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Structural Transition of Lipopolysaccharide and Reduction in the Biological Activity by Amphiphilic Lipid with Cationic Amino Acid

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Lipopolysaccharide (LPS), the endotoxin of Gram-negative bacteria, is a strong elicitor in the immune system by interacting with lipopolysaccharide-binding protein and CD14 with high specificity. The removal of LPS contamination in protein drug products expressed by bacteria is essential in pharmaceutical products for human use. Although polymyxin B (PMB)-immobilized columns are mainly used for removal of LPS, there are some problems, such as high production cost, and the toxicity of ligands. We synthesized aromatic lipids bearing lysine or arginine at the headgroup. These lipids form a complex with LPS through electrostatic interaction between cationic amino acids and phosphate groups in the lipid A backbone. The resultant complexes induce the structural transition of LPS from a cylindrical structure to a vesicle. Addition of amino-lipid/LPS complexes to RAW264.7 cells, a macrophage-like cell line, decrease the LPS activity. The efficiencies are higher than commonly used cationic compounds, such as dioleoyltrimethylammoniumpropane (DOTAP) and PMB. These results show that amphiphilic lipids with cationic amino acids can be used for deactivation of LPS.

Innate immunity is the front line of biological defense against microbial infections. It is initiated by recognition of microbial components with a variety of pathogen sensors, such as Toll-like receptors (TLRs)[1,2] and CD14.[3] Lipopolysaccharide (LPS) is the major structural component of Gram-negative bacteria comprising of three distinct domains: O-antigen polysaccharide chains, core oligosaccharides, and a lipid (called lipid-A, Figure 1) decorated with phosphorylated glucosamine disaccharides.[4,5] The lipid-A hydrophobic chains anchor LPS into the bacterial membrane and bacteriolysis or external stimuli can cause the fragments containing the lipids to be released. In our biological system, these fragments induce furious immune response and thus lead to fever, diarrhea, and fatal shock. This is the historical origin that such fragments are called endotoxin and the related response is called endotoxic shock. According to recent immunology, the immune response to LPS is advocated as follows;[6–10] the lipopolysaccharide binding protein (LBP) binds to LPS and induces the transfer of LPS to CD14. LPS/CD14 complexes are recognized by TLR4/myeloid differentiation protein (MD2) heterodimer. The resulting complexes activate signal transduction and induce the secretion of proinflammatory cytokines. LPS can show a strong immune response even at very low concentrations. For instance, the intravenous administration of LPS at less than $1\,ng\,kg^{-1}$ in humans induces endotoxin shock or sepsis.[11] Proteins expressed by Gram-negative bacteria are often contaminated with endotoxin. This contamination must be removed completely before the proteins are used for in vivo studies with animal models or sold as pharmaceutical products. According to FDA recommendation, the general threshold level of endotoxin is defined as no more than 5.0 endotoxin units (EU) per kg body weight per hour for intravenous administration, where 1 EU is approximately 100 pg bacterial endotoxin.[12] Therefore the development of techniques to remove endotoxin from products is crucial for therapeutic usage.

One of the most common methods to remove endotoxin is affinity chromatography where ligands with a high biding affinity for LPS are attached on a solid phase. Polymyxin B (PMB, Figure 1) is a cyclic lipopeptide produced by *Bacillus polymyxa* and can be used for such purpose.[13] Since PMB has four primary amino groups and the lipid-A backbone has two phosphate groups (Figure 1), it is thought that the binding between LPS and PMB is driven by electrostatic and hydrophobic interactions.[5,14] It has been reported that the aggregated structures are essential for the expression of bioactivity.[15–18] When PMB binds to LPS, PMB leads to a strong fluidization of the acyl chains of LPS, resulting in the reduction in the endotoxin activity.[17] However, there are several drawbacks in use of PMB including high production cost, complexity to immobilize ligands on supports, and the toxicity of PMB itself.[19] Furthermore, since PMB is difficult to synthesize, it has to be exclusively prepared through biosynthesis. Therefore there is a strong desire for alternatives. The most representative examples are synthetic compounds and oligopeptides with cationic amino acids such as lysine and arginine.[20,21]

