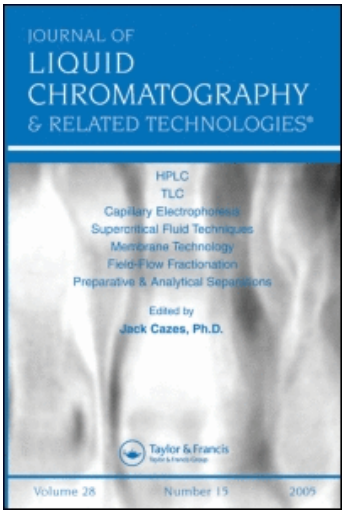

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ABSTRACT

Cross-linked *N,N*-dimethylaminopropylacrylamide (DMP) spherical particles for the selective removal of lipopoly-saccharides (LPS) from protein solution were prepared. When *N, N'*-buthylene-bis-methacrylamide (BBMA) and divinylbenzene (DVB) were each used as a cross-linking agent and the amino-group content was adjusted to 4.0 meq g^{-1} adsorbent or more, the DMP/BBMA and the DMP/DVB adsorbents showed good LPS adsorption at pH 7.0 and an ionic strength of $\mu = 0.05$ to 0.2. On the other hand, the adsorption of bovine serum albumin, an acidic protein, by each adsorbent increased with the increase in the

amino-group content to 4.5 mequiv. g^{-1} adsorbent or larger, but decreased with the increase in the ionic strength (μ) of the buffer to 0.2 or stronger. Only DMP/DVB specifically adsorbed aromatic proteins such as cytochrome *c* and myoglobin, over a wide ionic strength range of μ = 0.05 to 1.0. As a result, when the DMP/BBMA adsorbent which had an amino-group content of 4.0 meq g^{-1} was used in conditions of pH 7.0 and μ = 0.05, LPS was selectively removed from various protein solutions, naturally contaminated with LPS.

INTRODUCTION

Removal of endotoxin (lipopolysaccharides: LPS) from substances used as drugs is very important, since it causes pyrogenic and shock reactions in mammals upon intravenous injection even in nanogram quantities.[1,2] LPS, constituents of the cell wall of gram-negative bacteria, are potential contaminants of physiological fluids and aqueous solutions and very stable, resisting extreme temperatures and pH values. To remove LPS from solutions of high-molecular-weight compounds, such as proteins, the adsorption method has proved to be most effective.

We previously reported that aminated poly(γ-methyl L-glutamate) (PMLG-NH_2) spherical particles can selectively remove LPS from various protein solutions at a high ionic strength, μ = 0.2 to 0.8.[3-5] However, the adsorbing capacity of the particles was high for both LPS and acidic protein such as bovine serum albumin (BSA) at a low ionic strength, μ = 0.05.[5] We reported also that spherical copolymers of *N,N*-dimethylaminopropylacrylamide (DMP) and *N*-allylacrylamide (AA) were able to selectively adsorb LPS from a BSA-LPS solution even at a low ionic strength without affecting the recovery of BSA.[6] However, the LPS-adsorbing activity of DMP/AA was disappointingly low.

In this study, we developed novel cross-linked DMP spherical copolymers having N, N'-buthylene-bis-methacrylamide (BBMA) or divinylbenzene (DVB) as a cross-linking agent. BBMA and DVB are more hydrophobic cross-linking agents than AA. To increase the LPS-adsorbing activity, it was necessary to increase not only the cationic property but also the hydrophobic property of the adsorbent, because LPS is an amphipathic substance having both anionic and hydrophobic regions.[7] This report describes the synthesis of DMP/BBMA and DMP/DVB adsorbents by one-step polymerization. It then considers the hydrophobic effect of the cross-linking agent and the amino-group content on the selective removal of LPS from various protein solutions by DMP/BBMA and DMP/DVB adsorbents.

EXPERIMENTAL

Materials

Purified LPS (*Escherichia coli* UKT-B and O111:B4) were purchased from Wako Pure Chemical Ind. Ltd., Osaka (Japan). Limulus ES-II test Wako (Limulus amoebocyte lysate) was a product of Wako. DMP, AA, and BBMA monomers were kindly supplied by Kohjin Co., Ltd., Tokyo (Japan). DMP and AA were purified by vacuum distillation at 131°C / 1 mmHg and 115°C / 0.7 mmHg, respectively. DVB (Wako) was purified by extraction with 0.1 M sodium hydroxide. The structures of monomers used are shown in Figure 1. The histidine-immobilized Sepharose (Pyro Sep-C) was purchased from Daicel, Tokyo (Japan) and used as a standard adsorbent. Proteins were purchased from Nacalai Tesque, Kyoto (Japan). LPS-free water was kindly supplied by Chemo-Sero-Therapeutic Research Institute, Kumamoto (Japan).

