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The Effect of Divalent Cations on the Membrane Properties and Pharmacokinetics in Rat of the Lipid A Analogue E5531

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Abstract

To obtain information on the effects of Ca^{2+} on the membrane properties of the lipid A analogue E5531, we have determined the aggregate size, zeta potential, membrane fluidity, micropolarity and permeability of the E5531 membrane as a function of Ca^{2+} levels.

Within the molar ratios of $[Ca^{2+}]/[E5531] = 1$ and 3, Ca^{2+} increased the zeta potential of the E5531 membrane but had no effect on aggregate size (approximately 20 nm). Within the above ratios, Ca^{2+} decreased the membrane fluidity, as measured by micropolarity of E5531 and increased the phase transition temperature.

The pharmacokinetics in rats for these samples with different membrane fluidity, prepared by changing the pre-dose formulation concentration of Ca^{2+}, was determined and a correlation between membrane fluidity and pharmacokinetics was clearly observed.

It thus appears that Ca^{2+} effects the membrane fluidity of E5531 as well as its pharmacokinetics in rats.

Lipid A, a lipid anchor in lipopolysaccharide, is located on the outer membrane of Gram-negative bacteria. Lipid A is a potent biologically active site (Morrison & Ryan 1979) and is involved in the induction of prostaglandins and cytokines such as interferon (Homma et al 1985) in mammalian macrophages and lymphocytes. The compound is also involved in inducing certain undesirable toxic effects such as fever and the Schwartzmann bleeding reaction (Vogel et al 1984; Galanos et al 1985).

Numerous attempts have been made to synthesize low toxicity lipid A analogues. Recent work in our laboratory indicates that some lipid A analogues may be potent lipopolysaccharide antagonists. The synthetic disaccharide lipid A analogue E5531 (Figure 1) has a low toxicity, while retaining a variety of useful biological activities (such as reduction of production of tumour necrosis factor (TNF)) (Christ et al 1995). This compound has been found to be a specific lipopolysaccharide antagonist, as evidenced by a lipopolysaccharide-binding assay, and inhibits lipopolysaccharide-induced TNF production in monocytes/macrophages. The anticipated use for this compound is as a drug for the treatment of septic shock.

In this study, a new pH-jump method for dispersing E5531 has been developed for the preparation of an injectable formulation (Asai et al 1998). E5531 aggregates have vesicles with diameters of approximately 20 nm (Asai et al 1999a). In addition, the membrane fluidity was determined in the dispersing process in the pH-jump method and the pharmacokinetic profile was found to be dependent on membrane fluidity (Asai et al 1999b).

In the case of cationic phospholipids, membrane fluidity is affected by divalent cations which results in an increase in the phase transition temperature (Hammoudah et al 1979; Dluhy et al 1983; Hauser & Shipley 1984). E5531 is also classified as a cationic lipid and, hence, it would be expected that the membrane fluidity of E5531 aggregates would be altered by the addition of cations. In this study, the following hypotheses were examined: E5531 aggregates will rapidly interact with macromolecules and other factors in biological fields to form reaction products; the resulting pharmacokinetic properties of these reaction products would

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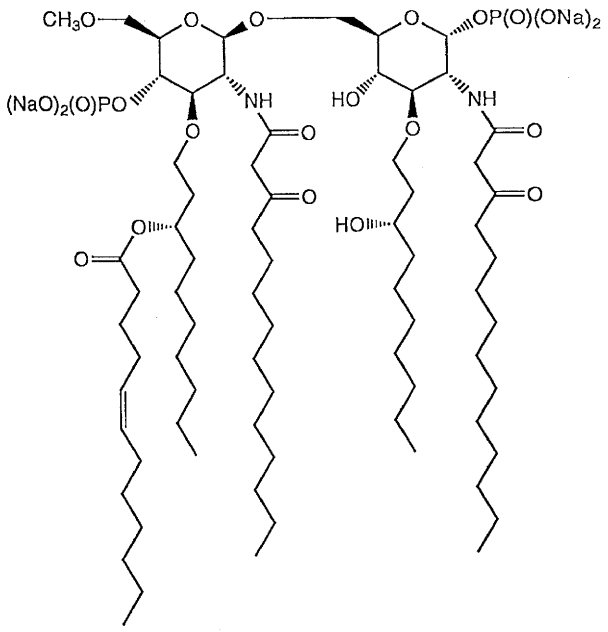

Figure 1. Chemical structure of the synthetic lipid A analogue, E5531.

likely be a function of the membrane properties of the original particles, which in turn would be functions of the formulation conditions, particularly [Ca^{2+}].

