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Micelles Consisting of Choline Phosphate-Bearing Calix[4]arene Lipid

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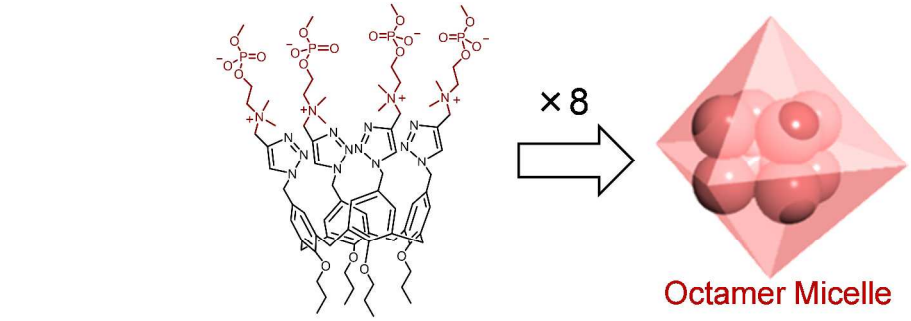

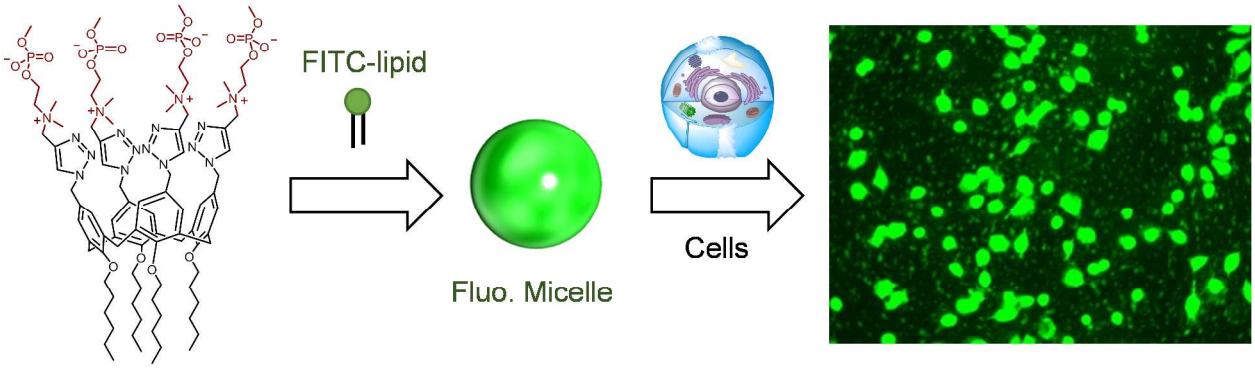

Choline phosphate bearing calix[4]arene based lipids form monodispersed micelles, and the micelles are taken up by cells through CP-PC interactions.

Abstract

We synthesized new calix[4]arene-based lipids, denoted by CPCaL*n*, bearing choline phosphate (CP) group which is inverse phosphoryl choline (PC) structure. Small-angle X-ray scattering and multi-angle light scattering coupled with field flow fractionation showed that these lipids form monodisperse micelles with a fixed aggregation number and diameters of 1.9 and 2.6 nm for lipids bearing C_3 and C_6 alkyl tails, respectively. Furthermore, when CPCaL*n* was mixed with the fluorescein isothiocyanate (FITC)-bearing lipids and added to cells, the strong fluorescence was observed at 37 °C, but not at 4 °C, indicating that the micelles were taken up by the cells through endocytosis. Recent studies have shown that replacement of polymer-attached PC groups with CP groups markedly promotes cellular uptake, even though the surface charge is neutral. On the basis of the idea, CPCaL*n* micelles interacted with cells in the same way, suggesting that the micelles bearing CP groups are expected to use as carriers in drug delivery system.

Introduction

Various amphiphilic compounds, such as surfactants, lipids, or diblock copolymers, form micelles in aqueous solutions and, consequently, have attracted great interest as drug carriers. [1] [2] Among these drug carriers, cationic micelles that interact with cellular surface through electrostatic interactions have been most frequently used *in vitro*. [3] [4] [5] However, when cationic micelles are used *in vivo*, they also interact with proteins and other negatively charged substances through cation–anion interactions, eventually leading to cytotoxicity and other unfavorable side effects. As a result, their practical applications are quite limited. [6] As a matter of fact, most micelle carriers that can be used *in vivo* contain neutral hydrophilic groups, for example poly(ethylene glycol) (PEG), or zwitterions, and it is well known that such neutral groups show little cytotoxicity. [2] [7] [8] [9] [10] In particular, phosphoryl choline (PC) is recognized as a highly biocompatible substance. This high biocompatibility of PC, even when attached to a synthetic substrate, is unsurprising, as phospholipids containing PC are major components of natural cell membranes. [9] [11] [12] [13] [14] Such neutral particles are expected to use in targeting for tumor tissues as drug carriers because particles of certain sizes do tend to accumulate to a greater extent in the tissues than in normal ones. This is called the enhanced permeability and retention (EPR) effect, and is the major strategy of delivering drugs to tumor tissues, especially for PEG-coated polymeric micelles. [15] However, the EPR effect is not enough to achieve the effective delivery since the neutral particles are not readily taken up by cells, and hence further improvement of the particles as drug carriers is required.

