis, Biochem. J., 186, 591 (1980).
21. H. Kiwada, I. Nakajima, H. Matsuura, and M. Tsuji, Chem. Pharm. Bull., 36, 1841 (1988).
22. F. Szoka, Jr., and D. Papahadjopoulos, Proc. Natl. Acad. Sci. USA, 75, 4194 (1978).
23. U. Seydel, K. Brandenburg, M. H. J. Michel, E. Th. Rietschel, Eur. J. Biochem., 186, 325 (1989).
24. H. Sezaki, *Drug Delivery System*, Nanko-do Press, Kyoto, Japan, 1986, pp. 137–145.