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Formation of spherical micelles composed of the novel platelet activating factor receptor antagonist, E5880

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E5880, a novel platelet activating factor receptor antagonist, was dispersed in water for use in an injectable formulation and the physicochemical properties of the preparation were characterized. The critical concentration for formation of micelles was 0.12 mM. Using area per molecule data, the critical packing parameter was calculated, indicating that the structure of the micelles was spherical and that each micelle containes 49 molecules. The diameter of the micelles was 8.1 nm. Attractive interactions occurred between E5880 molecules in the micelle. The hydrocarbon region in the micelle was more rigid and less hydrated than that of other surfactants, stearyltrimethylammonium chloride and cetyltrimethylammonium chloride.

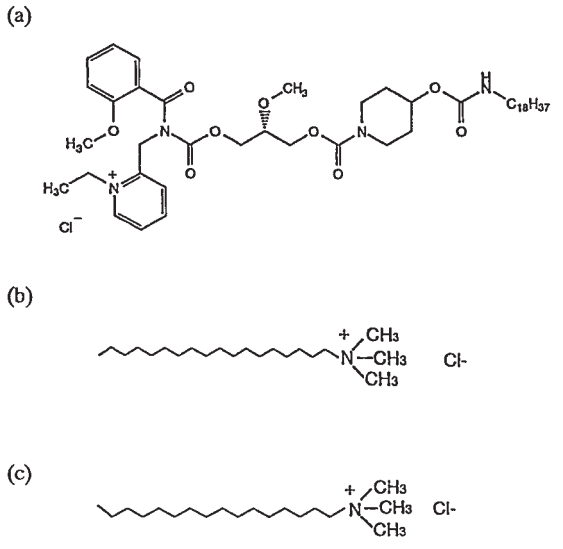
1. Introduction

Platelet activating factor (PAF), 1-alkyl-2-acetylglyceryl-phosphocholine, comprises a group of biologically active phosphoglycerides with actions more diverse than those of eicosanoids (Hanahan 1986). PAF exhibits a variety of biological activities including the activation of platelets (Benveniste et al. 1972), neutrophils (Shaw et al. 1981), bronchonstriction (Vargaftig et al. 1980), hypermeability in peripheral veins (Humphery et al. 1982), hypotension (Blank et al. 1979), and cardiac dysfunction (Bessin et al. 1983). Because these biological activities represent mediators of inflammation (Saeki et al. 1985), they play important roles in the pathology of thrombosis, asthma or hypotension in shock (Oh-ishi et al. 1986; Imura et al. 1986; Takizawa et al. 1988). Consequently, it would be expected that septic PAF receptor antagonists may be beneficial for the treatment of these diseases, and many efforts to develop potent and specific PAF antagonists have been made.

Several PAF antagonists, such as CV-3988 (Terashita et al. 1983), CV-6209 (Terashita et al. 1987), SRI63-072 (Handley et al. 1986), U66985 (Tokumura et al. 1985) have been synthesized and their biological activities were evaluated. These compounds have hydrocarbon chains ($C_{18:0}$) and are amphililic, indicating that some aggregates would be expected to be formed in aqueous media. They were dissolved in an aqueous medium and injected into animals for evaluation of their biological activities. It is noteworthy, however, that their physicochemical properties have not been reported.

E5880, a newly synthesized PAF antagonist (Formelschema), is more potent in terms of PAF receptor binding than PAF (Nagaoka et al. 1991). This compound is amphiphilic and would be expected to form micelles in aqueous media. For the treatment of the above mentiened diseases, an injectable formulation would be extremely useful. In order to develop an injectable formulation, it is important to clarify the characteristics of the physicochemical properties of E5880 micelles.

