Selective Adsorption of Lipopolysaccharide from Protein Solutions by Porous Supports Bearing Cationic Lipid Membranes

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Cationic lipid membranes of *N*-octadecylchitosan consisting of 2-deoxy-2-octadecylamino-D-glucopyranose, 2-amino-2-deoxy-D-glucopyranose, and 2-acetamido-2-deoxy-D-glucopyranose were covalently immobilized to carboxylated porous supports and used for the adsorption of pyrogenic lipopolysaccharide (LPS). When bovine serum albumin (BSA) solution, including 5 mg mL^{-1} of BSA and 100—148 ng mL^{-1} of LPS, was passed through a column packed with the porous supports bearing the cationic lipid membranes, LPS was selectively adsorbed and removed to as low as 0.1 ng mL^{-1} with quantitative recovery of BSA. LPS seemed to be dissociated from molecular complexes with BSA, and adsorbed selectively to the cationic lipid membranes by both an ionic interaction and a hydrophobic one.

Lipid membranes have been receiving considerable attention as models for various chemical investigations. Densely packed alkyl chains with hydrophilic head groups can have both a hydrophobic interaction and a hydrophilic one with guest substrates. This specific environment provided by lipid membranes is essential for molecular recognition in biological membranes, and seems to be promising in the areas of chemical sensing[1] and separation.[2]

Proteins or other biologically active substances, especially those produced by recombinant microorganisms, are usually contaminated with lipopolysaccharide[3] (LPS). LPS, which originates from an outer membrane of Gram-negative bacteria, consists of a polysaccharide and a terminal lipid A moiety. Lipid A is composed of a diglucosamine that is highly substituted with amide- and ester-linked long-chain fatty acids and negatively charged with phosphate groups. For pharmaceutical uses of those active substances, LPS has to be removed to not higher than 0.1 ng mL^{-1} because of its strong pyrogenicity.[4] Intensive studies have been done on chromatographic removal of LPS from protein solutions.[5] For example, LPS was selectively removed by ion-exchange chromatography using DEAE-Shepharose CL-6B,[6] and affinity chromatography using adsorbents bearing polymixin B,[7] or histidine.[8] Recently it has been suggested that the removal of LPS is extremely difficult when LPS is associated with the protein to be purified, and use of a hydrophobic interaction has been getting attention.[9]

We have covalently immobilized cationic lipid membranes of *N*-octadecylchitosan consisting of 2-deoxy-2-octadecylamino-D-glucopyranose (GlcNC18, Chart 1), 2-amino-2-deoxy-D-glucopyranose (GlcN), and 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) to carboxylated porous supports.[10] In this paper, we would like to report the selective adsorption of LPS from protein solutions by the resulting porous supports bearing cationic lipid membranes. LPS was selectively adsorbed and removed to as low as 0.1 ng mL^{-1} from a BSA solution that was highly contaminated with LPS.

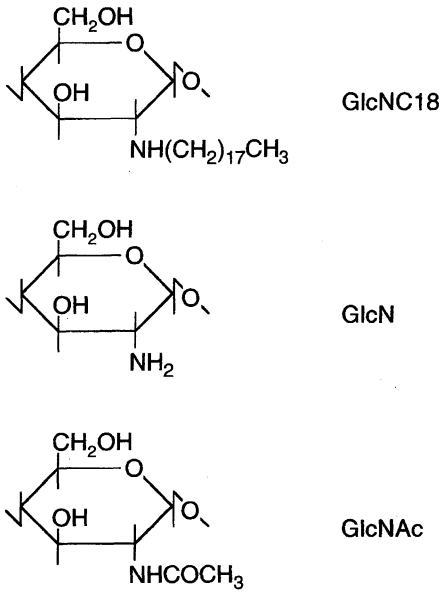

Chart 1.

Experimental

Materials and Assay Methods. BSA (fraction V) and LPS (*E. coli* serotype 0127:B8) were products of Seikagaku Kogyo (Tokyo, Japan) and Difco Laboratories (Detroit, MI, USA), respectively and used as obtained.

Toxicolor (Seikagaku Kogyo), which is a chromogenic *Limulus* Amebocyte Lysate test, was used as an assay method for LPS. Samples containing LPS were diluted with Tris-HCl buffer (pH 8.0) to lower than 0.085 ng mL^{-1} of LPS and assayed by the method recommended by the manufacturer. Detection limit of LPS in this test was as low as 0.020 ng mL^{-1}, which corresponded to 0.06 endotoxin unit. BSA concentration was measured by UV at 280

nm.