We have reported that a series of lipids that have an aromatic linker connected with primary amine, quaternary ammonium salt, or ethylenediamine can be used as a transfection reagent for DNA with better efficiency.[22,23] It is expected that these cationic compounds can form electrostatic complexes with the negatively charged LPS as well as plasmid DNA. In this study, we synthesized two lipids that have an aromatic linker

Published on the web March 23, 2013; doi:10.1246/bcsj.20120353

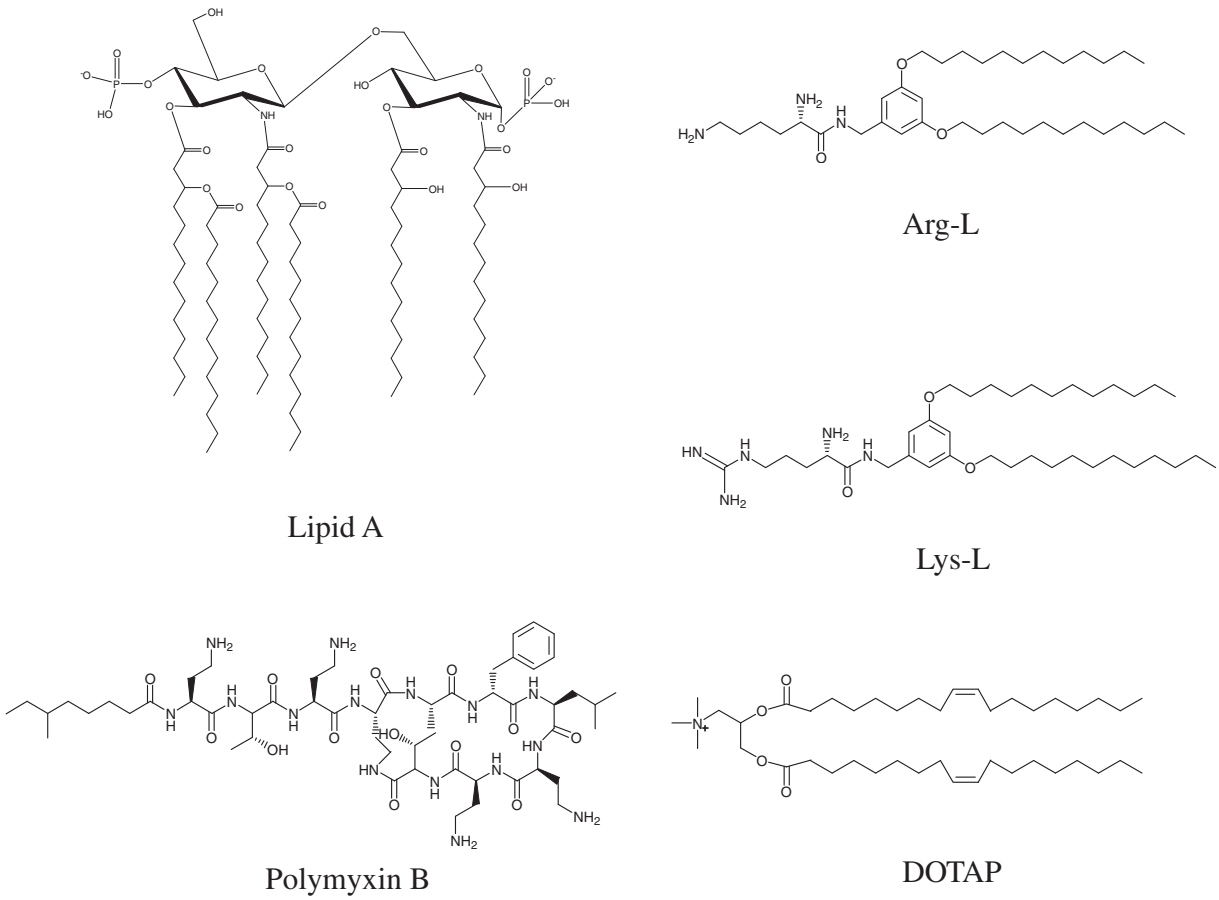


Figure 1. Chemical structures of lipid A and the cationic compounds used in this work.

connected with lysine or arginine and evaluated the LPS deactivation performance of them.