Choline phosphate (CP) contains amino and phosphate groups in the reverse order to that present in PC, and polymers containing CP have been found to be capable of interacting with cell

surfaces, presumably through binding between CP and PC groups. [16] [17] [18] [19] [20] The proposed mechanism for this interaction involves the formation of a quadrupole from two quaternary nitrogen–phosphorus pairs. This mechanism has been named the 'CP–PC interaction'. Yu and co-workers have demonstrated that polymers bearing CP groups are taken up to a marked extent, and we can presume that CP–PC interactions are less toxic than are cation–anion interactions. Although CP-bearing polymers have been studied by Yu and co-workers, there have been few reports on micelles formed from CP-bearing lipids. Perttu and co-workers synthesized a lipid with a single CP moiety and they found that it formed liposomes in aqueous solution, the surface potential of which remained negative across a broad pH range. [21] It is well known that the lipids with multiple head groups, such as a gemini-type lipids, [22] [23] show a synergistic function associated with the head groups. Furthermore, Yu and co-workers have shown that the number density of CP groups per macromolecule is important in increasing cellular uptake. [16] [17] On the basis of these ideas, we designed a calix[4]arene-based lipid with four CP groups attached on the upper rim and four alkoxy groups on the lower rim, as shown in Fig. 1. Furthermore, we have reported that similar calix[4]arene-based lipids with primary amine or cysteine attachments form shape-persistent micelles whose aggregation numbers are fixed. [24] [25] This research reported in this paper is a continuation of that work.

Experimental Section

Materials. All starting materials were purchased from Tokyo Chemical Industry Co., Sigma-Aldrich Co., or Wako Chemical Industries, and were used without further purification. 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 2-[(2,3-bis(oleoyloxy)propyl)dimethylammonio]ethyl ethyl phosphate (DOCPe), and (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(carboxyfluorescein) ammonium salt (FITC-LP) were purchased from Avanti Polar Lipids (Birmingham, AL, USA).

Synthesis of 5,11,17,23-Tetrakis{[1-(7,7-dimethyl-3,3-dioxido-2,4-dioxa-7-azonia-3λ^5-phosphaoctan-8-yl)-1*H*-1,2,3-triazol-4-yl]methyl}-25,26,27,28-tetrapropoxycalix[4]arene (IV; CPCaL3). Methyl prop-2-yn-1-ylcholine phosphate (p-CP, **I**) (0.304 g, 1.37 mmol), copper(II) sulfate pentahydrate (6.00 mg, 24.3 μmol), and sodium ascorbate (7.00 mg, 35.3 μmol) were dissolved in dry DMSO (2 mL), and then a solution of calixarene **II** (0.103 g, 0.127 mmol) in dry DMSO (2 mL) was added to the mixture. The mixture was stirred at 80 °C for 24 h, and then cooled to room temperature. A 50 mL of dichloromethane was added into the mixture to precipitate the product. The product was collected by filtration, washed with dichloromethane, and then collected with methanol. After removal of the solvent, the product was purified by dialysis with Spectra/Por Float-A-Lyzer (cellulose membrane; cut off 0.1–0.5 kDa) for 3 days. (yield: 0.163 g, 76%). ^{1}H NMR (400 MHz, methanol-d_4): δ (ppm) = 8.40 (s, 4H), 6.72 (s, 8H), 5.40 (s, 8H), 4.71 (s, 8H), 4.45 (d, J = 13.3 Hz, 4H), 3.90–3.86 (m, 16H), 3.90–3.86 (m, 8H), 3.84–3.82 (m, 8H), 3.56 (d, J = 10.8 Hz, 12H), 3.16 (s, 28H), 1.94–1.89 (m, 8H), 1.00 (t, J = 7.48 Hz, 12H). ^{13}C NMR (100 MHz, methanol-d_4): δ (ppm) = 156.8, 135.9, 135.5, 128.6, 128.2, 128.0, 76.8, 66.6, 61.6, 60.0, 53.6, 52.0, 51.6, 30.3, 23.0, 9.31.

Synthesis of 5,11,17,23-Tetrakis{[1-(7,7-dimethyl-3,3-dioxido-2,4-dioxa-7-azonia-3λ^5-phosphaoctan-8-yl)-1*H*-1,2,3-triazol-4-yl]methyl}-25,26,27,28-tetra(hexyloxy)calix[4]arene (V; CPCaL6). CPCaL6 was synthesized by a similar process to that used for CPCaL3; yield: 0.140 g (70%). ^{1}H NMR (400 MHz, methanol-d_4): δ (ppm) = 8.45 (s, 4H), 6.72 (s, 8H), 5.40 (s, 8H), 4.72(s, 8H), 4.44 (d, J = 13.2 Hz, 4H), 3.90–3.85 (m, 16H), 3.91–3.85 (m, 8H), 3.69–3.66 (m, 8H), 3.56 (d, J = 10.8 Hz, 12H), 3.17 (s, 24H), 1.92–1.87 (m, 8H), 1.43–1.36 (m, 24H), 0.927 (t, J = 6.96 Hz, 12H). ^{13}C NMR (100 MHz, methanol-d_4): δ (ppm) = 156.8, 135.9, 135.5, 128.6, 128.2, 128.0, 75.2, 66.6, 61.6, 59.8, 53.6, 52.0, 51.6, 31.9, 30.2, 25.9. 22.6. 13.1.

The intermediate compounds **I**, **II**, and **III** were synthesized as described elsewhere. [16 24]

Small angle X-ray scattering (SAXS) measurements. The concentration of the stock solutions of CPCaL*n* micelles was adjusted to 5.0 mM with ultrapure water, and then further diluted to 2.0 mM with 150 mM aqueous NaCl. The resulting samples were left for at least one day to equilibrate at room temperature. Small angle X-ray scattering (SAXS) measurements were carried out on the BL-40B2 beamline at the SPring-8 facility, Hyōgo Prefecture, Japan. We used a 30 × 30 cm imaging plate (Rigaku R-AXIS VII) detector placed 1.8 m from the sample. The wavelength of the incident beam (λ) was adjusted to 0.10 nm. This setup provided a q range of 0.1−4.0 nm^{-1}, where q is the magnitude of the scattering vector, defined as $q = 4\pi \sin\theta/\lambda$ with a scattering angle of 2θ. The X-ray transmittance of the samples was determined by using ion chambers located in front of and behind the sample. The detailed experimental procedures are reported elsewhere. [26]