Preparation of DMP/BBMA Adsorbents

DMP, BBMA, 20 vol-% of chloroform as a diluent, and 2 wt-% azobisisobutyronitrile as an initiator were mixed at room temperature. The mixture was added to a 25 wt-% anhydrous sodium sulfate solution containing 1% sodium carboxymethyl cellulose (Figure 1). The suspension was stirred and heated at 80°C for 20 h. The DMP/BBMA copolymer particles obtained were washed successively with cold and hot water, methanol, and ethanol. These particles with diameters of 44 to 105 μm were used as adsorbents.

Preparation of DMP/DVB Adsorbents

DMP, DVB, 20 vol-% of diethylbenzene as a diluent, and 2 wt-% azobisisobutyronitrile as an initiator were mixed at room temperature. Using the mixture obtained, the DMP/DVB adsorbents were prepared by the method used for DMP/BBMA (Figure 1).

Preparation of Other Adsorbents

DMP/AA copolymer particles were prepared by one-step polymerization as reported before.[6] PMLG-NH NH_2 spherical particles were prepared by the suspension-evaporation method[8] and aminolysis[3] with diaminoethane as described previously.

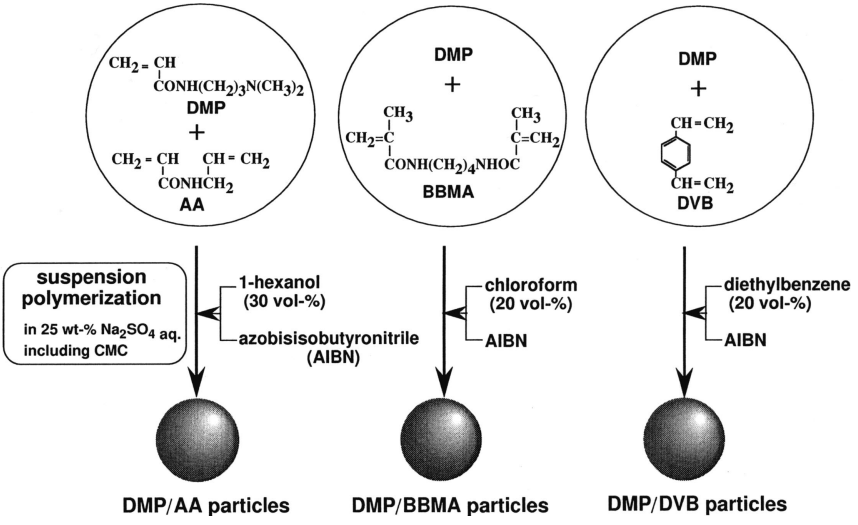

Figure 1. Preparation of various cross-linked DMP spherical particles.

Determination of Pore Size, Hydrophobicity, and Amino-Group Contents

The pore size (molecular mass exclusion, M_{lim}) of the matrix in the adsorbent was estimated from calibration curves by aqueous size exclusion chromatography (SEC) as previously described.[9,10] The particles used as adsorbents were packed into a stainless column (4.6 X 100 mm). The chromatograph was equipped with a JASCO 880-PU pump and a Shodex refract monitor SE-51. Pullulan standards were used as permeable substances. The hydrophobicity of the adsorbent was estimated from the elution behavior of 1-alcohol obtained by SEC with the adsorbent-packed column as previously described.[11] 1-Methanol, 1-ethanol, 1-propanol, and 1-butanol were used as permeable substances. The amino groups were quantified by pH titration and elemental analysis.

Adsorption of LPS and Other Substances

LPS was dissolved in the following buffers: 0.02 M sodium acetate (pH 4, 5); 0.02 M phosphate (pH 6, 7, 8); and 0.02 M Tris (pH 9, 10). The ionic strength of the buffer was adjusted by changing the sodium chloride concentration. The adsorption of endotoxin was measured by a batchwise method as follows: the adsorbent was washed and equilibrated with one of various buffers with different ionic strengths. A 0.1- to 0.4-g portion of wet adsorbent was suspended in 2 to 4 mL of an LPS solution. The suspension was shaken for 2 h at

25°C and filtered through a Millipore filter (0.8 μm) to remove the adsorbent. The filtrate was measured for LPS content. The apparent dissociation constant ($K_{d,app}$) between LPS and adsorbent was estimated by adsorption isotherm as previously described.[5,12,13] The adsorption of other substances was estimated by the method used for LPS-binding assay. The adsorbing activities for LPS and other substances were determined as the average of five measurements.

LPS and Protein Assay

LPS was assayed by the Limulus test involving turbidimetric time assay at 660 nm with a Toxinometer ET-201 (Wako).[14] Purified LPS (*E. coli* UKT-B) was used as the standard. Limulus ES-II test Wako was used as the reagent for the reaction. The protein concentration was measured at 280 nm with a Spectrophotometer UV-160 (Shimadzu).

