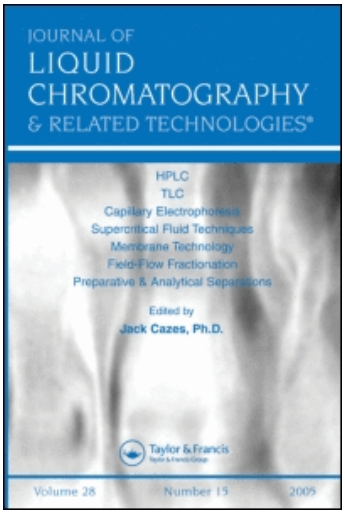

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 17 April 2002

To cite this Article Todokoro, Masami , Sakata, Masayo , Matama, Satoshi , Kunitake, Masashi , Ohkuma, Kunio and Hirayama, Chuichi(2002) 'PORE-SIZE CONTROLLED AND POLY(ε-LYSINE)-IMMOBILIZED CELLULOSE SPHERICAL PARTICLES FOR REMOVAL OF LIPOPOLYSACCHARIDES', Journal of Liquid Chromatography & Related Technologies, 25: 4, 601 — 614
To link to this Article: DOI: 10.1081/JLC-120008814
URL: http://dx.doi.org/10.1081/JLC-120008814

J. LIQ. CHROM. & REL. TECHNOL., 25(4), 601–614 (2002)

PORE-SIZE CONTROLLED AND POLY(ε-LYSINE)-IMMOBILIZED CELLULOSE SPHERICAL PARTICLES FOR REMOVAL OF LIPOPOLYSACCHARIDES

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ABSTRACT

Poly(ε-lysine) was covalently immobilized onto cellulose spherical particles and used for selective adsorption of pyrogenic lipopolysaccharides (LPS) from protein solutions. The resulting poly(ε-lysine)-immobilized cellulose particles (PL-cellulose), which had diameters of 44 to 105 μm and matrix's pore-sizes of 2×10^3, 1×10^4, and $>2 \times 10^6$ as molecular mass exclusions (M_{lim}), were used as adsorbents. The adsorption of LPS and protein to the adsorbent were determined using a batchwise

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method. The larger the pore size (M_{lim}) of the adsorbent, the larger is the LPS-adsorbing activity of the adsorbent.

The apparent dissociation constant between the LPS (*E. coli* O111:B4) and the adsorbent decreased from 3.8×10^{-10} to 1.1×10^{-11} M with an increase in the M_{lim} from 2×10^3 to $>2 \times 10^6$ at an ionic strength of $\mu = 0.05$ and a pH of 7.0. On the other hand, the adsorbing activity of bovine serum albumin also increased with the increasing M_{lim} of the adsorbent, but sharply decreased with increasing ionic strength of the buffer.

When PL-cellulose-10^3, having a small M_{lim} of 2×10^3, was used as the adsorbent at a wide ionic strength of $\mu = 0.05$ to 0.4 and a pH of 7.0, it selectively reduced LPS in various protein solutions, including 1 mg mL^{-1} of protein and 1500 to 32,000 pg mL^{-1} of natural LPS. When PL-cellulose-10^6, having a large M_{lim} of over 2×10^6, was used as the adsorbent, it only selectively removed LPS at a high ionic strength of $\mu = 0.4$ and a pH of 7.0. The LPS-removing activity of PL-cellulose-10^6 was always stronger than that of PL-cellulose-10^3. PL-cellulose-10^6 decreased the concentration of LPS in each protein solution to less than 10 pg mL^{-1}. On the other hand, the recovery rate (99%) of protein for PL-cellulose-10^3 was higher than that for PL-cellulose-10^6 (96 to 97%) in all cases.

INTRODUCTION

Cellular products, such as proteins and other biologically active substances, are usually contaminated with lipopolysaccharide (LPS) (1,2). LPS, originating from cell walls of gram-negative bacteria, consists of a polysaccharide and a terminal Lipid A moiety. Because of the potent biological activities of Lipid A that cause pyrogenic and shock reactions in mammals, LPS has to be reduced to lower than 100 pg mL^{-1} in those cellular products used as drugs administered by intravenous injection (3).

Recently it has been suggested that the removal of LPS is extremely difficult when the LPS is associated with the protein to be purified (3,4). Already, various cationic polymer adsorbents, such as polymyxin-immobilized Sepharose (4,5) and histamine-immobilized (6) or histidine-immobilized Sepharose (3,7), have been used for removing LPS from protein solutions. However, it has been reported (7,8) that these adsorbents cannot selectively remove LPS from acidic protein solutions, such as bovine serum albumin (BSA), because of the high adsorption for both LPS and BSA. We previously reported that cross-linked *N,N*-dimethylaminopropylacrylamide particles (cross-linked DMP) (8,9) can selec-

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tively adsorb LPS from a BSA solution, but only at a low ionic strength, $\mu = 0.05$. However, their LPS-adsorbing activities were not satisfactorily high at ionic strengths higher than $\mu = 0.05$. Antigenic proteins sometimes aggregate themselves during purification at an ionic strength as low as $\mu = 0.02$ to 0.1. Therefore, the development of novel LPS adsorbents capable of retaining high selectivity for LPS under physiological conditions (a wide ionic strength of $\mu = 0.05$ to 1.0 and neutral pH) is keenly pursued.