Multi-angle light scattering coupled with field flow fractionation (FFF-MALS) measurements. We prepared a micellar solution of 10 mg/mL CPCaL*n* in 150 mM aqueous NaCl. The solution was optically purified by using an ultracentrifuge to remove large dust particles and other impurities whose signals often overlap those of the micelles. Aliquots (60 μL) of the sample solution were immediately injected into an Eclipse 3+ separation system (Wyatt Technology Europe GmbH, Dernbach, Germany) for field-flow fractionation (FFF) at 22–28 °C. The output from FFF was then passed sequentially through a Dawn Heleos II multiangle light-scattering (MALS) detector (Wyatt Technology) and an Optilab rEX DSP differential refractive index (RI) detector (Wyatt Technology), operating at a wavelength of 658 nm. We used a Wyatt channel (Eclipse 3 channel LC) attached to a membrane (polyether sulfone membrane; 1 kDa LC) at the bottom of the channel. The cross-flow and channel-flow rates were fixed at 4.0 and 1.0 mL/min, respectively. Detailed experimental procedures are reported elsewhere. [27] We determined the specific refractive index increments ($\partial n/\partial c$) and the extinction coefficients (ε at 270 nm) of the micelles in aqueous solution with a DRM-1021 differential refractometer (Otsuka Electronics, Osaka) and a Jasco V-630 spectrometer, respectively (see Fig. S1).

Atomic force microscopy (AFM) observations. AFM was carried out with an SPA 400 microscope [SII NanoTechnology Inc. (now Hitachi High-Tech Science Corp., Tokyo)] fitted with a silicon tip [SI-DF20(AL)] and operated in the tapping mode at room temperature. The frequency of the tapping mode was 124 kHz, and the radius of the tip apex was about 10 nm.

Critical micelle concentration (CMC) measurements. The concentration of the stock solutions of CPCaL*n* was adjusted to 10 mM in water, and then it was diluted in 150 mM aqueous NaCl. We used sodium 8-Anilino-1-naphthalenesulfonic acid (ANS) as fluorescence probe. The stock

solution of ANS was prepared to the concentration of 0.1 mM in water, and then it was diluted to 10 μM in each solution. Before fluorescence measurement, all samples were incubated for 30 min in the dark at the room temperature. The fluorescence measurements were carried out with fluorescence spectrometer (JASCO FP-6600). The ANS was excited at 350 nm, and then the emission spectra of ANS was recorded at 400 – 700 nm. The scan speed was 240 nm min^{-1}. The fluorescence intensity of ANS sensitively reflects the polarity of its environment, therefore, we can determined CMC from the plot of the fluorescence intensity vs. CPCaL*n* concentration. [28 29]

Dynamic light scattering (DLS) and zeta potential measurements. DLS and zeta potential measurements were carried out with Zetasizer Nano ZS (Malvern, U.K.) instrument at a wavelength of 633 nm from solid-state He-Ne laser at a scattering angle of 173°. The concentration of CPCaL*n* micelles and fluorescence micelles with were adjusted to 8.0 mM in 150 mM NaCl solution. The liposome solution of DOPC or DOCPe were prepared by thin film hydration method. [21]

Cellular Interaction Study. Powdered CPCaL*n* and FITC-LP were dissolved in methanol and chloroform, respectively, and then the solutions were mixed in a [CPCaL*n*]/[FITC] molar ratio of 5.0. The mixed solution was dried to produce a thin film in a glass vial. Fluorescent micellar solutions were prepared by adding 150 mM aqueous NaCl to the lipid films and sonicating for 5 min. DOPC and DOCPe liposomes containing FITC-LP were prepared by the same method. A549 cells were seeded in collagen-coated 96-well plates at the density of 1×10^4 cells/well, and incubated at 37 °C under 5% CO_2 for 24 h. The cells were cultured in DMEM containing 10% FBS and 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The wells were replaced with fresh serum free medium containing fluorescent micelles at 15 μM. After 6 hours, we washed the cells

with phosphate buffered saline twice and observed the fluorescence image using a BZ-9000 digital fluorescence microscope (Keyence, Osaka, Japan).

Results

Synthesis and Molecular Characterization of CPCaL*n*.

Fig. 1 shows the synthetic route to the CP-bearing calix[4]arene lipid (compounds **IV** and **V**). The chemical structures of the products from each step were confirmed by means of ^{1}H and ^{13}C NMR spectroscopy. Compounds **IV** and **V** are denoted as CPCaL*n*, where the prefix CP signifies that the choline phosphate groups are attached to the head group to form a hydrophilic region, and the suffix *n* denotes the number of carbon atoms in each alkyl chain. Calix[4]arene can exist in four possible conformations: cone, partial cone, and 1,2- and 1,3-alternates. [30] The cone conformation is retained when alkyl chain groups larger than propyl are present on the lower rim. The method that we used yields the cone conformation exclusively. [24] This form retains flexibility in the methylene bonds between aromatic rings, which permits the existence of equilibrium structures with C_{2v} and C_{4v} symmetries. [30] In the C_{2v} structure, one pair of opposite aromatic rings are parallel to one another, with the planes of the other opposite pair of rings almost at right angles. In contrast, in the C_{4v} structure the four rings are tilted equally along the methylene bonds. The C_{4v} form is less prevalent than the C_{2v} form in crystals, but the two forms rapidly interconvert in solution. However, the electrostatic repulsions between partially negatively charged oxygen atoms can be suppressed when an alkaline metal such as sodium or potassium is trapped between the four lower-rim oxygen atoms, which leads to the C_{4v} form being the more stable structure. Among the alkaline metals, sodium ions have the highest binding affinity for calix[4]arene derivatives. [24] We therefore carried out all our experiments in the presence of an excess of Na^{+} ions (150 mM). The critical micelle concentration (CMC) of CPCaL*n* micelles was determined with 8-(phenylamino)-1-naphthalenesulfonic acid as a

fluorescence probe (Fig. 2), and the results are listed in Table 1. Owing to its greater hydrophobicity, CPCaL6 showed a lower CMC than did CPCaL3. The CMC of CPCaL*n* was much lower than typical surfactants such as CTAB (hexadecyltrimethylammonium bromide, CMC = 0.92 mM [31]). We presume that neutralized charges of the phosphobetaine headgroup might provide the relaxation of the electrostatic repulsion among the lipids in the same manner as normal phospholipids whose CMC is in the micro molar range.[32] All subsequent experiments were carried out above the CMC.