RESULTS AND DISCUSSION

Preparation of Adsorbents

We reported previously that acidic protein, such as BSA, was hardly adsorbed onto DMP/AA particles when the M_{lim} of the particles was below the molecular weight of the protein.[6] This result showed that adsorption of BSA was caused mainly by entry of the BSA into the pores of the particles. On the other hand, it was clear that the particles showed good LPS-adsorbing activity even at a small M_{lim} of 2,000 (as a molecular weight of polysaccharide).[6] These results[3,6] suggest that the cross-linked DMP particles with a small pore size would adsorb LPS selectively from protein solution. The pore size (M_{lim}) and amino-group content were easily adjusted by changing the diluent ratio and DMP (monomer) ratio, respectively, in the suspension for copolymerization. Therefore, as shown in Table 1, various cross-linked DMP adsorbents with a pore size of M_{lim} 2,000 and an amino-group content of 2.5 to 5.1 meq g^{-1} were prepared. The amino-group content of the various copolymer particles prepared was independent of the molar ratio of DMP in polymerization, because the cross-linking agents used (AA, BBMA, and DVB) have a different molecular weight. When the molar ratio of DMP (in polymerization) increased from 50 to 80%, the amino-group content increased from 3.6 to 5.1 meq g^{-1} in DMP/AA, from 2.5 to 4.0 meq g^{-1} in DMP/BBMA, and from 3.2 to 5.0 meq g^{-1} in DMP/DVB.

Figure 2 shows the elution behaviors of 1-alcohols determined by SEC with the various copolymer columns. The cross-linked DMP particles with 50% of the molar ratio of cross-linking agents (DMP/AA-3.6, DMP/BBMA-

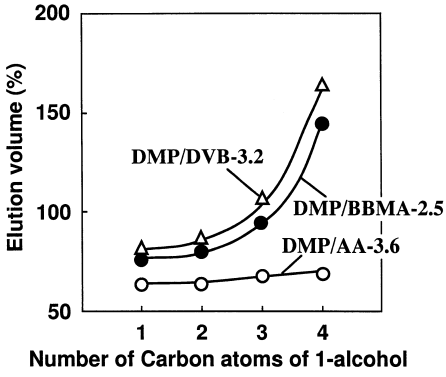

Figure 2. Elution behaviors of 1-alcohols with various cross-linked DMP columns (DMP/AA-3.6, DMP/BBMA-2.5, and DMP/DVB-3.2). A 5-μL portion of 1-alcohol dissolved in water (2 mg mL^{-1}) was injected to the column (100 X 4.6 mm I.D.) at 0.5 mL min^{-1} and at 25°C. The elution volume of 1-alcohol is expressed as a percentage of the column volume. Number of carbon atoms of 1-alcohol: 1 = methanol; 2 = ethanol; 3 = propanol; 4 = butanol. Pore size and content of the cross-linking agent of the adsorbent: M_{lim} 2 X 10^3 and 50 mol-%.

Table 1

Properties of Cross-Linked DMAPAA Adsorbents

Adsorbent	Molar Ratio (mol-%) DMP	AA	BBBA	DVB	Amino-Group Content[a] Meq g^{-1}	S_d[b] Wet-mL Dry-g^{-1}
DMP/AA-3.6	50	50	0	0	3.6	2.7
DMP/AA-4.1	60	40	0	0	4.1	4.0
DMP/AA-4.5	70	30	0	0	4.5	4.3
DMP/AA-5.1	80	20	0	0	5.1	7.9
DMP/BBMA-2.5	50	0	50	0	2.5	2.2
DMP/BBMA-3.1	60	0	40	0	3.1	2.5
DMP/BBMA-3.7	70	0	30	0	3.7	3.2
DMP/BBMA-4.0	80	0	20	0	4.0	3.8
DMP/DVB-3.2	50	0	0	50	3.2	2.5
DMP/DVB-4.0	60	0	0	40	4.0	3.5
DMP/DVB-5.0	80	0	0	20	5.0	4.0

[a] Content of amino groups in the adsorbent. [b] Degree of swelling in water.

2.5, and DMP/DVB-3.2) (Table 1) were used in the columns. The hydrophobicity of 1-alcohol increases with the increase in its carbon number from 1 (methanol) to 4 (butanol).[11] The elution volume of 1-alcohol increases with the increase in the hydrophobicity of the adsorbent, and the difference in the elution volume between 1-methanol and 1-butanol ($V_{BuOH-MeOH}$) also increases. The DMP/BBMA and the DMP/DVB columns always showed larger elution volumes of all 1-alcohols than the DMP/AA column. This result indicates that the adsorbent became hydrophobic on introduction of BBMA or DVB.

Effects of Various Factors on Selective Adsorption of LPS

For selective adsorption of LPS, it is necessary to check the interaction between the adsorbent and cell product (LPS, protein, and nucleic acids). The adsorbing activities of LPS and other cell products were examined by a batch-wise method with various kinds of buffers. The purified LPS (from *E. coli* O111:B4) was used as a standard LPS sample, and the various cross-linked DMP particles (DMP ratio: 50 - 80 unit-mol%) were used as adsorbents.