Material and Methods

Synthesis of the Amino-Lipid. Two types of a new lipid bearing arginine (Arg-L) or lysine (Lys-L) were synthesized as shown in Scheme 1 in a similar manner to a histidine bearing lipid described elsewhere.[24] The synthetic details and NMR characterization are described in Supporting Information. The chemical structures and their notations are shown in Figure 1.

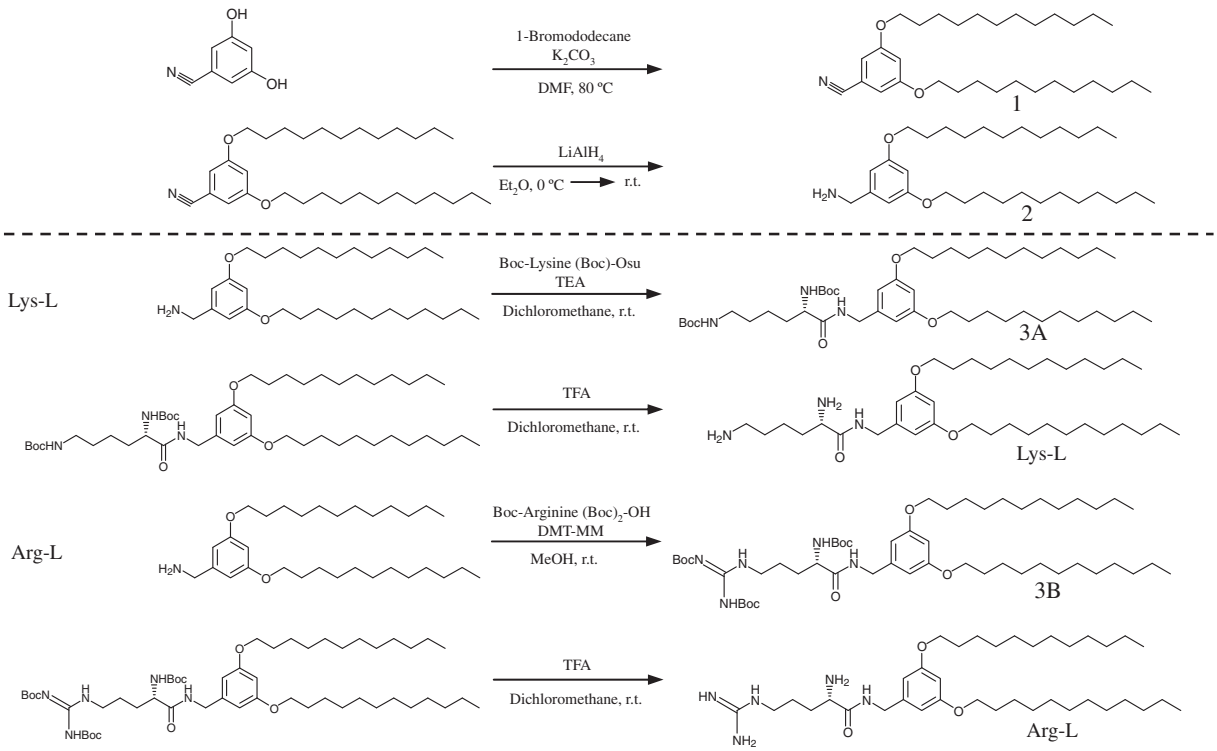
Preparation of the Amino-Lipid/LPS Complexes. The animo-lipid micelles were prepared with a common method described as follows. Each lipid was dissolved in dichloromethane and vacuum dried. An appropriate amount of 50 mM Tris buffer (pH 8.2) was added to hydrate the lipid. We obtained the lipid dispersion liquid with sonication for 10 min with a UH-50 (SMT Co., Tokyo, Japan). LPS (from *Escherichia coli* 055:B5; Sigma-Aldrich, St. Louis, MO) was dissolved in 50 mM Tris buffer (pH 8.2) at 1 mg mL^{-1} and added to the amino-lipid solution at various compositions. After mixing, the mixture was stored at room temperature overnight.

