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Concentric Nano-sized Vesicle Formation by Immunosuppressant FTY720 Revealed with Small-angle X-ray Scattering using Synchrotron Radiation Source

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Tertiary structure of nano-sized vesicle in aqueous solution of immunosuppressant FTY720 was studied. The molecular weight of FTY720 self-assembling in solution was determined by a laser light scattering method and was 20,800. Angular dependency of the intensities of the scattered light in small-angle X-ray scattering showed a typical pattern of the concentric spherical vesicle. The determined values through the theoretical curve fitting were 0.8 nm and 3.6 nm for the inner and our radius of the concentric vesicle, respectively. This means that the hydrophilic site of FTY720 situates at inner core and the hydrophobic group of FTY720 forms the dimer by end-to-end aggregation at the outer core.

Keywords: Tertiary structure in solution; immunosuppressant; FTY720; Small-angle X-ray scattering; synchrotron radiation source; concentric spherical vesicle shell; Kratky plot

Introduction

Immunosuppressants are clinically important for organ transplantations and the treatment of autoimmune diseases. Since cyclosporin A (CsA) [1] was introduced, the success rate in organ transplantations has increased remarkably. Immunosuppressant isolated from *Streptomyces* FK-506 [2] was found to be 10-100-fold more potent than CsA as an immunosuppressant. These compounds have very similar mechanisms of action, inhibiting the production of interleukin-2, a signal molecule that induces cytotoxic T cell, but higher doses of both compounds induce renal dysfunction and other side effects [3]. Therefore, less toxic drugs for the prevention of graft rejection are needed.

Several immunosuppressants, ISP-I, were isolated from culture broths of *Isaria sinclairii*, which is an imperfect stage of *Cordyceps sinclairii* and *Mycelia sterilia*, respectively [4, 5]. The immunosuppressive activity of ISP-I was 10-100 times more

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potent than that of CsA in terms of suppressing both lymphocyte proliferation in mouse allogeneic mixed lymphocyte reaction *in vitro* and generation of allo-reactive cytotoxic T lymphocytes in mice *in vivo* [4, 5].

FTY720, 2-amino-2-[2-(4-octylphenyl) ethyl]propane-1,3-diol hydrochloride, was synthesized with chemical modification technique as one of ISP-I derivatives. FTY720 prolonged rat skin allograft survival more effectively than CsA and showed activity in other administration routes [6]. FTY720 sequesters circulating mature lymphocytes into peripheral patches by acceleration of lymphocyte homing and thereby decreases the number of lymphocytes in peripheral blood [7]. FTY720 also had no inhibitory effect on serine palmitoyltransferase at a concentration of 1000 nmol/L or less, suggesting that FTY720 possess considerable activity and is expected to be useful as an immunosuppressive drug for organ transplantation [6].

FTY720 consists of a hydrophilic group (2-amino-2-ethylpropane-1,3-diol) and a hydrophobic group (hydrocarbon chain and phenyl ring) in a molecule as is shown in Fig. 1.

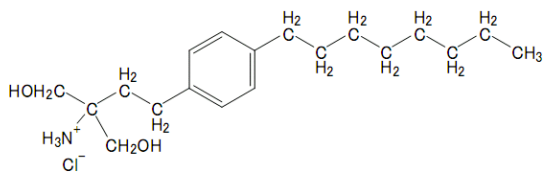

Fig. 1 Monomeric structure of FTY720

The characteristics of an amphiphile of FTY720 may induce nano-sized micelle-like vesicle formation in aqueous solutions. In fact FTY720 in aqueous solution showed higher molecular weight by gel filtration method than monomer whose molecular weight was 344 according to mass analysis of this sample. However, the detailed structure of FTY720 in aqueous solution remains exclusive.

In the present paper, we investigated the detailed molecular structure of vesicle of FTY720 in aqueous solution with Small-angle X-ray Scattering (SAXS) using Synchrotron Radiation Source.

2. Materials and Methods

2. 1. Materials

FTY720 was prepared according to the method by Fujita *et al.* [6]. The sample was converted to hydrochloride salt by treatment with ethanol and 1N HCl solution in diethyl ether and was recrystallized as white solid from ethanol and ethyl acetate mixture. The melting point of purified sample was 107-108℃ (decompose). The mass analysis (EI: Electron Ionization method) gave also as *m/z* 344 (M^+) and element analysis showed $C_{19}H_{33}NO_2 \cdot HCl$.