Figure 3a, b, c, and d show the effect of amino-group content of the adsorbent on the adsorption of LPS, BSA, γ-globulin, and cytochrome *c* at pH 7.0 and an ionic strength of $\mu = 0.05$. As shown in Figure 3a, the LPS-adsorbing activity of all adsorbents increased with an increase in the amino-group content. DMP/BBMA and DMP/DVB, with a high amino-group content (4.0 to 5.1 meq g^{-1}), showed excellent LPS-adsorbing activity (99%). DMP/AA had relatively poor LPS-adsorbing activity (34 to 86%) at the amino-group content of 3.6 - 5.1 meq g^{-1}. The BSA-adsorbing activity also increased with an increase in the amino-group content, as shown in Figure 3b. The BSA-adsorbing activity of DMP/DVB remarkably increased from <1 to 68% with an increase in the amino-group content from 4.0 to 5.0 meq g^{-1}. And that of DMP/AA increased from <1 to 44% with an increase in the amino-group content from 4.1 to 5.1 meq g^{-1}. The BSA-adsorption of all adsorbents was <1% at 2.5 - 4.0 meq g^{-1}. By contrast, the adsorption of γ-globulin by DMP/BBMA and DMP/DVB increased with a decrease in the amino-group content (with an increase in the content of cross-linking agent) (Figure 3c): the adsorption by DMP/BBMA increased from <1 to 20% with a decrease in the amino-group content from 4.0 to 2.5 meq g^{-1}. As shown in Figure 3d, cytochrome *c* was adsorbed only by DMP/DVB: its adsorption increased from 20 to 56% with a decrease in the amino-group content from 5.0 to 3.2 meq g^{-1} (with an increase in the content of DVB). The other adsorbents (DMP/AA and DMP/BBMA) hardly adsorbed cytochrome *c* (<1%) regardless of amino-group content.

The effect of ionic strength on LPS adsorption by various cross-linked DMP adsorbents was examined in phosphate buffer (pH 7.0, an ionic strength of $\mu = 0.05$ to 4.0), and the result is shown in Figure 4. DMP/BBMA and

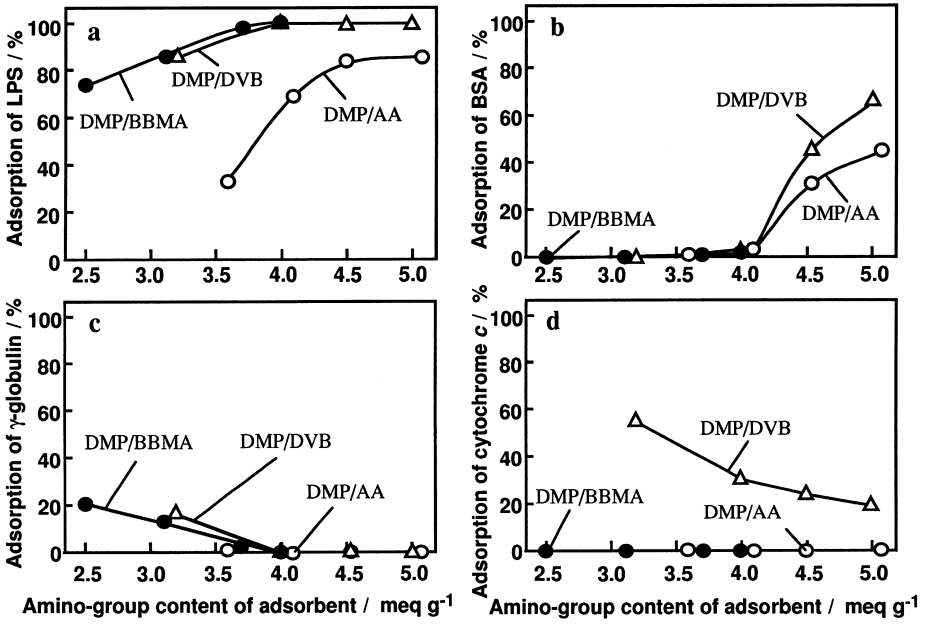

Figure 3. Effects of amino-group content of the adsorbent on adsorption of a cell product (LPS or protein) by various cross-linked DMP adsorbents. The adsorption of a cell product was determined by a batchwise method with 0.2 g of the wet adsorbent and 2 mL of a cell product solution (100 μg mL^{-1}, pH 7.0, ionic strength of μ = 0.05).