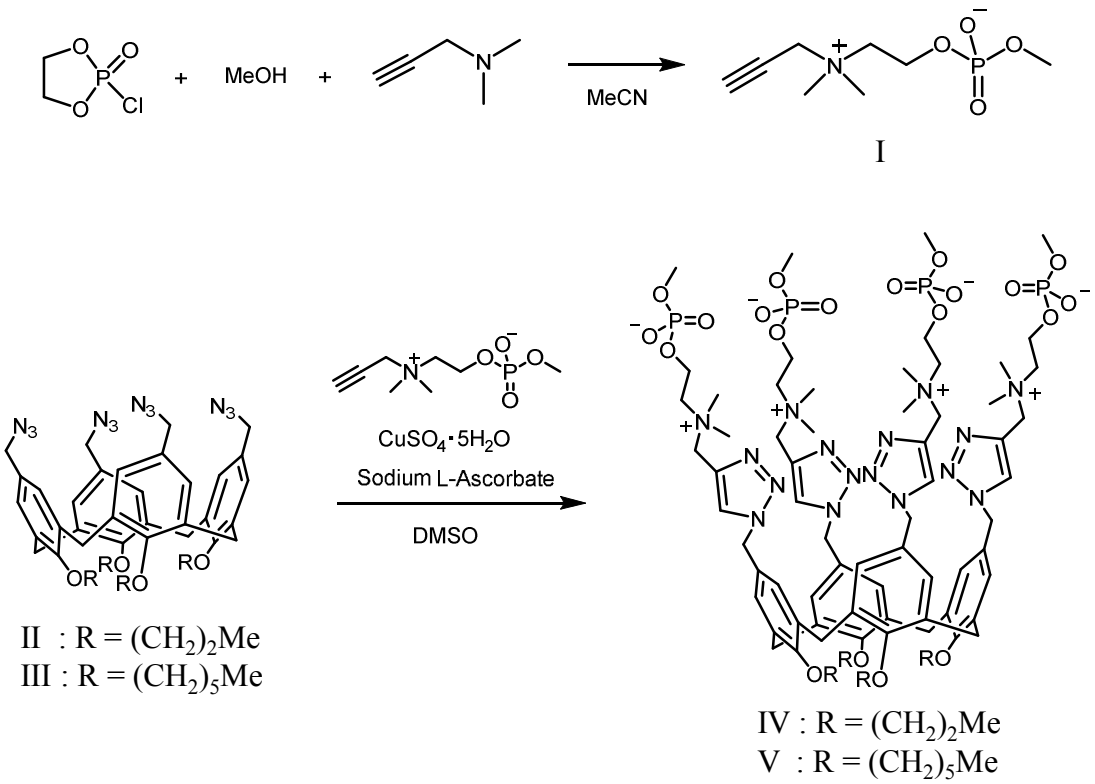

Fig. 1 Chemical structures of calix[4]arene-based lipids bearing choline phosphate (CP) groups, and the synthesis of CPCaL*n* (Compounds **IV** and **V**)

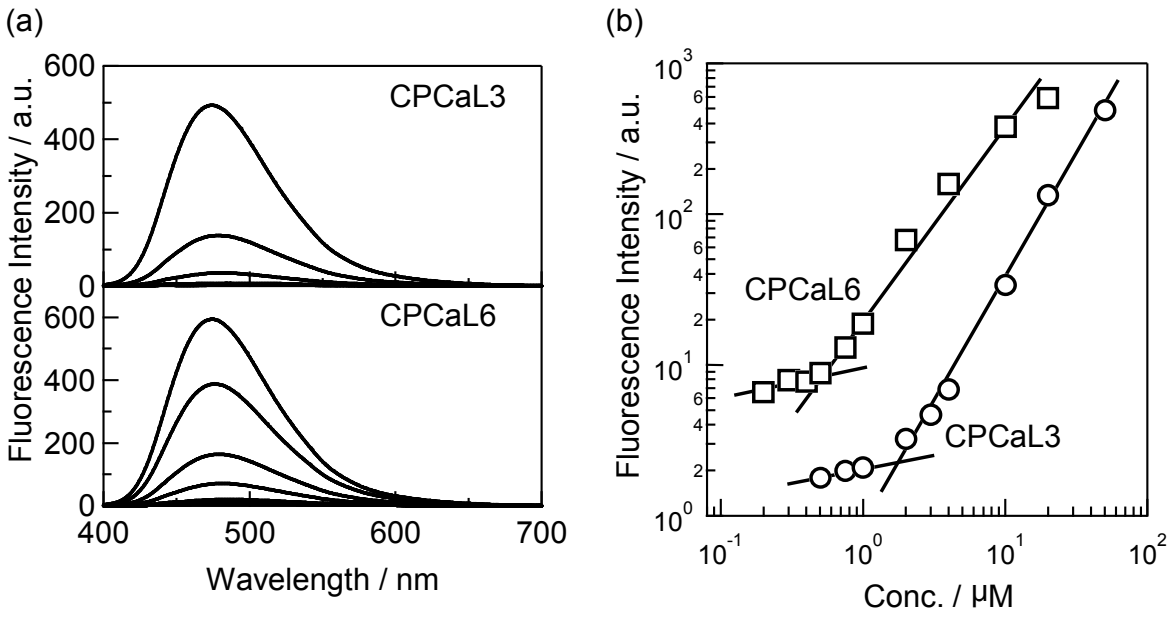

Fig. 2 (a) Fluorescence spectra of ANS in 150 mM aqueous NaCl at various concentration of CPCaL*n*. (b) The fluorescence intensity of the ANS spectra at 470 nm plotted against the concentration of CPCaL*n*.