2. 2. Determination of Molecular Weight

The apparent weight-average molecular weight of FTY720 in aqueous solution was measured by the intensity of light scattered at 90° angle and at a wavelength of 436 nm with the instrument of a modified ellipsometer, an automatic light scattering analyzer AEP-100 (Shimadzu Co. Ltd.) [8]. The apparatus constant was obtained by using a solution of bovine serum albumin whose molecular weight was taken as 65,800. The sample FTY720 solution and the solvent of distilled water for light

scattering measurements were optically clarified through a Gelman (Germany) filter (pore size 0.45 micro meter). The refractive index increment of FTY720 was measured as 0.140 ml/g.

2. 3. Small-angle X-ray Scattering (SAXS)

SAXS experiments were performed with the optics and detector system of SAXES (Small-angle X-ray Scattering Equipment for Solutions) installed at the 2.5 GeV storage ring in the Photon Factory of the National Laboratory for High Energy Physics, Tsukuba [9-12]. The X-ray beam is monochromatized by using a double-crystal monochromator and focused with a toroidal-focusing mirror. A wavelength, λ, of 0.149 nm was used. The Small-angle scattering from Q ($Q = 4\pi \sin\theta / \lambda$, where 2θ is the scattering angle) $=0.11$ nm^{-1} to 3.8 nm^{-1} was registered at 512 different angles by using the one-dimensional position-sensitive proportional counter with an effective length of 200 mm (Rigaku Denshi Co. Ltd). The scattered intensities were corrected with regard to the variation in the incident X-ray flux by monitoring the beam with an ionization chamber placed in front of the temperature controlled specimen chamber. The counting time was 1800 sec for each measurement. The net scattering intensities were calculated by subtracting the scattering intensities of a blank buffer solution from those of the sample after the correction of the effect of the X-ray beam transmissions.

3. Results

3. 1. Molecular weight of FTY720 aggregate in solution

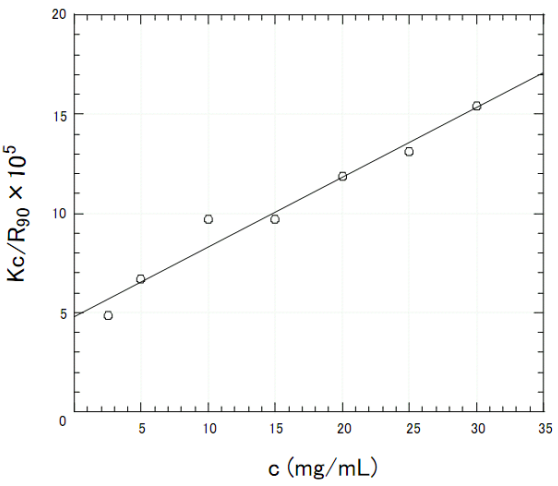

Fig. 2 Concentration dependence of inverse scattering intensity for FTY720 in aqueous solution.

Figure 2 showed the light scattering pattern of FTY720 in aqueous solution, where Kc/R_{90} was plotted against FTY720 concentration c (mg/ml). Here K is the optical constant and R_{90} is the reduced scattering intensity observed at the scattering angle 90°. Considering no effect of the internal interference factor because of small molecular size of FTY720 the following equation is derived as [13],

$$Kc/R_{90} = 1/M + 2A_2c \qquad (1)$$

Where M is the molecular weight and A_2 the second virial coefficient.

As was shown in Fig. 2, the inverse scattering intensity plot was almost linear. Therefore, the vesicle size is almost the same between 3 to 30 mg/mL concentration. The molecular weight of FTY720 in solution was obtained from the intercept of this straight line and was 20,800 which suggested that FTY720 in aqueous solution was situated in an aggregate state and formed a micelle-like vesicle structure, because monomeric molecular weight of FTY720 was 344.

3. 2. Size and shape of FTY720 aggregates

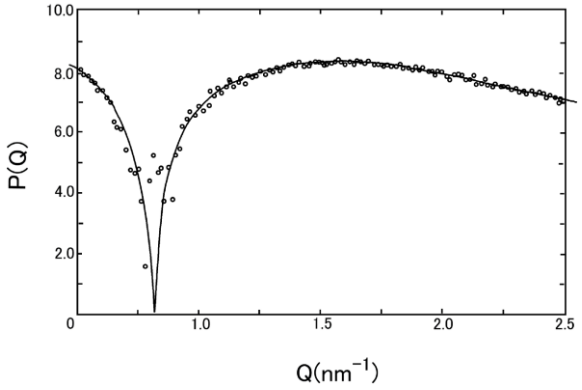

Fig. 3 Angular dependency of the scattering intensity profile of SAXS in 20 mg/mL FTY720 solution in distilled water. Open circles indicate the experimental values. The theoretical curve as the two concentric spherical vesicle shell model is shown with the solid line taking account of the spherical factor, P(Q).