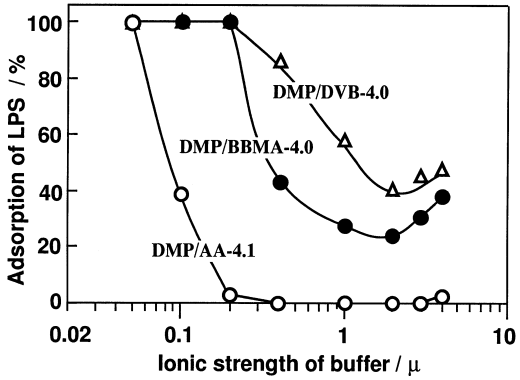

Figure 4. Effect of ionic strength on adsorption of LPS by various cross-linked DMP adsorbents. The adsorption of LPS was determined by a batchwise method with 0.2 g of the wet adsorbent and 2 mL of a LPS solution (1 μg mL^{-1}, pH 7.0, μ = 0.05 - 4.0). Amino-group content of adsorbent: 4.0 - 4.1 meq g^{-1}.

DMP/DVB showed strong LPS-adsorbing activity (99%) at a low ionic strength (μ = 0.05 to 0.2). When the ionic strength (μ) was increased from 0.2 to 2.0, the activity of each adsorbent decreased. However, when the ionic strength (μ) was higher than 2.0, the activity of each adsorbent increased only slightly. DMP/DVB-4.0 showed the highest LPS-adsorbing activity (99 to 48%) at an ionic strength of μ = 0.05 to 4.0. The activity of DMP/AA-4.1 decreased markedly when the ionic strength was increased to μ = 0.1 or higher.

The effect of pH on LPS adsorption by various cross-linked DMP adsorbents was examined in phosphate buffer (pH 4.0 to 10.0, an ionic strength of μ = 0.05). As shown in Figure 5, DMP/DVB-4.0 and DMP/BBMA-4.0 showed strong LPS-adsorbing activity (73 - 99%) over a wide pH range from 4.0 to 10.0. The highest level of activity was shown by DMP/DVB-4.0 at all pH values. On the other hand, DMP/AA-4.1 showed good LPS adsorption (>80%) only at pH 5 to 8.

Table 2 shows the adsorption activities of the cell products by the various adsorbents. The cross-linked DMP adsorbents with an amino-group content of 4.0 - 4.1 meq g^{-1}, which have high selectivity for LPS but do not adsorb BSA and γ-globulin (as shown in Figure 3a ,b and c), were used. The binding force of the adsorbents to the LPS was stronger than that to the protein (pI: 4.6 - 10.6): the proteins (pI: 4.6 - 10.6) were hardly adsorbed to DMP/AA-4.1 and DMP/BBMA-4.0 at pH 7.0 and μ = 0.05. However, under these conditions, only DMP/DVB adsorbed myoglobin (22%) and cytochrome *c* (22%).

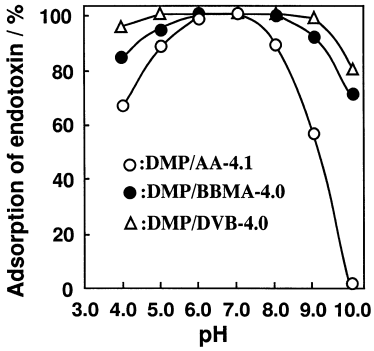

Figure 5. Effect of pH on adsorption of LPS by various cross-linked DMP adsorbents. The adsorption of LPS was determined by a batchwise method with 0.2 g of the wet adsorbent and 2 mL of a LPS solution (1 μg mL^{-1}, pH 4.0 - 10.0, μ = 0.05). Amino-group content of adsorbent: 4.0 - 4.1 meq g^{-1}.

Table 2

Adsorption of Various Cell Products by Cross-Linked DMP Adsorbents

Cell Product	pI	Adsorption[a] (%) DMP/AA-4.1	DMP/BBMA-4.0	DMP/DVB-4.0
Pepsin	<1	81	85	90
BSA	4.9	3	2	2
Insulin	5.3	5	4	3
Myoglobin	5.5	<1	<1	22
γ-globulin	6.8	2	3	2
Cytochrome C	10.6	<1	<1	34
DNA		70	83	70
RNA		68	78	68
E. coli O111:B4 LPS		99	99	99
E. coli UKT-B LPS			98	99
S. typhosa 0901 LPS		98	99	99

[a] The adsorption of cell product was determined by a batchwise method with 0.2 mL of wet adsorbent and 2 mL of a cell product solution (100 μg/mL, pH 7.0, $\mu = 0.05$).