In this study, to better understand the behavior of E5880 micelles in water, the critical micelle concentration was determined by surface tension measurements. From this, the surface density was calculated, and a surface pressure-molecular area curve could be obtained. The lateral interaction between the molecules was also evaluated, and the micropolarity in the vicinity of the hydrocarbon region of the micelles was determined using fluorescence



Chemical structures; (a) Novel platelet activating factor receptor antagonist, E5880, (b) Stearyltrimethylammonium chloride (STAC), (c) Cetyltrimethylammonium chloride (CTAC)

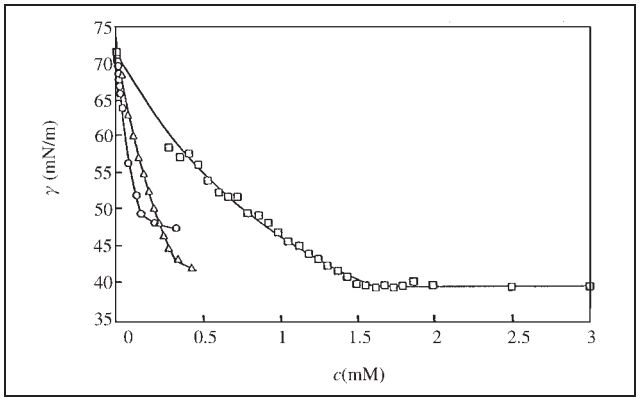


Fig. 1: Surface tension versus concentration curves for aqueous solutions of E5880, STAC and CTAC at 25 °C (○) E5880, (△) STAC, (□) CTAC

techniques. These features were compared with the surfactants, stearyltrimetylammonium chloride (STAC), which has the same length of hydrocarbon chains ($C_{18:0}$) as E5880, as well as with cetyltrimetylammonium chloride (CTAC), which has a fewer carbon number ($C_{16:0}$) than either E5880 or STAC.

2. Investigations, results and discussion

2.1. Surface tension

In order to determine the cmc, the surface tension of the lipids were measured. Fig. 1 shows the surface tension γ vs. concentration c curves for the three lipids, aqueous E5880, STAC and CTAC solution. The γ value decreased with increasing molarity c, and passed through a break point at the molarity corresponding to critical micelle concantration (cmc). The cmc values of E5880, STAC and CTAC were found to be 0.12, 0.33 and 1.6 mM, respectively. The cmc values of STAC and CTAC were similar to previously reported values (Rosen 1978).

2.2. Surface density and surface pressure vs. molecular area curve

Surface density Γ is calculated from the slope of the γ-c curve using the following equation (Handa et al. 1990);

$$\Gamma = -(c/iRT)(\partial\gamma/\partial c)_{T,P} \qquad (1)$$

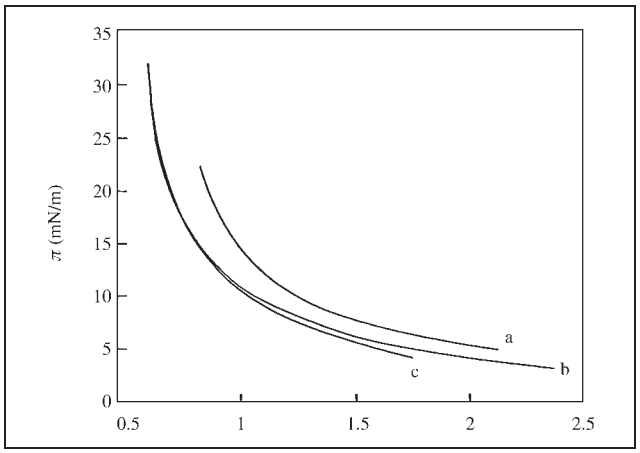


Fig. 2: Surface pressure versus molecular area of aqueous solutions of E5880, STAC and CTAC at 25 °C (a) E5880, (b) STAC, (c) CTAC

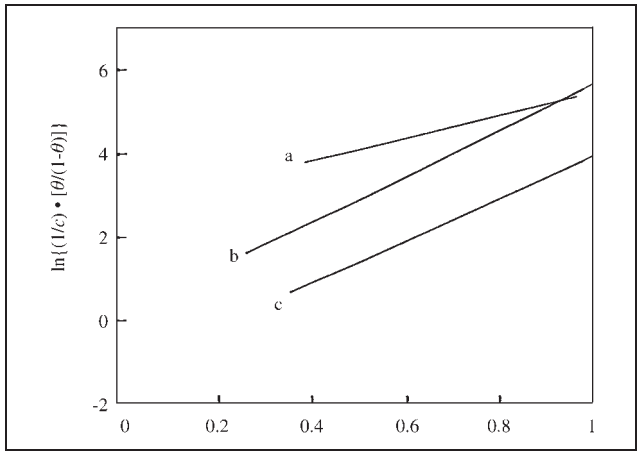


Fig. 3: Relationship between θ and ln{(1/c) · [θ/(1−θ)]} for the evaluation of lateral interaction parameters of the lipids. (a) E5880, (b) STAC, (c) CTAC