The purpose of this study was to verify the above hypotheses by investigating the effects of Ca^{2+} on E5531 membrane properties such as aggregate size, zeta potential, membrane fluidity, micropolarity and permeability and to correlate these physicochemical properties with pharmacokinetics in rats.

Materials and Methods

Materials

E5531 (MW = 1543·80) was obtained from Eisai Chemical Co., Ltd (Ibaraki, Japan). Calcium chloride ($CaCl_2$) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Wako Pure Industrial Ltd (Osaka, Japan). Calcein (3,3′-bis[*N*,*N*-bis (carboxymethyl)aminomethyl]-fluorescein) was purchased from Dojin Co., Ltd (Kumamoto, Japan). *N*-Dansylhexadecylamine (DSHA) was obtained from Lambda Co., Ltd (Graz, Austria).

Preparation of E5531 dispersions

Samples were prepared using the pH-jump method (Asai et al 1998). A 1·3-g sample of E5531 was dispersed in 650 mL 0·003 M NaOH solution by stirring at 50°C for 60 min. This solution was mixed with phosphate–NaOH buffer containing lactose and the volume adjusted to 13 L by adding water to achieve the formulated solution, which was E5531: 100 $\mu g\,mL^{-1}$ (64·7 μM), 4·25 mM phosphate–NaOH and 10% lactose solution, pH 7·3. This formulated solution was passed through a 0·22-μm filter and lyophilized. After reconstitution with water to an E5531 concentration of 100 $\mu g\,mL^{-1}$, the solution was ready for use in the experiments. Solutions (10 mM) of $CaCl_2$ were prepared and added to the E5531 reconstituted solutions to achieve [Ca^{2+}]/[E5531] molar ratios of 0, 1, 3, 5, 7 and 10.

Effect of Ca^{2+} on E5531 aggregate size

The size of the E5531 aggregates was determined after the addition of Ca^{2+} to the reconstituted solutions at 25°C by dynamic light scattering (DLS) techniques using a laser particle analyser (model DLS-7000DL, Ohtsuka Electronics Co., Ltd, Osaka, Japan). The data were analysed by the histogram method (Gulari et al 1979) and the weight-averaged size evaluated.

Effect of Ca^{2+} on zeta potentials

Zeta potentials of the E5531 aggregates after the addition of Ca^{2+} to the reconstituted solutions were measured at 25°C using a zeta potential analyser (model ELS-800, Ohtsuka Electronics Co., Ltd, Osaka, Japan). The data are given as the mean value of triplicate measurements.

Effect of Ca^{2+} on membrane fluidity of E5531 aggregates

The membrane fluidity of E5531 aggregates, after the addition of Ca^{2+} to the reconstituted solutions, was determined using a fluorescence polarization technique (probe: DPH) as reported by Iwamoto et al (1982). DPH has been well established as a probe for evaluation of the fluidity of the hydrocarbon regions in lipid membranes (Shinitzky 1984). DPH has an *all-trans*-polyene structure and a rod-like shape. The adsorption and fluorescence transition moments lie along the major axis of the molecule. Therefore, when DPH is excited by pulsed light at the last absorption band, time dependent emission anisotropy reflects the molecular motion of the hydrocarbon chains around DPH. DPH was added at 1 mol% of total lipids. The excitation and emission wavelengths used were 360 nm and 428 nm, respectively. All fluorescence measure-

ments 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 steady-state anisotropy (r_s) is defined by the following equation:

$$r_s = (I_{VV} - C_f \cdot I_{VH})/(I_{VV} + 2C_f \cdot I_{VH}) \quad (1)$$

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. The order parameter, S, was calculated using the following equation (Heyn 1979):

$$S = (r_s/r_o)^{1/2} \quad (2)$$

where r_o represents the maximal and limiting fluorescence anisotropy. For DPH, r_o has been estimated to be 0·398 using nanosecond time-resolved fluorescence techniques (Heyn 1979). In this work, equation 2 was used to estimate the order parameter, S. The data are given as the mean value of triplicate measurements.