SAXS experiments were performed with the solution of FTY720 from 15 to 20 mg/ml in distilled water. The scattering curve showed the well-defined maximum at about Q=l.5 nm^{-1}, as was shown in Fig. 3. The shape and size of the aggregates were determined by comparing the experimental angular scattering intensity profiles with the calculated theoretical values for suitable models. The theoretical equations of the shape factor, P(Q), are given as a function of scattering angle Q for various models [13, 14]. As was shown in Fig. 3, the scattering pattern indicated typical two concentric vesicle shell molecules. Therefore, FTY720 aggregates in solution could be approximated as two concentric vesicle shells model with different scattering length as in the case of dodecyl sulfate micelles (SDS) or glucose lipid micelles [15]. In the larger Q region the scattering curve mostly reflects the particle size of FTY720 aggregates.

3. 3. Kratky plot

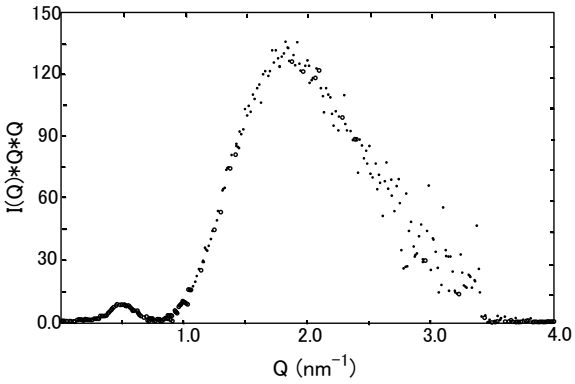

Fig. 4 Kratky plot of FTY720 solution of SAXS pattern shown in Fig. 1.

In synthetic polymer chemistry the Kratky plot is usually employed to evaluate the shape and the order of extension of a linear or branched chain polymer. Even if polymers have the same radius of gyration value in solution, Kratky plot reveals the difference according to their shape and chain extension, e.g., a spherical shape yields a sharp peak, a Gaussian chain a horizontal line and a rigid rod a straight line with a slope one [16]. As was shown in Fig. 4, the Kratky plot showed one sharp peak of scattering curve, indicating that FTY720 aggregate is approximated as vesicle shape. The peak in Kratky plot also indicated symmetrically and therefore the size distribution of the vesicle formed may be mono distribution.

4. Discussion

Total scattering intensity I(Q) is generally proportional to P(Q). For the two concentric vesicle shell model, P(Q) is given

$$P(Q) = \left\{\frac{1}{(R_2^3 - R_1^3)^2}\right\} \times \left\{R_2^3 F(QR_2) - R_1^3 F(QR_1)\right\}^2 \quad (2)$$

$$F(QR_i) = \frac{3 \times (\sin QR_i - QR_i \cos QR_i)}{(QRi)^3} \quad (3)$$

as [14] and R_i is the inner (R_1) or outer (R_2) radius, respectively. Each spherical portion of the two concentric vesicle shell model is expressed with the normal spherical particle factor.

The best fitted values of the theoretical curve shown with solid line in Fig. 3 as the two concentric spherical vesicle shell model were 0.8 nm for the inner radius R_1 and 3.6 nm for outer R_2. As is shown in Fig. 3, the theoretical curve is controlled with a position of the sharp valley and the moderate peak. Therefore, the radii values are determined uniquely and the errors of the determined values may be appreciably zero.

According to the atomic molecular model calculated with the computer using the energy minimum method, FTY720 monomer is approximated as two short rods bent at a phenyl ring as is shown in Fig. 1. The one part consists of a hydrophilic group (2-amino-propane-1,3-diol and phenyl ring) whose length is 0.89 nm. The other part consists of a hydrophobic group of the hydrocarbon chain whose length is 1.0 nm. The stretched end-to-end distance of FTY720 monomer is 1.8 nm. The region between the inner and the outer shell has the radius of 2.4 nm as is shown with the schematic model in Fig. 5 whose length agrees almost with the twice value of the stretched end-to-end distance of FTY720 monomer. This means that the hydrophilic parts of FTY720 situates at inner core and the hydrophobic group of FTY720 molecules forms dimer by end-to-end aggregation through hydrophobic interaction and situates at the outer shell core.

On the other hand, the volume of hydrophobic region is calculated as 193 nm^3 from the values of the two concentric vesicle shell model. If FTY720 molecule is assumed as a conic cylinder whose height is 1.7 nm and the diameters are 0.38 nm as the upper surface and 7.0 nm as the bottom surface according to the atomic molecular model because the bond angle between the hydrophilic and the hydrophobic parts is about 140 degree, the volume of one molecule of FTY720 is calculated as 2.9 nm^3. Therefore, about 67 molecules of FTY720 are included within the hydrophobic region whose value is consistent well with the result of molecular weight shown in Fig. 2.