Here, T is temperature and R is the gas constant, and i indicates the number of ions from electrolytes. The Γ value increases with increasing concentration of c and approaches the saturated surface densities Γ_s at cmc.
The surface pressure is expressed by

$$\pi = \gamma_0 - \gamma \qquad (2)$$

and surface area per lipid molecules *A* is calculated by

$$A = 1/N_A\,\Gamma \qquad (3)$$

where N_A is Avogadro's number. Fig. 2 shows the surface pressure vs. molecular area (π − A) curves. The limiting areas for E5880, STAC and CTAC were determined to be 0.840, 0.633 and 0.627 nm^2, respectively. The molecular structure of the head group of E5880 is larger than those of STAC and CTAC, as a result, the limiting area of E5880 is the largest among the three lipids.

2.3. Lateral interaction between the lipid molecules

A theory for equilibrium between the monolayer and the solution has been reported using chemical potential values (Handa et al. 1992). The adsorption isotherms of the lipids were evaluated by eq. (4):

$$\ln\{(1/c)\cdot[\theta/(1-\theta)]\} = 2\omega\theta + \ln K \qquad (4)$$

The fraction of the interface, θ, is equal to Γ/Γ_s. K and ω are parameters that correspond to the magnitude of adsorption and lateral interactions in the monolayer, respectively. The attractive and repulsive interactions are represented by negative and positive values of the ω parameter. When the ω values exceed 2, phase separation occurs in the monolayer.
Fig. 3 illustrates the relationship between θ and $\ln\{(1/c)\cdot[\theta/(1-\theta)]\}$ for evaluating the lateral interaction parameters of the lipids. The K and ω values are summarized in Table 1. The magnitude of adsorption of E5880 was the smallest among the lipids tested. These results indicate the existence of attractive interactions between the molecules.

Table 1: Lateral interaction parameter K and ω values of E5880, STAC and CTAC

	K (L/mol)	ω (RT)
E5880	15.6	1.33
STAC	1.19	2.70
CTAC	0.33	2.49

2.4. Critical packing parameters for the micelles

The critical packing parameters (Israelachvili et al. 1977, 1980) for E5880, STAC and CTAC were calculated based on the area per molecule results (Fig. 2), the volume of the hydrophobic position and the length of the alkyl chain. For the formation of closed lamellar bilayer structures, the effective cross-section of the hydrocarbon moiety must be lower than that of the hydrophobic headgroup region. This assumption can be confirmed by estimating the critical packing parameter according to eq. (5);

$$x = v/a \cdot l \quad (5)$$

where v is the volume of the hydrophobic part, a the area of the hydrophobic head group and l the length of the alkyl chain. When $x < 1/3$ spherical micelles are formed, while $1/3 < x < 1/2$ gives tubular micelles form, $1/2 < x <,1$ vesicles and $1 < x$, hexagonal H_{II} structures. The volume of the hydrocarbon domain (v) and the length of hydrocarbon chain (l) were calculated using the following equations;

$$v = (27.4 + 26.9n) \times 10^{-3}\ (nm^{-3}) \quad (6)$$

$$l = 0.15 + 0.1265n\ (nm) \quad (7)$$

where n represents the number of hydrocarbon chains. For E5880, $v = 0.5116\ nm^3$, $l = 2.427$ nm, $a = 0.843\ nm^2$. For STAC, $v = 0.5116\ nm^3$, $l = 2.427$ nm, $a = 0.633\ nm^2$. For CTAC, $v = 0.4578\ nm^3$, $l = 2.174$ nm, $a = 0.627\ nm^2$. The x values for E5880, STAC and CTAC were found to be 0.25, 0.33 and 0.33, respectively. The above values indicate that these three lipids exist as spherical inverted micelles. The radius (R) and the number of molecules in the micelles (N) were calculated using the following equations:

$$R = 3v/a \quad (8)$$

and

$$N = 4\pi R^2/a \quad (9)$$

For E5880, $R = 1.82$ nm and $N = 49$. For STAC, $R = 2.42$ nm and $N = 116$. For CTAC, $R = 2.19$ nm and $N = 96$. The values of N for CTAC was reported to be 102 (Milliaris et al. 1986), consistent with our results reported here.