Effect of Ca^{2+} on the micropolarity around DSHA

The effect of Ca^{2+} on the micropolarity of E5531 aggregates was determined using a fluorescence technique (probe: DSHA). The fluorescence characteristics of DSHA were dependent on the micropolarity around the probe (Waggoner & Stryer 1970). In the case of phospholipid liposomes, the dansyl fluorophore is located in the glycerol backbone of liposomal bilayers (Iwamoto et al 1982) and the hydration increases greatly in phospholipid liposomes above the phase transition temperature (Iwamoto & Sunamoto 1981; Iwamoto et al 1982). Therefore, it is expected that the emission maxima of DSHA in E5531 aggregates would provide information on the micropolarity around their surface. DSHA was added at 1 mol% of the total lipids. The fluorescence spectra were measured by excitation at 330 nm as a function of the incubation temperature. The micropolarity of DSHA incorporated into the E5531 membranes was evaluated using the wavelength of maximum intensity of emission. DSHA (3 mg) was dissolved in 10 mg methanol, ethanol, propanol, butanol, acetone, tetrahydrofuran, benzene or hexane. Samples of each solution (5 μL) 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 (Dimorth & Reichardt 1963). The micropolarity around the probe was determined using this standard curve. The data are given as the mean value of triplicate measurements.

Effect of Ca^{2+} on the pharmacokinetics of E5531 aggregates in rats

E5531 reconstituted solutions (100 μg mL^{-1}, pH 7·3, $[Ca^{2+}]/[E5531] = 0$, 1 and 3) were administered as a bolus injection (0·3 mg kg^{-1}) via a femoral vein. Blood samples were collected before and 2, 10 and 30 min and 1 and 2 h after dosing. The concentration of E5531 in plasma (100 μL) was measured by HPLC analysis with fluorescence detection (Reynaud et al 1994). The area under the plasma concentration–time curve (AUC) was calculated by the trapezoidal method from the plasma concentration for 2 h after administration ($AUC_{0-2\,h}$). For the AUC calculations the plasma concentration at time zero was estimated to be equal to the concentration at the first time-point measured (2 min).

Determination of permeability in rat plasma for E5531 aggregates with Ca^{2+}

To investigate the stability of E5531 aggregates ($[Ca^{2+}]/[E5531] = 0$, 1 and 3) after intravenous injection into rats, the leakage of calcein from the aggregates in plasma was evaluated using previously described methods (Kirby et al 1980; Kiwada et al 1988). E5531 (50 mg) was dispersed in 25 mL 70 mM calcein solution (pH 11·0), stirred at 50°C for 60 min and then cooled to 25°C. The pH of the solution was then adjusted to 7·3 by a 1 M HCl solution. The solutions of $CaCl_2$ were added to the E5531 solutions to give $[Ca^{2+}]/[E5531] = 0$, 1 and 3.

The untrapped calcein was eluted from Sephadex G-50 gel with 4·25 mM phosphate–NaOH, 10% lactose buffer solution (pH 7·3) and 0·5 mL of this 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 percent leakage of calcein was calculated using the following equation:

$$\text{Leakage (\%)} = [(F - F_o)/(F_\infty - F_o)] \times 100 \quad (3)$$

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 via the addition of 0·1 mL 10% Triton X-100.

Results

Determination of E5531 aggregate size and zeta potential

Table 1 shows the weight-averaged size of E5531 aggregates in the presence of different molar ratios of $[Ca^{2+}]/[E5531]$, as evaluated by DLS measurements.

At molar ratios $[Ca^{2+}]/[E5531] = 0$, 1 and 3, the mean diameters were close to 20 nm. At molar ratios of 5, 7 and 10, the aggregate size increased with increasing $[Ca^{2+}]$. The size distribution of E5531 aggregates in the absence of Ca^{2+} was a single population. After the addition of Ca^{2+} ($[Ca^{2+}]/[E5531] = 10$), the shape of the histogram (not shown) was a normally distributed single population, indicating that the distribution did not change to the multi-model as the result of added Ca^{2+}. To evaluate the effect of Ca^{2+} on the physicochemical properties at the conditions where the size of the aggregates was similar (approximately 20 nm), the zeta potentials, fluidity and micropolarity of the membrane, pharmacokinetics in rats and stability in the plasma were determined using the samples having molar ratios $[Ca^{2+}]/[E5531] = 0$, 1 and 3. Zeta potentials were negative and increased slightly with increasing $[Ca^{2+}]$ (Table 1). Since E5531 is negatively charged in neutral aqueous solution, the phosphate group at the head sugar moiety has a net negative charge and minus values for the zeta potentials. The addition of Ca^{2+}, in part, neutralizes the negative charge of the head phosphate group, thus causing the zeta potential to be increased. An increase of the zeta potential, in some cases, induces instability of the colloidal particles (Kamo et al 1978) and their fusion (Nakagaki et al 1982). However, at the molar ratio $[Ca^{2+}]/[E5531] = 0$, 1 and 3, these phenomena were not observed.