Figures 6a, b, c, and d show the effects of ionic strength on the selective removal of LPS from a BSA-containing solution with various adsorbents. A BSA solution (500 μg mL^{-1}) to which was added purified LPS (100 ng mL^{-1}) was used as a sample solution at pH 7.0 and various ionic strengths. As shown in Figure 6-a, DMP/BBMA selectively adsorbed LPS from the BSA solution at an ionic strength of $\mu = 0.05$ to 0.2: the adsorption of LPS was >99% (the residual concentration of LPS after treatment was <100 pg mL^{-1}) and that of BSA was <2%. DMP/DVB also selectively adsorbed LPS under similar conditions (Figure 6-b). PMLG-NH_2 selectively adsorbed LPS at $\mu = 0.2$ to 0.4 (Figure 6c). By contrast, the histidine-immobilized Sepharose, which is commercially sold as LPS adsorbent,[12,13] had strong adsorbing activities for both LPS and BSA at low ionic strength, $\mu = 0.05$, and each adsorbing activity decreased remarkably with increase in ionic strength (Figure 6d). The histidine-immobilized Sepharose, therefore, could not selectively remove LPS from a BSA-LPS solution at any ionic strength. DMP/AA had relatively poor LPS-adsorbing activity (81 - 62%) at all ionic strengths although its recovery of BSA was high (>98%) (data not shown).

From these results (Figures 2-6 and Table 2), we assumed that the adsorbing-activities of the cross-linked DMP adsorbents for LPS and protein were due to both a cationic property which originated from DMP and a hydrophobic or

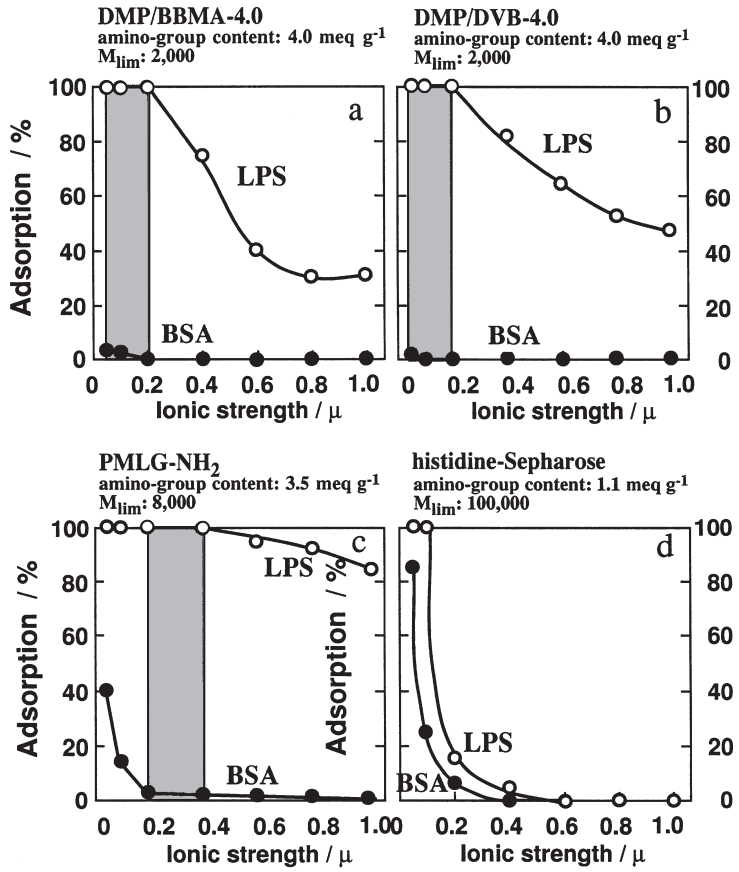

Figure 6. Effect of ionic strength on removal of LPS from a BSA solution (BSA: 500 μg mL^{-1}, LPS (*E. coli* O111:B4): 100 ng mL^{-1}, pH: 7.0) by various adsorbents. The removal of LPS was determined by a batchwise method with 0.2 mL of the wet adsorbent and 2 mL of a sample solution.

other property which originated from the cross-linking agent. LPS is an amphipathic substance having both anionic (phosphate groups) and hydrophobic regions (lipophilic groups),[7] and this characteristic also suggests that the LPS-adsorbing activity of the adsorbent was due to both effects. As shown in Figures 3a, 4, and 5, the LPS-adsorbing activity of various cross-linked DMP adsorbents was dependent on amino-group content, ionic strength (at $\mu = 0.05$ - 2.0), and pH (at pH 4.0 - 10.0). These results suggest a cationic interaction.

Furthermore, DMP/DVB and DMP/BBMA similarly adsorbed LPS (40 to 45%) at a high ionic strength of $\mu = 4.0$ (Figure 4). Matsumae et al.[12] reported that a hydrophobic bond was formed between LPS and histidine-immobilized Sepharose under the conditions of high ionic strength ($\mu > 3$). It was found that DMP/DVB and DMP/BBMA were more hydrophobic adsorbents than DMP/AA (Figure 2). These results suggest that DMP/DVB and DMP/BBMA adsorb LPS also by hydrophobic effects which originate from the cross-linking agent (DVB and BBMA).