2.5. Determination of the size of the micelles

Using the DLS techniques, the weight-averaged diameters for E5880, STAC and CTAC were determined to be 8.1, 6.3 and 5.1 nm, respectively. These values are in agreement with values calculated using critical packing parameters.

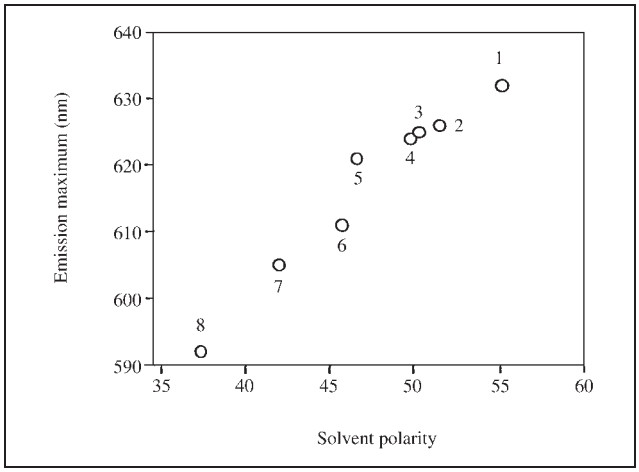


Fig. 4: Relationship between solvent polarity and emission maximum of NR (excitation: 549 nm) at 25 °C. 1, methanol; 2, ethanol; 3, propanol, 4, butanol; 5, isobutanol; 6, acetone; 7, tetrahydrofuran; 8, acetonitrile

2.6. Micropolarity around NR in the micelles

The micropolarity around NR (Nile Red) in the micelles was determined, as were the emission maxima of NR embedded in the micelles. It has been reported that the fluorescence characteristics of NR are dependent on the micropolarity around the probe, which is located in a hydrophobic region in the micelles (Greenspan and Flower 1985; Sackett and Wolff 1987). Therefore, the emission maxima of NR in the micelles are generally thought to provide information on the micropolarity around the hydrocarbon chains. Fig. 5 shows the relationship between solvent polarity (Dimth and Reichardt 1963) and the emission maximun of NR at 25 °C. The emission maxima of E5880, STAC, CTAC micelles were 621, 636, 636 nm, respectively, indicating that the micropolarity around the probe in the micelles is similar to that of isobutanol, methanol, methanol, respectively. This indicates that the hydrocarbon regions around NR in STAC and CTAC micelles are more hydrated than that of E5880 micelles.

2.7. Conclusions

The physicochemical properties of E5880, STAC and CTAC micelles are shown in Table 2. E5880 readily forms micelles in water. The cmc of E5880 at 25 °C was determined to be 0.12 mM. Using area/molecule results, the micelles were determined to have a spherical structure and to contain 49 molecules per micelle. Attractive interactions occurred between E5880 molecules in the micelle. The hydrocarbon region in the micelle was more rigid and less hydrated than that of either STAC, or CTAC.

Table 2: Physicochemical characteristics of E58880, STAC and CTAC

	E5880	STAC	CTAC
Cmc (mM)	0.12	0.33	1.6
Saturated surface density: Γ_s (mol/m^2)	2.0	2.6	2.7
Area/molecule (nm^2)	0.840	0.633	0.627
Critical packing parameter	0.25	0.33	0.33
Shape of micelle	spherical	spherical	spherical
The Number of molecules per micelle	49	116	96
Size of micelles (nm)	8.1	6.3	5.1
Emission maximum (nm) at 25 °C	621	636	636
Micropolarity (comparable to organic solvent)	isobutanol	methanol	methanol

3. Experimental

E5880 was obtained from Eisai Chemical Co., Ltd. (Ibaraki, Japan). Stearyltrimethylammonium chloride (STAC) was purchased from Tokyo-kasei Co., Ltd (Tokyo, Japan). Cetyltrimethylammonium chloride (CTAC) was purchased from Wako Pure Chemical Industrial Ltd. (Osaka, Japan). Nile Red (NR) was purchased from Lambda Co., Ltd (Graz, Austria).