Critical Micelle Concentration (CMC) Measurement. We used 8-anilino-1-naphthalenesulfonic acid (ANS; Wako Pure Chemical Industries, Ltd., Osaka, Japan) as a fluorescence probe. After we prepared 2.0×10^{-5} M ANS solution containing the amino-lipid at indicated concentrations, we measured the fluorescence intensities with a fluorescence spectrophotometer (Hitachi F-4500; Hitachi, Ltd., Tokyo, Japan). Excitation was at 385 nm and the emission range was set between 400–700 nm.

ζ-Potential Measurement. We prepared 0.25 mg mL^{-1} LPS solution containing the amino-lipid or dioleoyltrimethylammoniumpropane (DOTAP; Sigma) at indicated concentrations. The ζ-potentials for amino-lipid/LPS complexes were measured with a Malvern Zetasizer nano (Malvern Instruments, Malvern, U.K.) at room temperature.

Small-Angle X-ray Scattering (SAXS) Measurement. SAXS measurements from the amino-lipid/LPS, DOTAP/LPS, and PMB (Sigma)/LPS solution were carried out at 40B2 SPring-8 with a 1.8 m camera using a Rigaku imaging plate (30 × 30 cm, 3000 × 3000 pixels) as a detector. The wavelength of the beam was 1 Å, and the exposure time was 30 s. The obtained two-dimensional image was circularly averaged to give an intensity $I(q)$ vs. q plots, where q is the magnitude of the scattering vector defined by $q = 4\pi \sin\theta/\lambda$ with the scattering angle of 2θ. The concentration of cationic compounds and LPS were fixed at 4.5 mM and 0.5 mg mL^{-1}, respectively.

Cytokine Assay. RAW264.7 cells were plated at 1.0×10^5 cells/well in 96-well plates, and incubated for 24 h. To the cells were added amino-lipid/LPS, DOTAP/LPS, or PMB/LPS complexes (LPS; 100 ng mL^{-1}) followed by incubation for 12 h at 37 °C. The concentration of cationic compounds was fixed at 9.0 μM. Tumor necrosis factor (TNF-α) released into



Scheme 1. Synthetic routes of Arg-L and Lys-L.

the supernatants was measured with a Murine TNF-α ELISA Development Kit (R&D System, Inc., Minneapolis, MN).

Results

Preparing Cationic-Lipid/LPS Complexes and the ζ-Potential. We have been studying several types of aromatic cationic lipids bearing two alkyl chains and showed that CMC for these lipids is almost one or two orders of magnitude lower than conventional cationic lipids such as DOTAP.[25] This is because of the synergetic effects of the hydrophobicity and π-stacking of the aromatic ring. Based on our previous studies, we can suppose that when the chain length is longer than C12, the aromatic lipids were expected to show a low CMC. We presume that it is essential for the lipids to maintain micellar structures to disturb the LPS activity. Figure 2 shows the lipid concentration dependence of the ANS fluorescence intensity. Since the fluorescence intensity of ANS sensitively reflects the polarity of its environment, ANS is suitable for a probe to determine CMC. The CMC was determined from the breakpoint on each plot. The obtained CMCs for Arg-L and Lys-L were almost the same values (2–3 μM) and are much lower than DOTAP (CMC; 70 μM).[26]

LPS has a relatively large molecular weight and contains many different lengths of polysaccharide chains. To study mixtures containing LPS, it may be inappropriate to determine the molecular weight and then carry out discussion based on the molar composition. Alternatively, we determined the electrostatically stoichiometric composition (isoelectric point) at which the negative charge of LPS is canceled by added cations. We measured the ζ-potential and the hydrodynamic radius (R_h) for the mixture for different compositions. LPS alone shows −18 mV because of the presence of negative phosphate groups (Figure 3a). With increase of the cationic lipid, the ζ-potentials increased for all of the samples and eventually crossed the zero line at the same concentrations, and further addition of the cations caused positive charge owing to off-stoichiometry. Essentially, there was no difference for all samples. These results indicate that the lipids are interacting with LPS and can conceal the negative charge of LPS in the same manner for the quaternary, primary, and guanidine amines.