Characterization of CPCaL*n* Micelles.

Fig. 3a shows SAXS profiles of the CPCaL*n* micelles. The asymptotic behavior of $q = 0$ in the low q region in both samples satisfies $I(q) \sim q^0$, indicating that CPCaL*n* micelles behave as isolated objects, and no appreciable secondary aggregates are formed. The radius of gyration (R_g) was determined from the Guinier plot (shown in Fig. S2) to be 1.76 for CPCaL3 and 2.46 nm for CPCaL6. Both of the SAXS profiles were fitted to a core–shell spherical model, as follows:

$$I(q) \sim \left(\frac{V_c(\rho_c - \rho_s)(\sin(qR_c) - qR_c cos(qR_c)}{(qR_c)^3} + \frac{V_s(\rho_s - \rho_{sol})(\sin(qR_s) - qR_s cos(qR_s)}{(qR_s)^3} \right)^2 \quad (1)$$

where R_c and R_s are the radii of the core and shell, respectively, V_c and V_s are the volume of the core and shell, respectively, and ρ_c, ρ_s, and ρ_{sol} are the electron densities of the core, shell, and solvent, respectively. The values of ρ_{sol} and ρ_c were chosen as 334 and 270 e nm^{-3}, respectively, in accordance with our previous paper. [24] [25] The solid lines in Fig. 3a represent the calculated values that were the best fit to the results shown in the figure. The agreement is good, showing that the core–shell model is a good representation of the present system. Fig. 3b shows typical AFM images of the CPCaL*n* micelles. Both the AFM images showed the existence of spherical objects; however, their heights were smaller than corresponding sizes of the micelles determined by SAXS. Sample preparation for AFM required a dry process that might have caused shrinkage of the micelles, leading to disagreement between the SAXS and AFM results. Because of the longer alkyl chain of CPCaL6 than that of CPCaL3, the micellar core size (R_c) of CPCaL6 was larger than that of CPCaL3. This is consistent with the size difference in AFM.

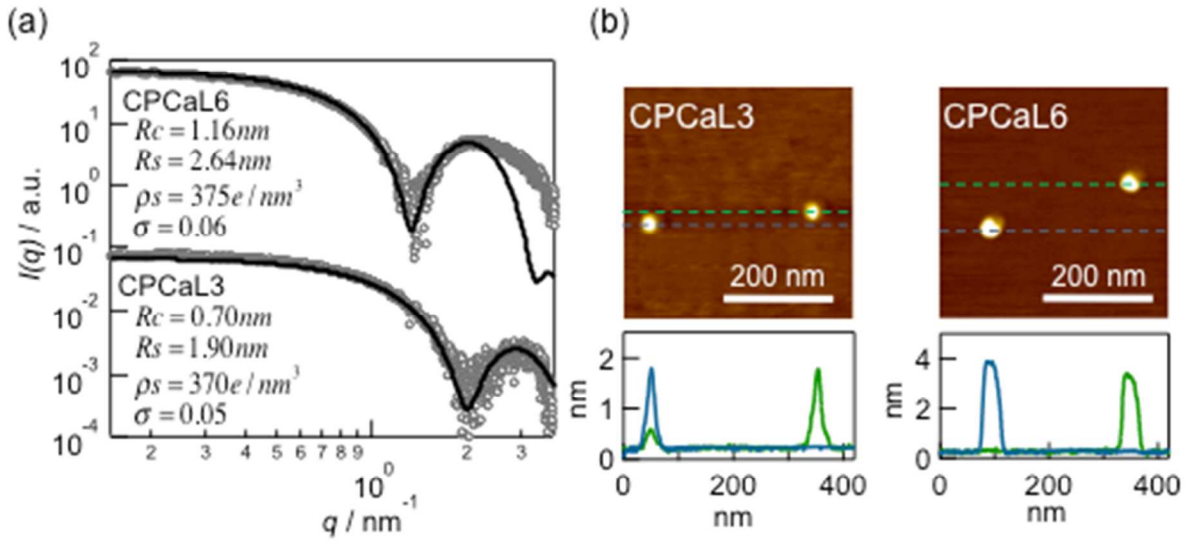

Fig. 3 (a) SAXS profiles of 2.0 mM CPCaL*n* micelles in 150 mM aqueous NaCl. The solid line was calculated from the core–shell spherical model in Eq. 1, and the fitting parameters are shown in the figure. The parameter of σ is the standard deviation of the Gauss function for the radii of

the core and shell. (b) Typical AFM images of CPCaL*n* micelles; the height profiles along the dashed lines are shown in the lower panels.