The tip length of the hydrophilic region of FTY720 shown in Fig. 1 is calculated as 0.24 nm according to the atomic molecular model and the radius of chloride ion is assumed as 0.17 nm. The inner core of the hydrophilic region of the two concentric vesicle shell models is compatible well with the experimental value. This indicated that the tip hydrophilic region of FTY720 molecule in the core regions situates as the mixtures of dissociative and undissociative states.

In physiologically, it is recognized that the effect of FTY720 was continuous [17,18] as was already shown in Introduction. Clinically, results of a short-term trial indicated that FTY720 in combination with steroids and CsA was efficient in preventing acute rejection [17].

As is shown in Fig. 5, FTY720 has a self-assembling property in physiological solution and can form the two concentric vesicle shell molecule which consists of the large external hydrophobic core and the

small internal hydrophilic core.

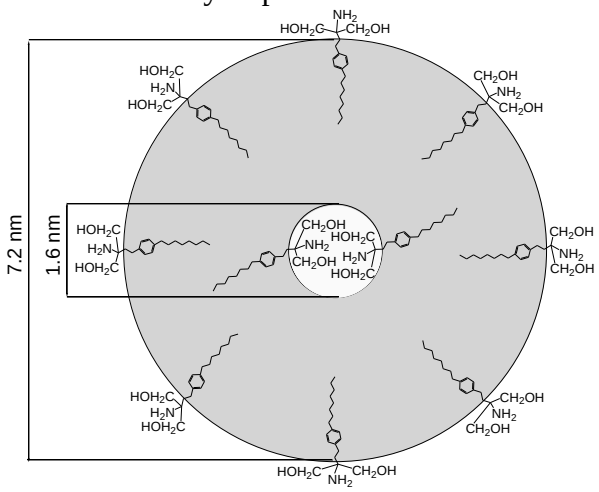

Fig. 5 Schematic two concentric vesicle shell model of FTY720 aggregates in aqueous solution. Closed circles indicate hydrophilic parts (blank part at center) and zigzag line and benzene signals show hydrocarbon chains and phenyl rings of hydrophobic parts (gray zone) of FTY720 molecule shown in Fig. 1.

Under the physiological condition at neutral pH, the monomeric FTY720 is fairly in soluble in water and forms the water soluble aggregate molecule which may act as a reservoir. The anti-inflammatory drug such as steroids or the other immunosuppressant drug such as CsA is simultaneously prescribed with FTY720. These compounds are also fairly in soluble water and therefore may be solubilized into the hydrophobic part of the FTY720 aggregate, and then may be carried to an active site of membranes.

5. Summary

FTY720 molecule has an amphiphile structure and therefore formed micelle-like aggregates in aqueous solution. Angular dependency of scattered light intensity in SAXS showed a typical pattern of the two concentric vesicle shell. The best fitted values of the theoretical curves as the two concentric spherical vesicle shell model was 0.8 nm for the inner radius and 3.6 nm for outer radius, indicating that the hydrophilic sites of FTY720 situated at inner core and the hydrophobic group of FTY720 molecule forms dimer by end-to-end aggregation through the hydrophobic interaction and situated at the outer shell core. These dimensions were consistent with the values calculated from the molecular structure of FTY720. The two concentric vesicle shell structure of FTY720 aggregates may relate with the continuous physiological effect of FTY720.

Reference

[1] Borel, J. F. (1982) History of cyclosporin A and its significance in immunology. In cyclosporin A, Borel, J. F., Ed., Elsevier Biochemical Press: Amsterdam, 5-17.

[2] Tanaka, H., Kuroda, A., Marusawa, H., Hatanaka, H., Kino, T., Goto, T., Hashimoto, M., and Taga, T. (1987) Structure of FK506: a novel immunosuppressant (Ed- confirm the singular) isolated from Streptomyces. *J. Am. Chem. Soc.*, **109**, 5031-5033

[3] Kelly, P. A., Burckart, G. J., and Venkataramanan, R. (1995) Tacrolimus: a new immunosuppressive agent. *Am. J. Health- Syst. Pharm.*, **52,** 1521-1535.

[4] Fujita, T., Inoue, K., Yamamoto, S., Ikumoto, T., Sasaki, S., Toyama, R., Chiba, K., Hoshino, Y., and Okumoto, T. (1994) Fungal metabolites. Part 11. A potent immunosuppressive activity found in Isaria sinclairii metabolite. *J. Antibiot.* (Tokyo) **47**, 208-215.