When carefully examined the lipid concentration dependence, the ζ-potential was changed in a stepwise manner; rapid increase up-to 0.2 mM and slow increase in 0.2–0.6 mM. The ζ-potential is related to the electrical charge at the interfacial double layer and does not necessary reflect the total amount of change of the entire particle. The rapid increase at 0.2 mM may be ascribed to the added small amount of cations binding to the surface of the LPS micelle and then cancelling the surface charge. It can be related to the neutralization due to the ion-pair formation between 0.2 and 0.6 mM. Therefore, morphological transition of the micelles may occur in the second step. In contrast to the zeta potential, R_h did not change (Figure 3b) and we did not observe the formation of large aggregates at the isoelectric point, which is normally observed for polyion complexes. These features can be interpreted in that the solubility of LPS is mainly ascribed to its saccharide portions and thus elimination of its negative charge does not induce aggregation. Further addition of the cationic lipids above 0.8 mM showed that Arg-L gave larger positive charge than the others, simply, relating the difference in the electrophilicity between the cations.

Structural Changes of LPS Micelle by Addition of the Cationic Lipids. Figures 4 and 5b show the angular dependence of DLS and SAXS for our LPS micelle solution,

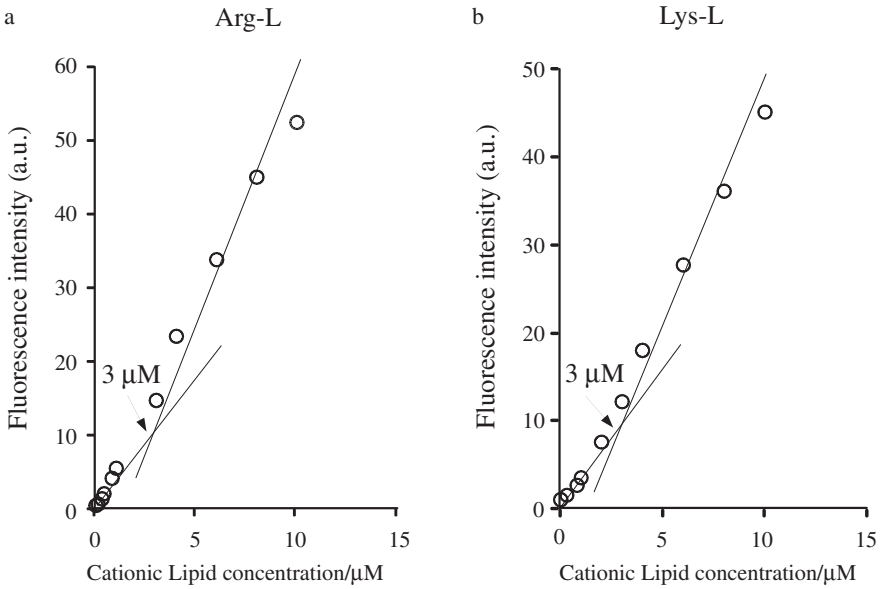


Figure 2. Arg-L (a) and Lys-L (b) concentration dependency of the ANS fluorescence intensity. The concentration of ANS was fixed at 2.0×10^{-5} M.