It is well known that the conformation of a polyelectrolyte in solution is usually dependent on the ionic strength, because both the magnitude of electrostatic interactions and the regions affected by such interactions are determined by the ionic strength. [33] [34] [35] Generally, the shapes of ionic micelles also change in a manner dependent on the ionic strength. [36] However, PC-bearing polymers do not show such a dependence on the salt concentration, because electrostatic interactions are canceled by the oppositely charged groups present in the PC. [37] When we examined CPCaL*n* micelles by SAXS at various salt concentrations (Fig. S3), all profiles were identical in the range of NaCl concentrations 50–300 mM, in a manner consistent with that of the polymeric system. [37]

Note that there are very sharp minima in the SAXS profiles of the CPCaL*n* micelles. This indicates that the size distribution was very narrow and that the micellar shape was highly symmetric. [38] We have previously reported shape-persistent micelles consisting of calix[4]arene-based lipids that also showed similar features in SAXS. [24] [25] To examine the shape persistency (monodispersity), we carried out light-scattering (LS) measurements for elutes from FFF. Fig. 4 shows the fractogram measured by LS and UV spectrophotometry. The micelles showed peaks at 8–11 min, and the LS and UV signals completely overlapped. The LS intensity is proportional to the sample concentration and the molecular weight, whereas the UV absorbance depends only on the concentration. Therefore, the overlap between the LS and UV profiles shows that the molecular weight was constant over the whole peak. By extrapolating the intensity at zero angle,

we calculated the molar mass of the micelles to be 13.8 × 10^3 for CPCaL3 and 59.5 × 10^3 g/mol for CPCaL6 (Fig. S4). From these values, the micellar aggregation numbers were determined to be 8 and 32, respectively. According to the SAXS analysis for the core size of CPCaL*n* micelles, the difference in the micellar core volume between CPCaL3 and CPCaL6 was about quadruple, which is consistent with the difference of their aggregation number. As shown in the figure, the molecular masses were almost constant over the peaks for both samples, and the molecular-weight distribution (M_w/M_n) was almost 1.0, as summarized in Table 1.

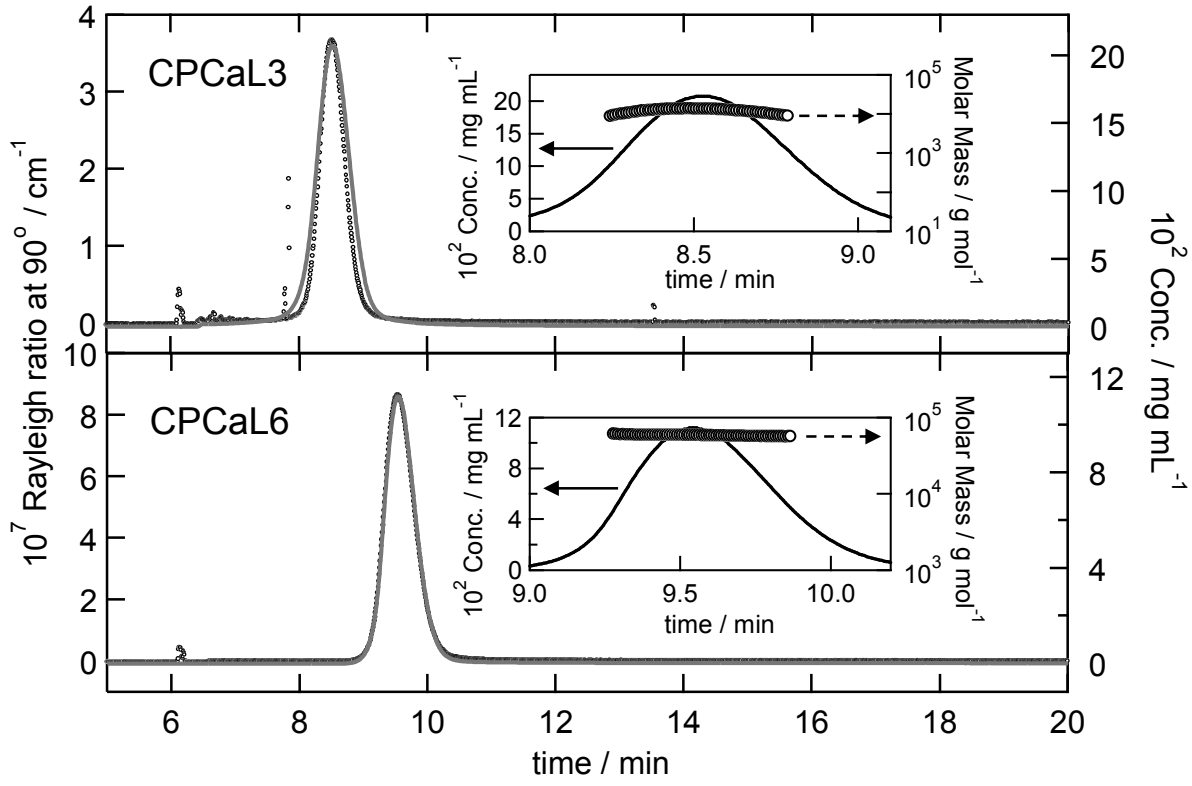

Fig. 4 FFF-MALS fractograms of CPCaL*n* micelles measured by LS at 90° (circle) and UV spectrophotometry at 270 nm (line) in 150 mM aqueous NaCl. The insets show the time dependence of the molar mass and the concentration of the CPCaL*n* micelles.

Table 1. Critical Micelle Concentration, Refractive Index Increment, and Extinction Coefficient of CPCaL*n* micelles in 150 mM NaCl solution, and the Micellar Molar Masses and Aggregation Number Determined with FFF-MALS

sample	*CMC* μM	$\partial n/\partial c$ mL g^{-1}	ε at 270 nm L g^{-1} cm^{-1}	M_w 10^3 g mol^{-1}	M_w/M_n	N_{agg}
CPCaL3	1.8 ($\pm$0.20)	0.144	1.45	13.8 ±0.20	1.04	$8._1 \fallingdotseq 8.0$
CPCaL6	0.50 ($\pm$0.10)	0.145	1.33	59.5 ± 0.90	1.00	$32._2 \fallingdotseq 32$

The molar masses of CPCaL3, 6 are 1.70×10^3 and 1.85×10^3g mol^{-1}, respectively.

CPCaL*n* Micelles – Cells Interaction *In Vitro*.