The charge of the protein is anionic at a pH over the pI value and cationic at a pH under it. Therefore, the charge of BSA is anionic at pH values greater than 4.9 (its pI), and also the adsorption of BSA by various cross-linked DMP adsorbents is dependent on amino-group content, as shown in Figure 3b. This suggests ionic interaction between the adsorbent and BSA. On the other hand, the ionic interaction of the adsorbent with γ-globulin (pI 7.4) and cytochrome *c* (pI 10.6) is not induced at pH 7.0 (lower than their pI). However, γ-globulin and cytochrome *c* were adsorbed by the cross-linked DMP, as shown in Figure 3c and d. γ-Globulin is a weak hydrophobic protein, and its adsorption by DMP/BBMA and DMP/DVB increased with an increase in the hydrophobicity (content of cross-linking agent) of the adsorbent (Figure 3c). These findings suggest the participation of hydrophobic binding. Cytochrome *c* and myoglobin are aromatic proteins, and only DMP/DVB, which has an aromatic property originating from DVB, adsorbed both: the cytochrome *c* adsorption increased with an increase in the content of DVB (with a decrease in the amino-group content) of the adsorbent, as shown in Figure 3d. These findings suggest π–π interaction between the aromatic protein and the aromatic adsorbent. It is also suggested that the binding force of the adsorbent to the LPS is stronger than that to the protein (pI: 4.6-10.6) (Table 2), because the LPS have a lower pK_a than the protein.

On the other hand, we previously reported that the adsorption of BSA is dependent on the pore size of the matrix.[3,5] DMP/BBMA and DMP/DVB, which have a small pore size (M_{lim}: 2,000), selectively adsorbed LPS from the BSA solution at an ionic strength of $\mu = 0.05$ to 0.2: little BSA was adsorbed by each adsorbent at any ionic strength (Figures 6a and b). We previously reported that LPS cannot enter pores with a M_{lim} <400,000, because it forms a supermolecular aggregate (M_{lim} >1,000,000).[3] LPS, however, is adsorbed very efficiently by the cross-linked DMP (M_{lim}: 2,000). Therefore, we assume that LPS is adsorbed also at the surface of the adsorbent, but BSA is not. By contrast, the adsorbing activities of PMLG-NH_2 (M_{lim}: 8,000) and the histidine-immobilized Sepharose (M_{lim}: 100,000), which have a large pore size, were strong for both LPS and BSA at $\mu = 0.05$ (Figures 6c and d). These results show that the adsorption of BSA is caused by its entry into the pores of each adsorbent. It is suggested that DMP/BBMA-4.0 and DMP/DVB-4.0 bind LPS more

strongly than BSA probably because the adsorbent can adsorb LPS not only through cationic properties but also through hydrophobic properties, and also because LPS have a lower pK_a and greater hydrophobicity than BSA.

Figures 6a, b, c, and d show that it is effective for the selective adsorption of LPS to adjust the amino-group content of the adsorbent to 4.0 meq g^{-1} and its pore size (M_{lim}) to 2,000, and to control its hydrophobicity. However, it is difficult to adjust the M_{lim} of the adsorbent when the ligand is introduced to the matrix already prepared, because the introduction of ligands such as amino groups often increases the degree of swelling of the adsorbent. For example, we observed that the M_{lim} increased from <500 to 8,000 on introducing 3.5 meq g^{-1} of amino groups in amination of PMLG particles. In contrast, cross-linked DMP particles were prepared by one-step polymerization without amination; therefore it was easy to adjust the M_{lim}.

These results (Figures 2-6, Table 2) suggest that only DMP/BBMA-4, which is non-aromatic and weakly hydrophobic, can adsorb LPS selectively without affecting protein recovery. And it was also found that DMP/DVB with aromatic properties originating from DVB cannot selectively remove LPS from an aromatic protein solution.

Dissociation Constant Between LPS and Adsorbent

When removing LPS from a solution by adsorption, it is necessary to check not only the adsorption capacity but also the dissociation constant (K_d) between LPS and adsorbent. The LPS-adsorption capacity and the apparent dissociation constants ($K_{d,app}$) of the DMP/BBMA-4.0 adsorbent was compared with those of other adsorbents (Table 3). The adsorption isotherms of LPS for various adsorbents were determined by a batchwise method in a phosphate buffer (pH 7.0, $\mu = 0.05$) by changing the concentration of LPS. The LPS-adsorption capacities and the apparent dissociation constants ($K_{d,app}$) were estimated based on the adsorption isotherms obtained, as previously described.[11,12] As shown in Table 3, PMLG-NH_2 had the greatest LPS-adsorption capacity of all the adsorbents. The adsorption capacity of DMP/BBMA (3.8×10^2 μg mL^{-1}) was similar to that of DMP/AA-4.1 (3.6×10^2 μg mL^{-1}) and DMP/DVB-4.0 (3.9×10^2 μg mL^{-1}). DMP/DVB-4.0 had the smallest $K_{d,app}$ value: the $K_{d,app}$ (4.5×10^{-11} M) was about 1/6 as large as that of DMP/AA. In the removal of LPS from a dilute LPS solution, the LPS-removing activity depends on the $K_{d,app}$ more than the adsorbing capacity. The smaller the K_d values between the LPS and the adsorbent, the stronger the removing activity.[5] The $K_{d,app}$ of DMP/BBMA-4.0 (7.2×10^{-11} M) was about 1/4 of DMP/AA, and was similar to that of PMLG-NH_2 (7.9×10^{-11} M). As a result, it was found that the LPS-