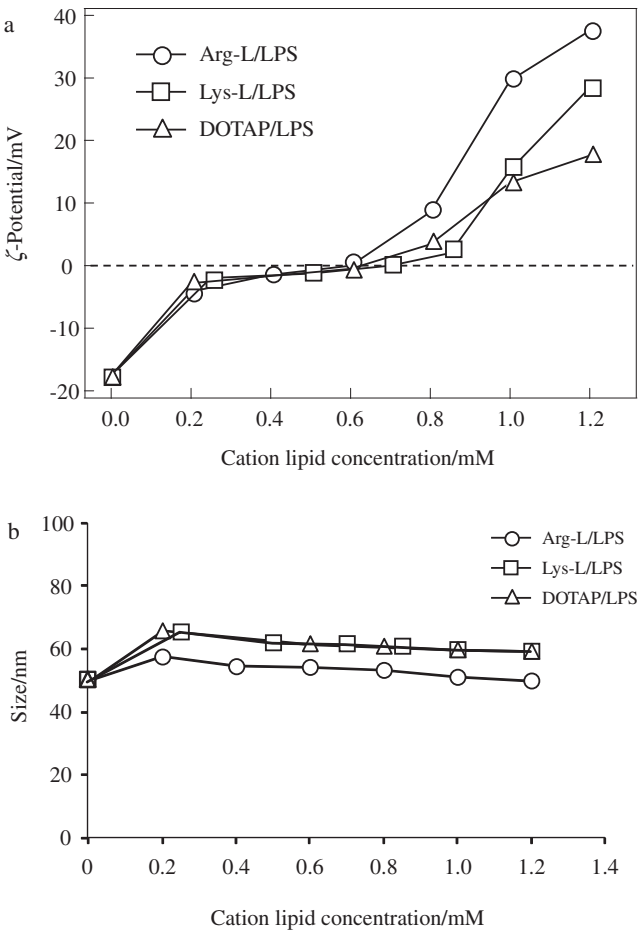


Figure 3. ζ-Potential (a) and hydrodynamic radius (b) for mixtures of LPS (0.25 mg mL^{-1}) and the indicated cationic lipid concentrations at 25 °C.

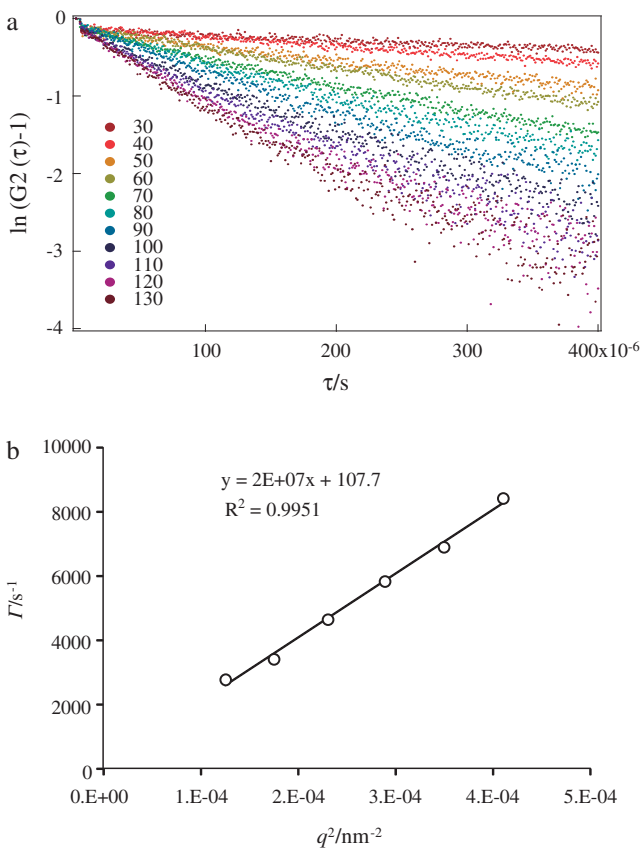


Figure 4. The first-order autocorrelation function (a) and the decay constant (Γ) against q^2 (b) for LPS measured with DLS.

respectively. As presented in Figure 4a, the first-order autocorrelation function can be expressed by a single exponential decay, suggesting monodisperse population. When we plotted the decay constant (Γ) against q^2 (Figure 4b), the data points