Calixarene have been used as scaffolds to introduce multi-functional groups to lipids and improve their interaction with cells. [39] Meanwhile, Coleman and coworkers have shown that para-sulfonato-calix[4]arene is not toxic and show no cellular uptake in any cells, [40] indicating calixarene backbone have little to do with interactions with cells. In addition to that, the preparation of PC-bearing calix[4]arene lipid is difficult. It is for these reasons that we used DOPC and DOCPe liposomes as control samples in this study.

Surface potential of particles is important in interaction with cells. To confirm the surface potential of CPCaL*n* micelles, the zeta potentials in 150 mM NaCl solution were compared with those of DOPC and DOCPe unilamellar liposomes whose sizes are less than 100 nm in diameter (Fig. S5). Fig. 5 shows that the average zeta potentials for CPCaL*n* and DOPC were slightly positive, whereas DOCPe showed a small negative value (~–5 mV). When these values are compared with that of a cationic calixarene-based spherical micelle that has four quaternary amines as head groups, the magnitudes for all the PC and CP micelles were quite small, and their

zeta potentials can be regarded as almost zero. The difference in sign between the charge on the CPe and that on the other micelles might be related to slight difference in the pK_a values of PC and CPe. [21]

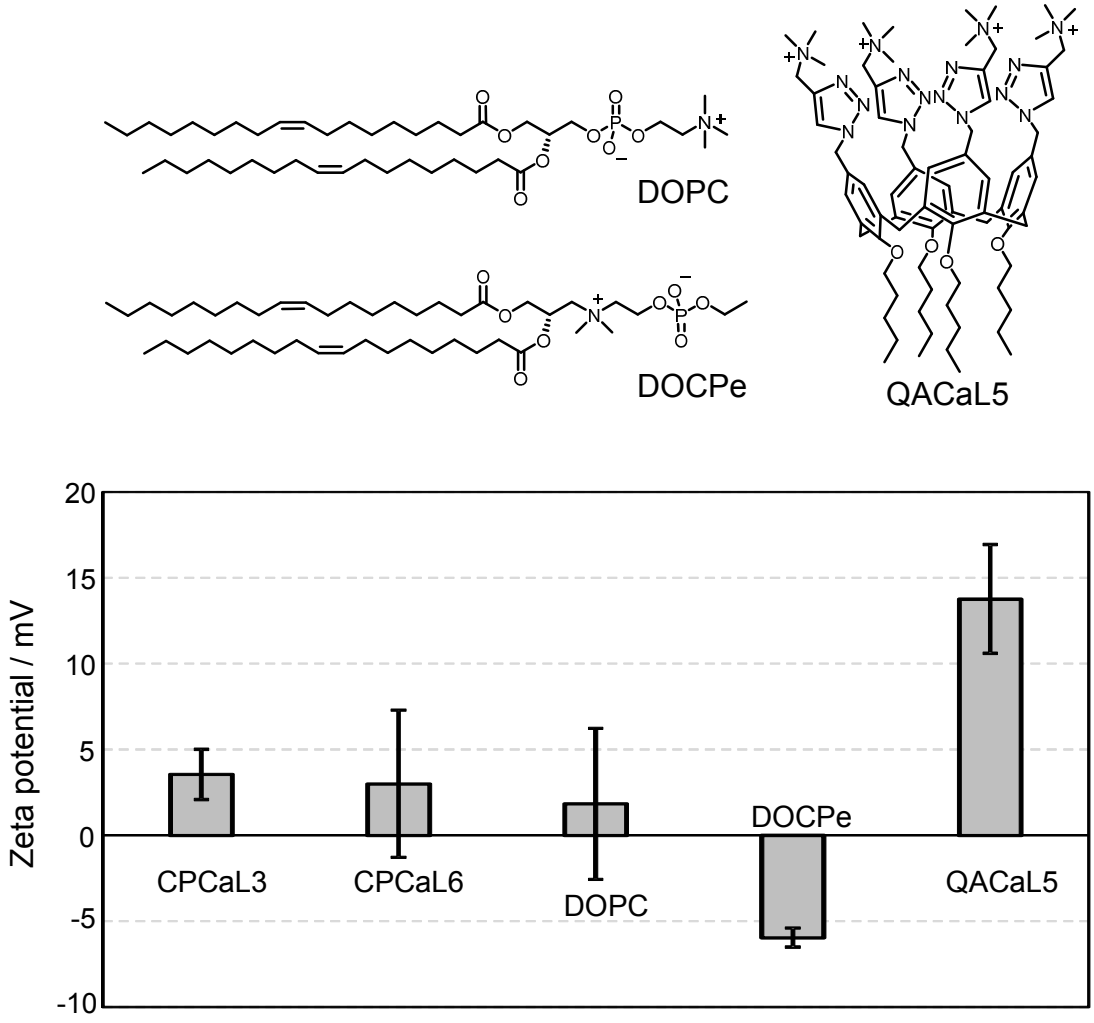

Fig. 5 Zeta potential of CPCaL*n* micelles in 150 mM aqueous NaCl. The chemical structures of the control samples (DOPC, DOCPe, and QACaL5) are shown above the graph. All samples were measured three times and the resulting values were averaged.

To trace the behavior of CPCaL*n* micelle, the fluorescent micelles were prepared with FITC-LP. After confirming that the addition of FITC-LP did not alter the size or the CMC for CPCaL*n* micelles (Fig. S6), we examined the interactions of CPCaL*n*/FITC-LP micelles with A549 cells (Fig. 6). Fig. 6 clearly shows that the fluorescence was observed in the cells treated with CPCaL*n*/FITC-LP micelles, and that a stronger fluorescence was observed with the longer alkyl chain. When we carried out the same assay for CPCaL*n* micelles at 4 °C, we could not observe the fluorescence in any cells, indicating that CPCaL*n* micelles were taken up by energy-dependent endocytosis. CPCaL*n* micelles showed similar features to CP-bearing polymers in cellular uptake behavior, [16] [17] whereas DOCPe/FITC-LP liposomes were hardly taken up by the cell, suggesting that the interaction between the micelles and cells should be CP-PC interaction.