[5] Fujita, T., Hirose, R., Yoneta, M., Sasaki, S., Inoue, K., Kiuchi, M., Hirase, S., Chiba, K., Sakamoto, H., and Arita, M. (1996) Potent immunosuppressants, 2-alkyl-2-aminopropane-1,3-diols. *J. Med. Chem*., **39** (22), 4451-4459.

[6] Kiuchi, M., Adachi, K., Kohara, T., Minoguchi, M., Hanano, T., Aoki, Y., Nishina, T., Arita, M., Nakao, N., Ohtsuki, M., Hoshino, Y., Teshina, K., Chiba, K., Sasaki, S., and Fujita, T. (2000) Synthesis and Immunosuppressive Activity of 2-Substituted 2-Aminopropane-1,3-diols and 2-Aminoethanols. *J. Med. Chem.*, **43,** 2946-2961 (2000).

[7] Chiba, K., Yanagawa, Y., Masubuchi, Y., Kataoka, H., Kawaguchi, T., Ohtsuki, M., and Hoshino, Y. (1998) FTY720, a novel immunosuppressant, induces sequest-ration of circulating mature-lymphocytes by acceleration of lymphocyte homing in rats. I. FTY720 selectively decreases the number of circulating mature lympho-cytes by acceleration of lymphocyte homing. *J. Immunol.*, **160**, 5037-5044.

[8] Sugiura, S., Ichikawa, S., Sano, Y., Nakajima, M., Liu, X. Q., Seki, M., and Furusaki, S. (2001) Formation and characterization of reversed micelles composed of phospholipids and fatty acids. *J. Colloid and Interface Sci.,* **240**, 566-572.

[9] Hirata, Y., Sano, Y., Aoki, M., Shoji, H., Kato, S., and Yamamoto, H. (2000) Small-angle X-ray scattering studies on nucleation formation of dextran precipitation in the presence of boron. *J. Colloid and Interface Sci.,* **223**, 139-141.

[10] Muroga, Y., Sano, Y., Inoue, H., Suzuki, K., Miyata, T., Hiyoshi, T., Yokota, K., Watanabe, Y., Liu, X., Ichikawa, S., Tagawa, H., and Hiragi, Y. (2000) Small angle X-ray scattering studies on local structure of tobacco mosaic virus RNA in solution. *Biophys. Chem.*, **83**(3):197-209.

[11] Sano, Y., Inoue, H., and Hiragi, Y. (1999) Differences of reconstitution process between tobacco mosaic virus and cucumber green mottle mosaic virus by synchrotron Small-angle X -ray scattering using low-temperature quenching. *J. Protein Chem.*, 18(7), 801-805.

[12] Hirata, Y., Sano, Y., Aoki, M., Kobatake, H., Kato, S., and Yamamoto, H. (1999) Structural change in dextran: mechanism of insolubilization by adsorption on the air-liquid interface. J. Colloid and Interface Sci., **212**(2), 530-534.

[13] Nakagawa, J., Kamogawa, K., Momozawa, N., Sakai, H., Kawase, T., Sawada, H., Sano, Y., and Abe, M. (1998) Molecular assemblies of fluorinated silicon oligomers with carboxylic acid groups: effects of chemical oligomer structure on assembly shape. *Langmuir*, **14**(8), 2061- 2067.

[14] Sun, C., Sano, Y., Kashiwagi, H., and Ueno, M. (2002) Characterization of aggregate structures of phospholipid in the process of vesicle solubilization with sodium cholate using laser light scattering method. *Colloid Polym. Sci.*, **280**(10), 900-907.

[15] Ishigami, Y., Gama, Y., Sano, Y., Lang, S., and Wagner, F. (1994) Interfacial and micellar behavior of glucose lipid. *Biotechnol. Lett.*, **16**(6), 593-598.

[16] Kratky, O. (1982) Small Angle X-ray Scattering (Glatter, O. and Kratky, O., Eds.), pp. 361-386, Academic Press, London.

[17] Koshiba, T., Van Damme, B., Rutgeerts, O., Waer, M., and Pirenne J. (2003) FTY720, an immunosuppressant that alters lymphocyte trafficking, abrogates chronic rejection in combination with cyclosporine A. *Transplantation*, **75**(7), 945-952.

[18] Kimura, T., Hasegawa, T., Nakai, H., Azuma, T., Usui, N., Sasaki, T., and Okada, A. (2003) FTY720 reduces T-cell recruitment into murine intestinal allograft and prevents activation of graft-infiltrating cells. *Transplantation*, **75**(9), 1469-1474.

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