Table 3

LPS-Adsorption Capacity and Apparent Dissociation Constant Between Adsorbent and LPS*

Adsorbent	Amino-Group Content Meq. g^{-1}	Pore Size M_{lim}	Adsorption Capacity of LPS μg mL^{-1} Adsorbent	$K_{d,app}$ of LPS M
DMP/AA-4.1	4.1	2×10^3	3.6×10^2	2.7×10^{-10}
DMP/BBMA-4.0	4.0	2×10^3	3.8×10^2	7.2×10^{-11}
DMP/DVB-4.0	4.0	2×10^3	3.9×10^2	4.5×10^{-11}
PMLG-NH_2	3.5	8×10^3	1.6×10^3	7.9×10^{-11}

* The LPS-adsorption capacity per mL adsorbent and the apparent dissociation constant ($K_{d,app}$) of LPS to adsorbent were estimated by adsorption isotherm, as described previously.[11,12] The adsorption isotherm of LPS was determined by a batchwise method with 0.1 mL of wet adsorbent and 4 mL of a LPS solution (from *E.coli* O111:B4 ($\overline{M}_n$: 1×10^6): 1 to 2 x 10^2 μg/mL, pH 7.0 μ = 0.05). The $K_{d,app}$ was expressed in mol L^{-1} (M) of molecular weight of LPS.

removing activity of DMP/BBMA-4.0 is superior to that of DMP/AA-4.1 although its activity is not much stronger than that of DMP/DVB-4.0.

Selective Removal of LPS from Various Protein Solutions

The LPS-removing activity of DMP/BBMA-4.0 was compared with that of DMP/DVB-4.0. The results are shown in Table 4. Various protein solutions, which were naturally contaminated with LPS at concentrations from 350 to 42,000 pg mL^{-1} were used as samples. It is essential to eliminate LPS to at least a concentration lower than 100 pg mL^{-1} from fluids used for intravenous injection,[12] since it elicits pyrogenic and shock reactions in mammals.[1,2] As shown in Table 4, each adsorbent was able to remove LPS from all protein solutions to a level below 100 pg mL^{-1}. The removing activity of DMP/DVB-4.0 was always higher than that of DMP/BBMA-4.0. The activity of DMP/DVB-4.0, however, was also high for aromatic protein such as myoglobin and cytochrome *c*. The recoveries of the proteins were 82 and 78%, respectively. In contrast, DMP/BBMA-4.0 showed high recoveries (97 to 99%) of all protein as well as efficient removal of LPS at pH 7.0 and μ = 0.05. For practical application, ease of regeneration is very important, and we reported previously that the cross-linked DMP column packings can be completely regenerated by frontal chromatography with 0.2 M sodium hydroxide followed by 2.0 M sodium chloride.[6]

Table 4

Removal of LPS from a Protein Solution by DMP/BMA-4.0 and DMP/DVB-4.0 Adsorbents*

		Sample Solution	DMP/BBMA-4.0 Adsorbent		DMP/DVB-4.0 Adsorbent	
Compound	pI	Conc. of LPS Before Treatment pg mL^{-1}	Conc. of LPS After Treatment pg mL^{-1}	Rec. of Protein After Treatment %	Conc. of LPS After Treatment pg mL^{-1}	Rec. of Protein After Treatment %
BSA	4.9	42000	85	98	15	99
Insulin	5.3	3800	55	97	22	96
Myoglobin	7.4	1200	<10	99	47	82
γ-Globulin	8.4	5800	34	98	8	96
Cytochrome C	10.6	350	<10	99	25	78

* The removal of LPS was determined by a batchwise method with 0.2 mL of wet adsorbent and 2mL of a protein solution containing natural LPS (protein: 1 mg mL^{-1}, pH 7.0, $\mu = 0.05$). Amino-group content of adsorbent: 4.0 meq g^{-1}.

In conclusion, the present results suggest that DMP/BBMA can reduce the concentration of LPS in drugs and fluids for injection to 100 pg mL^{-1} or lower, at an ionic strength of $\mu = 0.05 - 0.2$ and a neutral pH. This process did not affect the recovery of important compounds such as protein. This high LPS-selectivity of DMP/BBMA is due to:

(1) the simultaneous effect of the cationic properties of DMP and hydrophobic properties of BBMA;

(2) the exempting effects on protein molecules when a non-aromatic substance such as BBMA is used as a cross-linking agent and also the M_{lim} is adjusted to 2,000.

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