Dynamic Light Scattering. Dynamic light scattering measurements were carried out using a laser light scattering photometer, DLS-700, manufactured by Otuka Electronics Co. (Osaka, Japan). The light source was 5 mW He–Ne laser at a wave length of 632.8 nm and temperature was controlled to 20 °C by circulating water of constant temperature. Scattering angle was 90°.

Porous Supports Bearing Cationic Lipid Membranes. Preparation and characterization of the porous supports bearing cationic lipid membranes were reported elsewhere.[10] The following is a brief description. *N*-Octadecylchitosan consisting 70 mol % of GlcNC18, 17 mol % of GlcN, and 13 mol % of GlcNAc was prepared by *N*-alkylation of chitosan with 1-bromooctadecane in *N*,*N*-dimethylacetamide. Carboxylated porous supports were prepared by *N*-succinylation of Kurimover II (Kurita Water Industries, Tokyo), cross-linked porous chitosan gel particles, with succinic anhydride. Vesicles prepared from *N*-octadecylchitosan were reacted with the carboxylated porous supports in the presence of water-soluble carbodiimide and *N*-hydroxysuccinimide to form amide bonds from primary amino groups of *N*-octadecylchitosan and carboxyl groups of the supports. Resulting materials were further reacted with *N*-acetylglucosamine to block remaining carboxyl groups by amidation. The immobilized amount of *N*-octadecylchitosan was 4 mg/mL-particles.

Column-Wise Adsorption of LPS from Protein Solutions. Purified water and buffer solutions were sterilized by an autoclave at 115—121 °C for 15 min. Glass wares were also sterilized by the autoclave at 250 °C for 2 h. BSA, which was highly contaminated with LPS as obtained, was dissolved in water. The solution was filtered with a filter disk having 0.2-μm diameter pores, and diluted with buffer solutions to desired concentration for a column-wise adsorption experiments. Acetic acid buffer was used for experiments at pH 4.2, 4.3 and 5.3, and phosphate buffer was used for experiments at pH 7.0.

Column-wise adsorption was done at 20 °C. Porous supports bearing cationic lipid membranes were suspended in water and fed into a glass column (8 mm i.d.×100 mm L.) using a LC-6A pump (Shimadzu). The length of the gel bed was between 930 and 980 mm. The resulting column was washed with 0.5 M NaOH, (M = mol dm^{-3}) at 10 mL h^{-1} for 3 h, and left overnight filled with 0.5 M NaOH to decompose LPS in the column (depyrogenation). After washing with water for 3 h, 0.1 M acetic acid was passed through for 1.5 h to make *N*-octadecylchitosan immobilized on the supports become its ammonium form. After buffer solution was passed through for 6.5 h, BSA solution was passed through at 5 mL h^{-1} for 15—16 h. Eluted solution was collected immediately as ten fractions of 7.5 mL each. Fractions 2, 4, 6, 8, and 10 were analyzed for the concentration of LPS and BSA.

Results and Discussion

Interaction between LPS and BSA. It is known that LPS forms large molecular aggregates in aqueous solutions.[11] As mentioned in the introduction, an association of LPS with protein seems to influence the removal of LPS. We have reported the association of LPS with BSA at pH 6.8.[12] Since the isoelectric point (pI) of BSA is 4.8, both LPS and BSA are negatively charged at pH 6.8. In this report we studied an interaction between LPS and BSA at pH 4.2 where BSA is positively charged and LPS is negatively charged. Figure 1 shows the effect of BSA concentration on the scattering intensity of LPS solution and the average hydrodynamic diameter

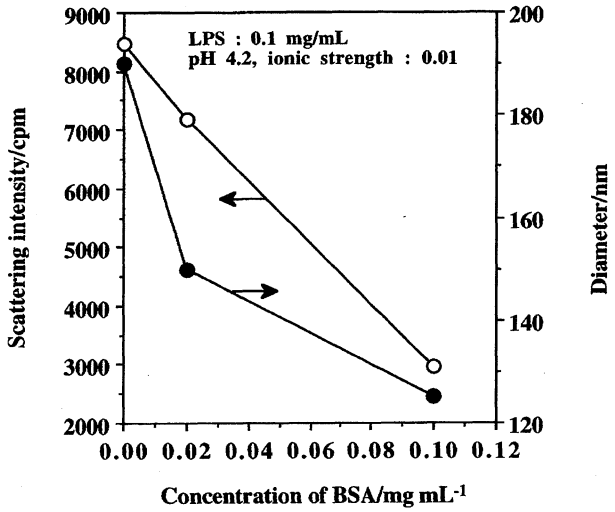

Fig. 1. Effect of BSA on the scattering intensity of LPS solution and the average hydrodynamic diameter of LPS aggregates. Scattering intensities and average hydrodynamic diameters of LPS solution (0.1 mg mL^{-1}, pH 4.2, ionic strength 0.01) including various concentrations of BSA were determined by DLS.

of LPS aggregates determined by dynamic light scattering measurements. The LPS solution exhibited a scattering intensity of 8500 and a diameter of 190 nm. In the presence of BSA the scattering intensity and the diameter decreased with the increase of BSA concentration, and reached 3000 and 125 nm, respectively. Since the concentration of LPS is constant in these solutions, the decrease of the scattering intensity indicates substantial interaction between LPS and BSA.