Effect of Ca^{2+} on E5531 membrane fluidity

The influence of Ca^{2+} on the membrane fluidity of E5531 aggregates was evaluated by fluorescence polarization (probe: DPH). Figure 2 illustrates the relationship between the temperature and order parameter as a function of the molar ratio of $[Ca^{2+}]/[E5531]$. A dramatic change in fluorescence polarization and an increase in the phase transition temperature occurred with increasing $[Ca^{2+}]$. In the absence of Ca^{2+}, a phase transition takes place at 30°C and, as $[Ca^{2+}]$ is increased, the phase transition is shifted to slightly higher temperatures. When $[Ca^{2+}]/[E5531] = 1$, the phase transition temperatures increased to 37°C and when $[Ca^{2+}]/[E5531] = 3$, the phase transition temperature increased and was not detected in the temperature range from 20°C to 50°C. This is probably due to the fact that the phase transition temperature is lost by the formation of a glass-like phase which has no distinct phase transition temperature.

Effect of Ca^{2+} on the micropolarity around DSHA in E5531 aggregates

The influence of Ca^{2+} on the micropolarity around DSHA in E5531 aggregates was evaluated by the

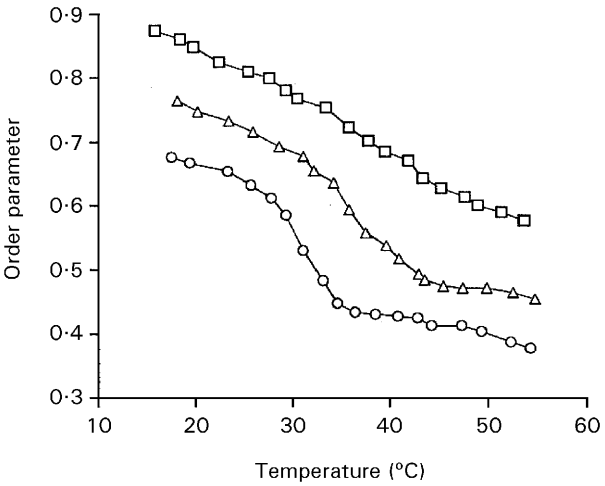

Figure 2. Relationships between temperature and order parameter of E5531 membranes (fluorescence probe: 1,6-diphenyl-1,3,5-hexatriene) at 39°C as a function of the molar ratio of $[Ca^{2+}]/[E5531]$. Each point represents the mean of three samples. $[Ca^{2+}]/[E5531] = 0$ (○), 1 (△), and 3 (□).

Table 1. Effect of Ca^{2+} on the physicochemical properties of E5531 aggregates and pharmacokinetics in rats.

$[Ca^{2+}]/[E5531]$	Aggregate size (nm)	Zeta potential (mV)	Order parameter at 37°C (probe: 1,6-diphenyl-1,3,5-hexatriene)	AUC_{0-2h} (ng h mL^{-1})	Plasma half-life ($t_{1/2}$ 0·5 h ~ (h)
0	20·9 ± 6·2	−48·7 ± 0·6	0·434	11384 ± 376	4·0 ± 1·0
1	21·2 ± 6·4	−39·3 ± 0·7	0·558	8582 ± 665	4·3 ± 1·3
3	20·6 ± 6·2	−35·1 ± 0·5	0·702	5321 ± 419	4·0 ± 1·2
5	54·8 ± 26·6	np	np	np	np
7	144·9 ± 78·0	np	np	np	np
10	169·0 ± 36·2	np	np	np	np

np = not performed.

fluorescence intensity taken from the shift in the maximum for the emission wavelength. The emission maximum wavelength as a function of temperature is illustrated in Figure 3 for E5531 aggregates at three different Ca^{2+} concentrations. 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 Ca^{2+}, the phase transition takes place at 30°C and is shifted to slightly higher temperatures with increasing Ca^{2+} concentrations. When $[Ca^{2+}]/[E5531] = 1$, the phase transition temperatures increased to 35°C and when $[Ca^{2+}]/[E5531] = 3$, phase transition temperature increased, but was not detected in the temperature range from 20°C to 50°C. These results are consistent with fluorescence polarization data.