3.1. Surface tension measurement

To determine the critical micelle concentration and nature of the interaction between molecules, the surface tension of aqueous solutions of E5880, STAC and CTAC was measured as a function of the concentration of these lipids. The measurements were performed by Whilhelmy's plate method using a surface tensiometer (model CBVP-A3, Kyowa Kaimenkagaku Co., Ltd., Tokyo) at 25 °C.

3.2. Determination of size of the micelles

The size of E5880, STAC and CTAC micelles (concentration: 10 mM: more than critical micelle concentration) was determined by a dynamic light scattering (DLS) technique using a particle analyzer equipper with an Ar laser (model DLS-7000DL, Ohtsuka Electronics Co., Ltd., Osaka) at 25 °C. The data was analyzed by the histogram method (Gulari et al. 1979) and the weight-averaged size of the micelles was evaluated.

3.3. Determination of the micropolarity around NR in the micelles

The micropolarity of the hydrocarbon regions in E5880, STAC and CTAC micelles was determined using a fluorescence technique (probe: NR). NR exhibits a strong environment-dependent blue shift, a high quantum yield and low fluorescence in water (Greenspan and Fowler 1985; Sackett and Wolff 1987). Fluorescence spectra were measured using a fluorescence spectrophotometer (model F-4500, Hitach Cp., Ltd., Tokyo) excited at 549 nm at 25 °C. The micropolarity of NR incorporated into micelles was evaluated using the wavelength corresponding to the maximum intensity of emission. Twenty-eight micrograms of NR were dissolved in 8.8 ml of acetone (0.1 mM). Five microliters of each solution was then diluted with 5 ml of 10 mM aqueous solutions of E5880, STAC and CTAC or methanol, ethanol, propanol, butanol isobutanol, acetone, tetrahydrofuran, acetonitrile, respectively (each concentration: 10 mM, more than critical micelle concentration). The wavelengths corresponding to the maximum fluorescence intensity of each solution were plotted against the polarity of each solvent (Dimorth and Reichardt 1963). The micropolarity around the probe was determined using this standard curve.

References

Benveniste J, Henso PM, Cochrance CG (1972) Leukicyte-dependent histamine release from rabbit platelets. The role of IgE, basophilis, and a platelet-activating factor. J Exp Med 136: 1356–1377.

Bessin P, Bonnet J, Apffel D, Soulard C, Desgroux L, Pelas I, Benveniste J (1983) Acute circulatory collapse caused by platelet-activating factor (PAF-acether). Eur J Pharmacol 86: 403–413.

Blank ML, Snyder F, Byers LW, Brooks B, Muirhead EE (1979) Antihypertensive activity of an alkyl ether analog of phosphatidylcholine. Biochem Biophys Res Commum 90: 1194–1200.

Dimroth K, Reichardt C et. al. (1963) Über Pyridinumphenol-betaine und ihre Verwendung zur Charakteriserung der Polarität von Lösungsmitteln. Liebigs Ann 661: 1–37.

Greenspan P, Fowler SD (1985) Spectrofluorometric studies of the lipid probe, nile red. J Lipid Res 26: 780–789.

Gulari E, Gulari E, Tsunashima Y, Chu E (1979) Photon correlation spectroscopy of particle distribution. J Chem Phys 70: 3965–3972.

Hanahan DJ (1986) Platelet activating factor: a biological active phosphoglycerid. Amnu Rev Biochem 55: 483–489.

Handa T, Saito H, Miyajima K (1990) Phospholipid monolayers at the triolein-saline interface: production of microemulsion particles and conversion of monolayers to bilayers. Biochemistry 29: 2884–2890.

Handa T, Saito H, Kakee A, Tanaka I, Miyajima K (1992) Lateral interactions of pig apolipoprotein A-1 with egg yolk phosphatidylcholine and with cholesterol in mixed monolayers at the triolein-saline interface. Biochemistry 31: 1415–1420.