With the results reported previously,[12] it is shown that LPS associates with BSA at pH both higher and lower than the pI of BSA. Considering BSA is an amphoteric electrolyte and has a hydrophobic domain to bind fatty acids,[13] the interaction between LPS and BSA in their complex seems to be both electrostatic and hydrophobic.

Selective Adsorption of LPS from BSA Solution. A column-wise adsorption was done by passing the BSA solution with a concentration of 5 mg mL^{-1} through the column packed with the porous supports bearing *N*-octadecylchitosan lipid membranes. A typical solution including 5 mg mL^{-1} of BSA had a LPS concentration of 100—148 ng mL^{-1}.

Table 1 summarizes the results of the column-wise adsorption of LPS and BSA. At pH 4.3—7.0 LPS was selectively adsorbed with 99.2—99.9% LPS removal and 91—100% BSA recovery. However, the concentration of LPS in the recovered solution varied with the pH and an ionic strength. For example, LPS was removed to as low as 0.1 ng mL^{-1} at pH 4.3 with an ionic strength of 0.01 and at pH 5.3 with an ionic strength of 0.05. As shown in the elution profile of LPS and BSA (Figs. 2 and 3), the concentration of LPS in the recovered fraction was almost constant. BSA recovery was almost 100% except that in the 2nd fraction at pH 5.3.

On the other hand, at pH 5.3 with an ionic strength of 0.01 or 0.1 and at pH 7.0, the average of LPS concentration

Table 1. Column-Wise Adsorption of LPS and BSA

Run	BSA solution applied[a]			BSA solution recovered[b]		
	pH	Ionic strength	LPS included / ng mL^{-1}	LPS detected / ng mL^{-1}	LPS removal / %	BSA recovery / %
1	4.3	0.01	148	0.10	99.9	98
2	5.3	0.01	112	0.30	99.7	91
3	5.3	0.05	104	0.11	99.9	93
4	5.3	0.10	102	0.26	99.8	97
5	7.0	0.01	113	0.87	99.2	100

a) Concentration of BSA is 5 mg mL^{-1}. b) LPS detected, LPS removal, and BSA recovery are averages of recovered fractions.

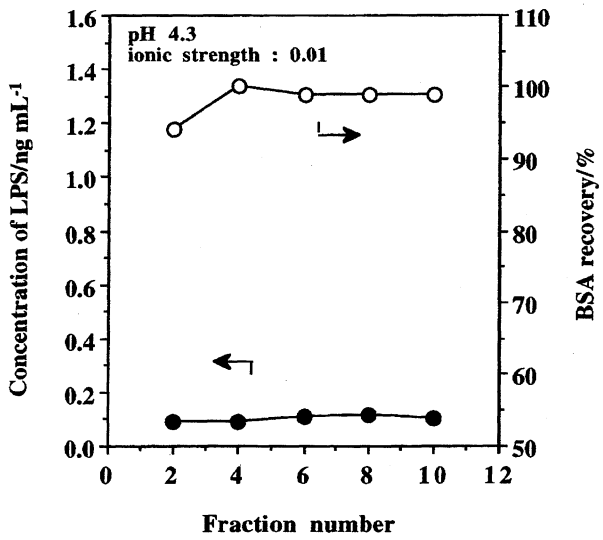

Fig. 2. Elution profile of LPS and BSA from the column packed with porous supports bearing *N*-octadecylchitosan lipid membranes. BSA, 5 mg mL^{-1}; LPS, 148 ng mL^{-1}; pH 4.3; ionic strength, 0.01.