Figure 4 represents the relationship between the micropolarity of the solvent and the emission maximum from DSHA at 25°C. Based upon the results of the emission maxima and the standard curve (Figure 4), information on the effect of Ca^{2+} on the micropolarity around DSHA in E5531 aggregates can be obtained. The emission maxima for E5531 aggregates with the molar ratio of $[Ca^{2+}]/[E5531] = 0$, 1 and 3 at 25°C were approximately 508·6, 503·8 and 499·5 nm, respectively. This indicates that the micropolarity around the probe in E5531 aggregates was comparable with that of butanol, acetone and acetone, respectively, and that the micropolarity around the probe was decreased.

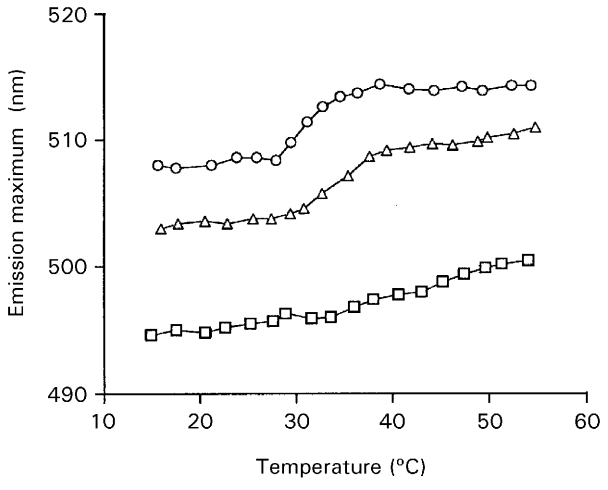

Figure 3. Relationships between temperature and fluorescence emission maximum of E5531 membranes (fluorescence probe: dansylhexadecylamine) as a function of the molar ratio of $[Ca^{2+}]/[E5531]$. Each point represents the mean of three samples. $[Ca^{2+}]/[E5531] = 0$ (○), 1 (△), and 3 (□).

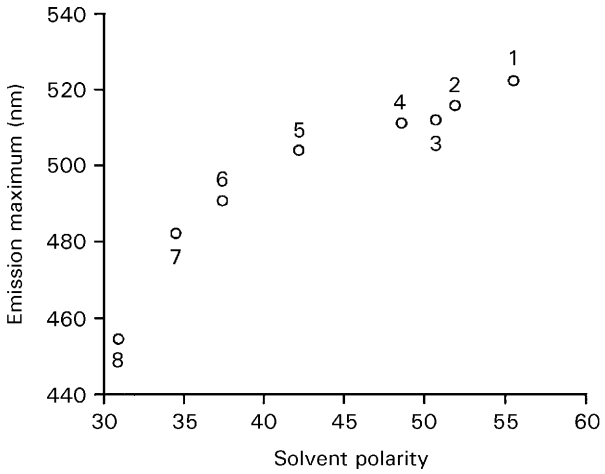

Figure 4. Relationships between solvent polarity and emission maximum of dansylhexadecylamine (64·8 nM) at 25°C. 1, Methanol; 2, ethanol; 3, propanol; 4, butanol; 5, acetone; 6, tetrahydrofuran; 7, benzene; 8, hexane.

Pharmacokinetics in rats for E5531 aggregates with different membrane fluidity

To investigate the relationship between the membrane fluidity of E5531 aggregates and the pharmacokinetic profile, a reconstituted E5531 solution ($100\,\mu g\,mL^{-1}$, pH 7·3) with different membrane fluidity, prepared by the addition of Ca^{2+}, was intravenously injected into rats. The plasma concentrations up to 2 h after dosing indicated linear kinetics as judged by $AUC_{0-2\,h}$ and plasma half-life (Table 1).

Different pharmacokinetic profiles in rats are observed for samples with different membrane fluidity. We believe that the fluidity of the E5531 membrane was decreased by the addition of Ca^{2+} and that this fluidity has a direct effect on the pharmacokinetics of the compound in rats.