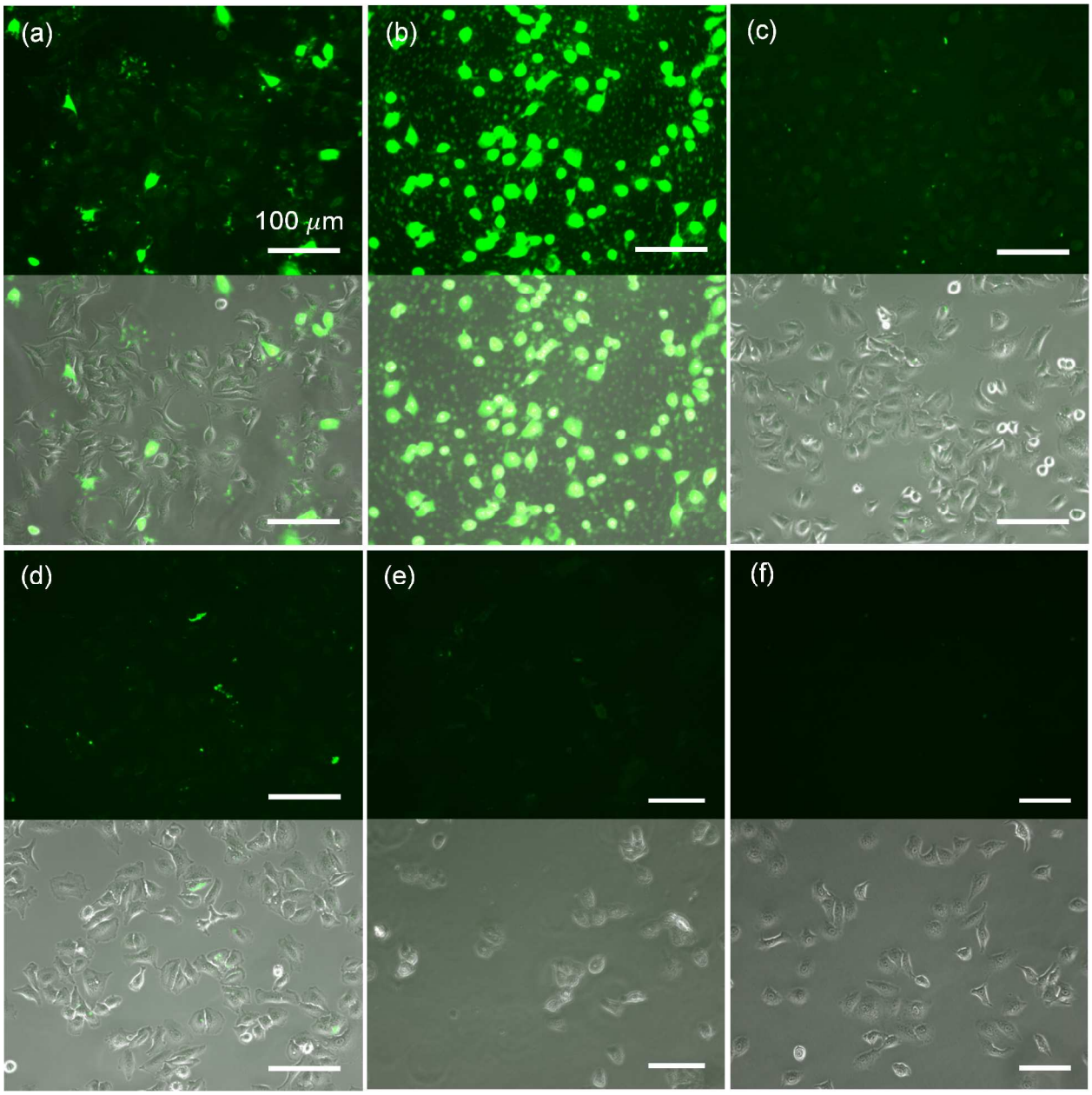

Fig. 6 Fluorescence images (upper panels) and the overlaid images on the bright-fields (lower panels) for A549 cells treated with (a) CPCaL3, (b) CPCaL6, (c) DOPC and (d) DOCPe micelles or liposomes containing FITC-LP at 37 °C. The same assay was carried out at 4 °C for (e) CPCaL3 and (f) CPCaL6 micelles.

Discussion and Concluding Remarks

We synthesized two new calix[4]arene-based lipids, CPCaL*n*, containing CP as the head group, and we found that these lipids formed spherical micelles in aqueous solution. Like other calix[4]arene micelles, [24] [25] CPCaL*n* showed monodisperse aggregation numbers, and the diameters of the micelles were 1.9 and 2.6 nm for CPCaL3 and CPCaL6, respectively. When CPCaL*n*s were mixed with the FITC-LPs and added to cells, the strong fluorescence was observed at 37 °C, but not at 4 °C, indicating that the micelles were taken up by the cells through endocytosis. The zeta potential of the micelles was negligible, and therefore their enhanced cellular interactions were not the result of electrostatic interactions. We can assume that the cellular interaction might be related to the formation of a quadrupole from two quaternary nitrogen–phosphorus pairs, termed the CP–PC interaction. It is interesting that DOPC and DOCPe did not show any cellular interaction. This suggests that the presence of multiple heads might be necessary to induce appreciable interaction. This speculation is consistent with the fact that when CPs are attached along polymer chains, the resultant polymer is capable of interacting with cells. Other lipids with multiple heads (and their micelles) are known to show enhanced cellular interactions, as exemplified by gemini lipids. [23] On these grounds, we propose that to extend the idea of CP–PC interaction to micelles, we need a system with multiple heads.

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