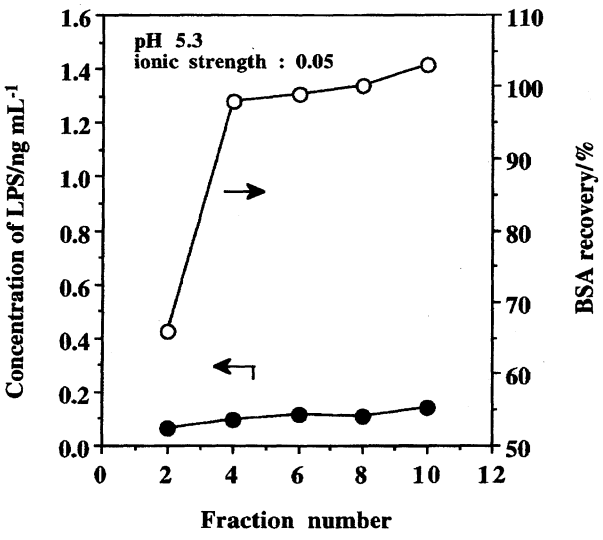

Fig. 3. Elution profile of LPS and BSA from the column packed with porous supports bearing *N*-octadecylchitosan lipid membranes. BSA, 5 mg mL^{-1}; LPS, 104 ng mL^{-1}; pH 5.3; ionic strength, 0.05.

in the fractions were 0.30 ng mL^{-1} (pH 5.3; ionic strength, 0.01), 0.26 mg mL^{-1} (pH 5.3; ionic strength, 0.1) and 0.87 ng mL^{-1} (pH 7.0), respectively. The concentration of LPS in the recovered fraction gradually increased with the elution volume (Figs. 4, 5, and 6). BSA recovery was almost 100% except those in the 2nd fractions at pH 5.3.

Mechanism of the Selective Adsorption of LPS. BSA used in this study is highly contaminated with LPS as obtained. These LPS, at least some of them, are considered to associate with BSA. The selective adsorption of LPS by the porous supports bearing cationic lipid membranes suggests that LPS is dissociated from the molecular complexes with BSA and adsorbed to *N*-octadecylchitosan lipid membranes. We have reported that fluorescein isothiocyanate–lipopolysaccharide (FITC–LPS) was selectively adsorbed to the polyion-complexed lipid membranes of dioctadecyldimethylammonium and dextran sulfate from the mixed solution of FITC–LPS and BSA.[12] The interaction between the polyion-complexed lipid membranes and FITC–LPS or BSA is a hydrophobic interaction. Since *N*-octadecylchitosan has a cationic charge at the pH range in this study, an ionic interaction between LPS and lipid membranes as well as the hydrophobic one is essential for the selective

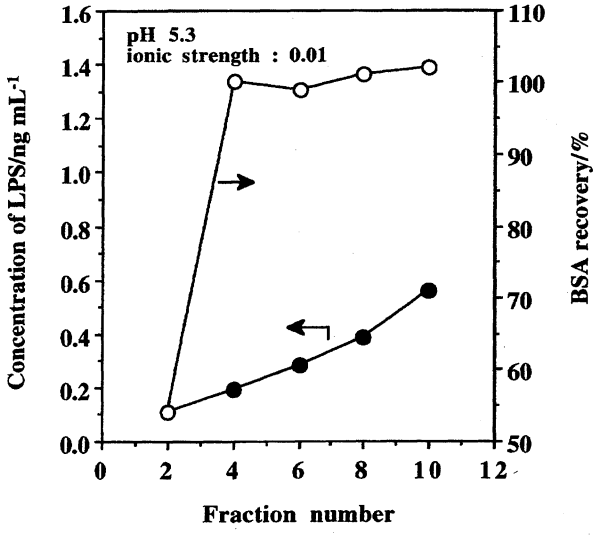

Fig. 4. Elution profile of LPS and BSA from the column packed with porous supports bearing *N*-octadecylchitosan lipid membranes. BSA, 5 mg mL^{-1}; LPS, 112 ng mL^{-1}; pH 5.3; ionic strength, 0.01.

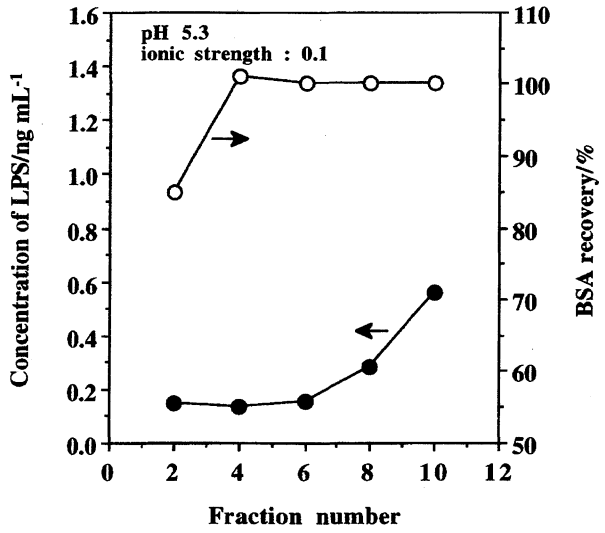

Fig. 5. Elution profile of LPS and BSA from the column packed with porous supports bearing *N*-octadecylchitosan lipid membranes. BSA, 5 mg mL^{-1}; LPS, 102 ng mL^{-1}; pH 5.3; ionic strength, 0.1.