We should note that the evaluation of AUC includes the effect of distribution and the elimination phases (or the plasma half-life, $t_{1/2}$). As shown in Table 1, the $t_{1/2}$ values of the three samples were similar and we believe that the fluidity will effect the distribution phase. Further investigation is necessary to clarify the effect of the fluidity on the distribution phase of E5531 and biodistribution in rats using the [^{14}C]E5531 compound.

Stability of E5531 aggregates in rat plasma

To investigate the stability of E5531 aggregates after intravenous injection in rats, the permeability of E5531 aggregates with different membrane fluidity ($[Ca^{2+}]/[E5531] = 0$, 1 and 3) in rat plasma was evaluated on the basis of the leakage profile of calcein at 37°C. Figure 5 shows the time course for the leakage of calcein from E5531 aggregates, and

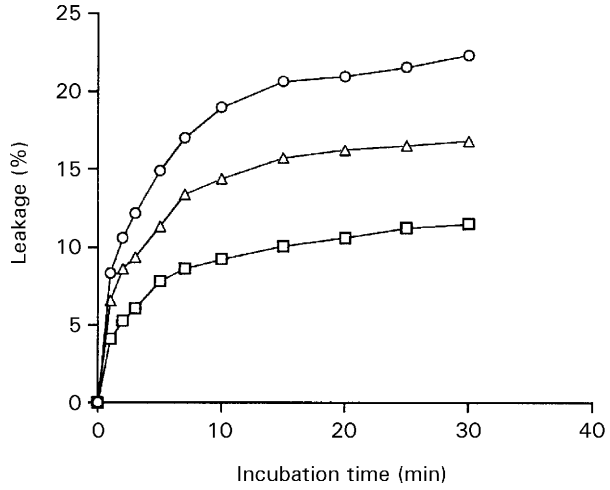

Figure 5. Leakage profiles of calcein from E5531 aggregates at 37°C as a function of the molar ratio of $[Ca^{2+}]/[E5531]$. $[Ca^{2+}]/[E5531] = 0$ (○), 1 (△), and 3 (□).

indicates that the permeability of E5531 aggregates for calcein decreased with increase in $[Ca^{2+}]$. Based on the data relative to membrane fluidity (Figure 2 and Table 1), the order parameters of E5531 aggregates at 37°C decreases with increasing $[Ca^{2+}]$, indicating that the decrease in fluidity, as the result of added Ca^{2+}, caused a lower permeability of the aggregates after intravenous injection in rats.

Discussion

Within the molar ratio of $[Ca^{2+}]/[E5531] = 0$, 1 and 3, aggregate size did not change but the membrane fluidity was clearly altered. We can speculate that within this range of molar ratios, Ca^{2+} exerts an intramolecular, but not an intermolecular effect on the E5531 aggregates.

Distinctly different pharmacokinetic profiles were obtained in rats using samples obtained via changing the Ca^{2+} concentration and the membrane fluidity. Hampton & Raets (1991) reported on the interactions between the metabolism of lipid A-like molecules by macrophages and their response to these molecules. They concluded that the uptake of the molecules is mediated by scavenger receptors. At this time, the metabolic pathway of E5531 is not known with certainty. As shown in Table 1, the $t_{1/2}$ values of the three samples were similar and the fluidity did effect the distribution phase. It is assumed that when E5531 molecules are bound to the receptors and incorporated into the macrophages in the distribution phase, the more rigid membrane is more easily bound to the receptor and the uptake of the molecules therefore, occurs more rapidly.

In addition, Ca^{2+} affected the stability of E5531 aggregates in rat plasma. Based on the membrane fluidity data (Figure 2), the order parameter of E5531 aggregates at 37°C increased with increasing $[Ca^{2+}]$. The rigidity of the E5531 membrane may increase with an increase in $[Ca^{2+}]$ and a lower permeability of calcein. These leakage data indicate that, on injection of the sample with Ca^{2+} into the blood, the affinity of Ca^{2+} for E5531 is large and the equilibrium between Ca^{2+}/E5531 will not be altered by rapid dilution of the Ca^{2+}.

In conclusion, within the molar ratios of $[Ca^{2+}]/[E5531] = 0$, 1 and 3, Ca^{2+} increased the zeta potentials of the E5531 membrane but had no effect on aggregate size (approximately 20 nm). The Ca^{2+} decreased the fluidity and micropolarity of E5531 membranes and these membrane properties of the aggregates could be correlated with the pharmacokinetics in rats.

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