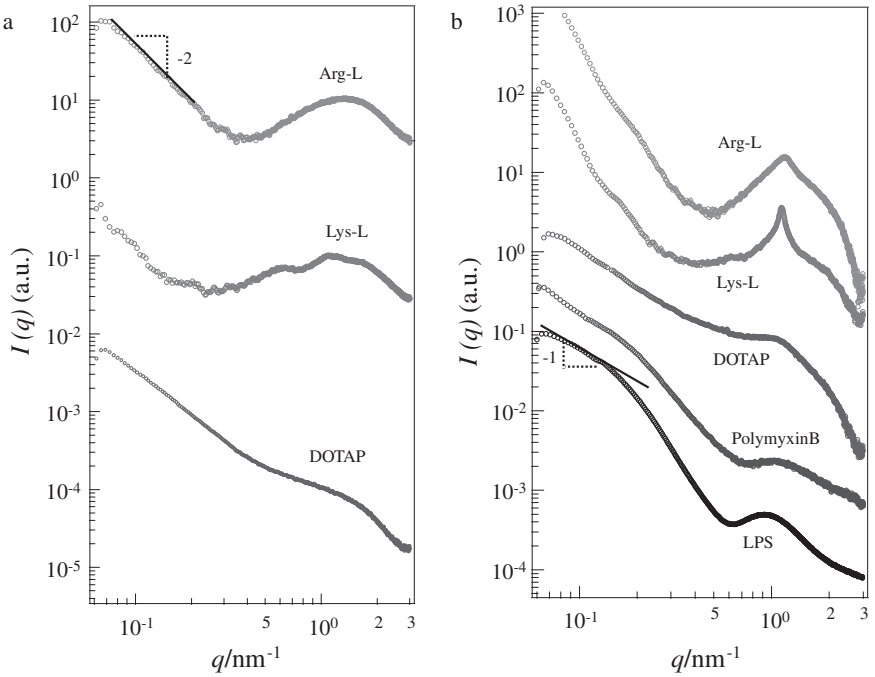


Figure 5. SAXS profiles of the Arg-L, Lys-L, and DOTAP at 4.5 mM (a) and the complexes composed of LPS (0.5 mg mL^{-1}) and the cationic compounds (b). The straight lines show the slopes of -2 and -1, representing the expected values for vesicle- and cylinder-like scattering objects, respectively.

can be fitted to a straight line with a nonzero intercept, indicating -1 dependence in SAXS observed at low-q, indicating the presence of cylinder-like structures, being consistent with the DLS. The present result agrees with previous morphological observations by transmission electron microscopy.[20,27] The second maximum at $q = 1.0\,\mathrm{nm}^{-1}$ in SAXS can be ascribed to cross-sectional structure of the LPS micelles.

Figure 5a shows SAXS profiles for Arg-L, Lys-L, and DOTAP micelles, respectively. All of the samples showed $I(q) \approx q^{-2}$ at the low-q region. This q-dependence indicates vesicle formation.[28,29] The SAXS profiles after mixing LPS and each cationic lipid is compared with LPS itself in Figure 5b. The mixture with PMB showed a very similar profile with that of LPS itself. There was subtle change observed for the second maximum; the mixing caused the peak to broaden. This is probably ascribed to disruption of the uniform cross-sectional structure due to PMB attaching to the micellar surface. Brandenburg et al. advocate the relation between the type of aggregate structure of LPS and the biological activity by use of SAXS.[15–18,30] They measured SAXS from LPS/PMB mixtures and reported that the mixing led to formation of multilamellae. They measured considerably concentrated solutions, i.e., 10%, probably owing to very week scattering intensity. We presume that the difference in the obtained morphology is due to the concentration effect. Although the data are not shown, when we added arginine or lysine to LPS, there was no major structural change.