Handley DA, Van Valen RG, Melden MK, Flury S, Lee ML (1986) Inhibition and reversal of endotoxin-, aggregated IgG- and PAF-induced hypotension in the rat by SRI 63-072, a PAF receptor antagonist. Immunopharmacology 12: 11–16.

Humphrey DM, McManus LM, Satouchi K, Hanahan DJ, Pinckard RN (1982) Vasoactive properties of acetyl glyceryl phosphorylcholine and analogues. Lab Invest 46: 422–427.

Imura Y, Terashita Z, Nishikawa K (1986) Possible role platelet activating factor (PAF) in disseminated intravascular coagularion (DIC), evidenced by use of a PAF antagonist. Life Sci 39: 111–117.

Israelachvili JN, Mitchell DJ, Ninham BW (1977) Theory of selfassembly of lipid bilayers and vesicle. J Chem Soc Faraday II 470: 185–201.

Israelachvili JN, Marceija S, Horn RG (1980) Physical principles of membrane organization. Q Rev Biophys 13: 121–200.

Milliaris A, Binana-Limbelew W, Zana R (1986) Fluorescence probing studies of surfactant aggregation in aqueous solutions of mixed ionic micelles. J Colloid Interface Sci 110: 114–120.

Nagaoka J, Harada K, Kimura A, Kobayashi S, Murakami M, Yoshimura T, Yamada K, Asano O, Katayama K, Yamatsu I (1991) Inhibitory effects of the novel platelet factor receptor antagonist, 1-ethyl-2-[*N*-(2-methyl)benzoyl-*N*-[(2*R*)-2-methyl-3-(4-octadecylcarbamoyloxy)piperidinocarbonyloxypropyloxy]carbonyl]aminomethyl-pyridinium chloride, in several experimentally induced shock models. Arzneim-Forsch Drug Res 41: 719–724.

Oh-ishi S, Tamaki K, Hayashi M, Tsushima S, Nomura H (1986) Suppression of phorbol myristate acetate-induced pleurisy by CV-3988. Chem Pharm Bull 34: 4896–4898.

Rosen MJ (1978) Surfactants and Interfacial Phenomena, Wiley-Interscience.

Sackett DL, Wolff J (1987) Nile red, as a polarity sensitive fluorescent probe of hydrophobic protein surfaces. Anal Biochem 167: 228–234.

Saeki S, Masugi F, Ogihara T, Otsuka A, Kumahara Y, Watanabe K, Tamura K, Akashi A, Kumagai A (1985) Effect of a-*O*-alkyl-2-acetyl-sn-glycero-3-phosphochoiline (platelet activating factor) on cardiac function in perfused guinea-pig heart. Life Sci 37: 325–329.

Shaw JO, Pinckard RN, Ferrigmi KS, McManus LM, Hanahan DJ (1981) Activation of human neutrophilis with 1-*O*-hexadecyl/octadecyl/-2-acetyl-sn-glycerol-3-phosphorylcholine (platelet activating factor). J Immunol 127: 1259–1255.

Takizawa H, Ishii A, Suzuki S, Shiga J, Miyamoto T (1988) Bronchoconstriction induced by platelet-activating factor in the guinea pig and its inhibition by CV-3988, a PAF antagonist: serial changes in findings of lung histology and bronchalveolar lavage cell population. Int Arch Allergy Appl Immunol 86: 375–382.

Terashita Z, Tsushima S, Yoshida Y, Nomura H, Inada Y, Nishikawa K (1983) CV-3988 – a specific antagonist of platelet activating factor (PAF). Life Sci 32: 1975–1982.

Terashita Z, Imura Y, Takatani M, Tsushima S, Nishikawa N (1987) CV-6209, a highly potent antagonist of platelet activating factor *in vitro* and *in vivo*. J Pharmacol Exp Ther 242: 263–268.

Tokumura A, Homma H, Hanahan DJ (1985) Structural analogs of alkylacetylglycerophosphocholine inhibitory behavior on platelet activation. J Biol Chem 260: 12710–12714.

Vargaftig BB, Lefort J, Chigrard M, Benveniste J (1980) Platelet-activating factor induces a platelet-dependent bronchoconstriction unrelated to the formation of prostagrandin derivatives. Eur J Pharmacol 65: 185–192.