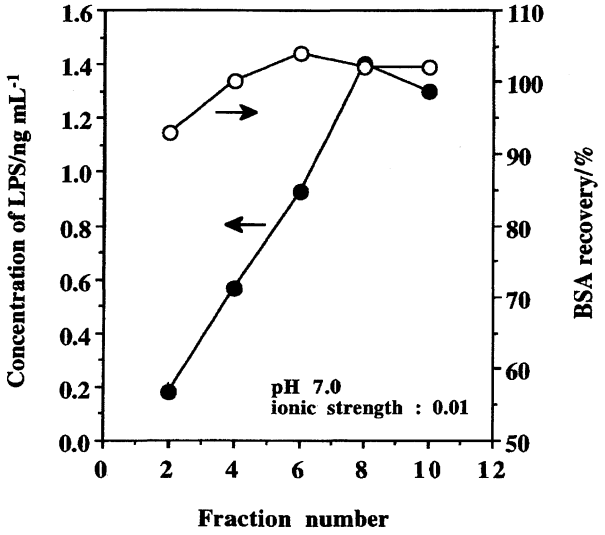

Fig. 6. Elution profile of LPS and BSA from the column packed with porous supports bearing *N*-octadecylchitosan lipid membranes. BSA, 5 mg ml^{-1}; LPS, 120 ng mL^{-1}; pH 7.0; ionic strength, 0.01.

adsorption of LPS to the porous supports bearing *N*-octadecylchitosan lipid membranes. The reason why BSA was not adsorbed can be explained by a relatively weak electrostatic interaction between charged lipid bilayer membranes and proteins,[14] and the difficulty of hydrophobic adsorption of BSA to lipid membranes in gel phase.[12] Lipid A moiety of LPS is a diglucosamine which is highly substituted with amide- and ester-linked long-chain fatty acids. The structural similarity of *N*-octadecylchitosan and lipid A seems to make their interaction more specific than that between LPS and polyion-complexed lipid membranes.

The influence of pH and ionic strength on the LPS removal is explained by the ionic interaction between cationic lipid membranes and LPS or BSA. Since *N*-octadecylchitosan is a polyelectrolyte consisting of secondary and primary amino groups, its cationic charge is stronger at lower pH. Therefore, the ionic interaction between LPS and *N*-octadecylchitosan lipid membranes is stronger at lower pH. The strong ionic interaction combined with the hydrophobic one results in the sufficient removal of LPS at pH 4.3 in spite of the interaction between anionic LPS and cationically charged BSA.

At pH 5.3 and 7.0, the ionic interaction between LPS and *N*-octadecylchitosan lipid membranes is weaker. Moreover, BSA, which is negatively charged, may exist near the surface of cationic lipid membranes and inhibit the adsorption of LPS. Therefore, the LPS concentration in the recovered solution is higher at pH 5.3 and 7.0 than that at pH 4.3. The shielding effect by BSA can yield the increase of LPS concentration with applied volume observed in Figs. 4 and 5. At pH 5.3, the influence of ionic strength was observed. Lower ionic strength is favorable for the ionic adsorption of LPS by *N*-octadecylchitosan lipid membranes. However, the shielding effect of BSA is also stronger at lower ionic strength. Therefore, it is likely that LPS was removed to the lowest concentration with the moderate ionic strength of 0.05 at pH 5.3.

Conclusion

Porous supports bearing cationic lipid membranes of *N*-octadecylchitosan adsorb LPS selectively from BSA solution at pH 4.3—7.0 with the ionic strength of 0.01—0.1. LPS was removed to as low as 0.1 ng mL^{-1} from BSA solution by a column-wise adsorption. Since LPS includes terminal diglucosamine which is negatively charged and highly substituted with long-chain fatty acids, LPS is adsorbed by both an ionic interaction and a hydrophobic one. The structural similarity of LPS and *N*-octadecylchitosan seems to make the interaction specific. In addition, a relatively weak interaction between BSA and the lipid membranes results in the selective adsorption of LPS. The porous supports bearing the cationic lipid membranes seem to be useful for the removal of pyrogenic LPS from pharmaceutical protein solutions.

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