DOTAP eliminated the second maximum of the original LPS, suggesting that considerable disruption of the local structure was induced. On the other hand, the low-q slope was almost maintained, indicating that rod-like shape still existed. There were rather drastic changes observed for Arg-L and Lys-L. The low-q slope became much steeper and it was almost 3, suggesting formation of random aggregates. A diffraction peak appeared at $q = 1.1\,\mathrm{nm}^{-1}$ for both addition of Arg-L and Lys-L, although Lys-L produced a shaper peak than Arg-L. Because of the absence of the second diffraction, we were not able to assign the structure. These SAXS data indicated that amino acid lipids potently bind to LPS and drastically disturb the original rod structure of LPS.

Inhibition of the LPS-Induced TNF-α in Macrophages. It is well-known that RAW264.7 cells secrete a large amount of TNF-α when stimulated by LPS.[31] In cells treated with 100 ng mL^{-1} of LPS, the TNF-α level in supernatant increased to 4.0 ng mL^{-1} (Figure 6). The addition of the cationic compounds led to a decreased secretion of TNF-α, but showed completely different efficiencies. The cells to which PMB/LPS or DOTAP/LPS complexes were added reduced TNF-α secretion less than one-third of that secreted from the cells treated with LPS alone. Further reductions in TNF-α secretion were observed in the cells to which Arg-L/LPS and Lys-L/LPS complexes were added. The values were almost tenth-one of that for LPS treatment. Although we expect higher suppression by Arg-L than Lys-L because of the high interaction with phosphate groups of LPS, in this study, we did not observe significant difference between Arg-L and Lys-L. When we prepared the complexes, where the concentration for Arg-L and Lys-L is lower than the CMC (Figure 2), the reductions in LPS activities were not observed (data not shown). These results indicate that cationic micelles or compounds binding LPS cannot fully activate cells and the addition of micellar form is essential to induce structural change in LPS. Among these cationic compounds, amino-lipids have higher suppression ability than commonly used compound PMB.

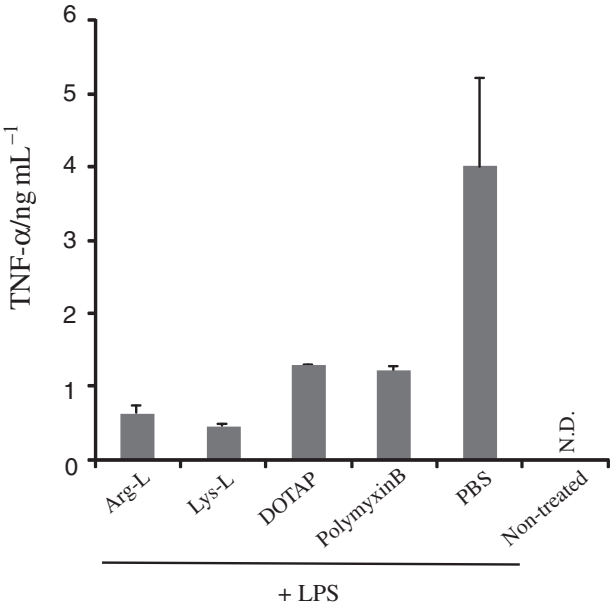


Figure 6. Reduction in LPS activity by cationic compounds on the production of TNF-α. RAW264.7 cells were stimulated with LPS with or without complexation for 12 h at 37 °C. Results represent the mean ± S.D. of triplicate wells.

Discussion and Concluding Remarks

When the micelles of Arg-L and Lys-L were mixed with LPS, drastic structural changes occurred and antagonistic cell activation by LPS was reduced. According to previous studies, the role of PMB or other LPS binding peptides in deactivating LPS is to block the signal transduction through TLRs or ion channels owing to the structural transition of LPS.[15] Accepting this interpretation, Arg-L and Lys-L more radically disturbed the original LPS structures than PMB and thus showed more efficient deactivation of LPS.

This work is financially supported by JST CREST program and SAXS measurements were carried out at SPring-8 40B2.

Supporting Information

The synthetic details and ^{1}H spectra of Lys-L and Arg-L. This material is available free of charge on the Web at: http://www.csj.jp/journals/bcsj/.

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