To increase LPS-binding of adsorbents, it is necessary to increase not only the cationic properties of adsorbents but also their hydrophobic ones and pore sizes. This is because, in an aqueous solution, LPS forms a super molecular aggregate (10) of M_w 4×10^3 to 1×10 (6) which has both an anionic region (the phosphoric acid groups) and a hydrophobic region (the lipophilic groups) (2). Thus, we developed new cationic adsorbents having poly(ε-lysine) as the ligand and cellulose spherical particles as the matrix. Polymyxin B and histamine, which have been used as ligands, are biologically active substances and thus are not suitable as ligands for removing LPS from an injection solution (7). By contrast, poly(ε-lysine) (11,12) produced by *Streptomyces albulus*, which has become commercially available as a safe food preservative, is more suitable as a ligand. For the matrix, we chose various cellulose spherical particles (13,14) with pore sizes (molecular mass exclusion, M_{lim}) of 2×10^3, 1×10^4 or $>2 \times 10^6$.

In the paper, we describe the effects of the pore size of adsorbents and ionic strength of the buffer on the adsorption of purified LPS by various poly(ε-lysine)-immobilized cellulose adsorbents having a range of pore sizes. We also describe the application of adsorbents to the removal of natural LPS associated with various water-soluble proteins under conditions of wide ionic strengths and neutral pH.

EXPERIMENTAL

Materials

A 25 wt-% poly(ε-lysine) aqueous solution, produced by *Streptomyces albulus*, and various Cellulose particles (Cellufine-GC15 (13), -GC700 (13), and -CPC (14)) were obtained from Chisso Co. Ltd. (Tokyo, Japan). The number-average molecular weight (M_n) of the poly(ε-lysine) was about 4.0×10^4 (degree of polymerization: 35, pK_a: 7.6) (11,12) Detoxi-Gel polymyxin-Sepharose) (4,5) was purchased from Pierce Chemical (Rockford, U.S.A.). Chloromethyloxirane was purchased from Nacalai Tesque (Kyoto, Japan). Purified LPS (*Escherichia coli* UKT-B) and Limulus ES-II test Wako (Limulus amoebocyte lysate) were purchased from Wako Pure Chemical (Osaka, Japan). Purified LPS from *E. coli* O111:B4 was purchased from Difco Laboratories (Detroit, U.S.A.). Albumin

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(from bovine serum), γ-globulin (from human serum), and cytochrome C (from horse heart) were purchased from Wako. All other chemicals were of analytical reagent grade.

Preparation of Adsorbents

In 150 mL of 3 wt-% sodium hydroxide solution, 10 mL of Cellufine-GC15, -GC700 or -CPC was suspended by stirring at 30°C for 1 h, and 21 mL of chloromethyloxirane then added to the suspension. The mixture was stirred at 30°C for 2 h and the resulting chloromethyloxirane-activated cellulose particles collected and washed with water, and then suspended in 22 mL of the 25 wt-% poly(ε-lysine) aqueous solution. The suspension was stirred at 45°C for 2 h. The resulting poly(ε-lysine)-immobilized cellulose particles (PL-cellulose) were collected and washed with 200 mL of 2 M sodium chloride solution and water. PL-cellulose particles with diameters of 44 to 105 μm were used as adsorbents. The schematic illustration of the preparation of the PL-cellulose adsorbent is shown in Figure 1.

Cross-linked DMP adsorbent was prepared in a suspension polymerization using *N,N*-dimethylaminopropylacrylamide and *N,N*-buthylene-bis-methacrylamide, as previously reported (9).

Determination of Pore Size and Amino-Group Contents

The pore size of the matrix in the adsorbent was estimated as molecular mass exclusion (M_{lim}) from the calibration curves by aqueous size exclusion chromatography as previously described (15). The M_{lim} values were reduced as the molecular mass of polysaccharide. Homogeneous series of the polysaccharides (pullulan and maltose) were used as the permeable substances. The amino-group content of the adsorbents was quantified by pH titration and elemental analyses.

LPS and Protein Assay

LPS was assayed by a Limulus test involving turbidimetric time assay(16) at 660 nm with a Toxinometer ET-201 (Wako). Purified LPS (*E. coli* UKT-B) was used as the standard. Limulus ES-II test Wako was used as reagent for the reaction. Protein concentrations in sample solutions were determined by absorbencies at 280 nm (albumin and γ-globulin) or 410 nm (cytochrome C) using a Spectrophotometer UV-160 (Shimadzu).

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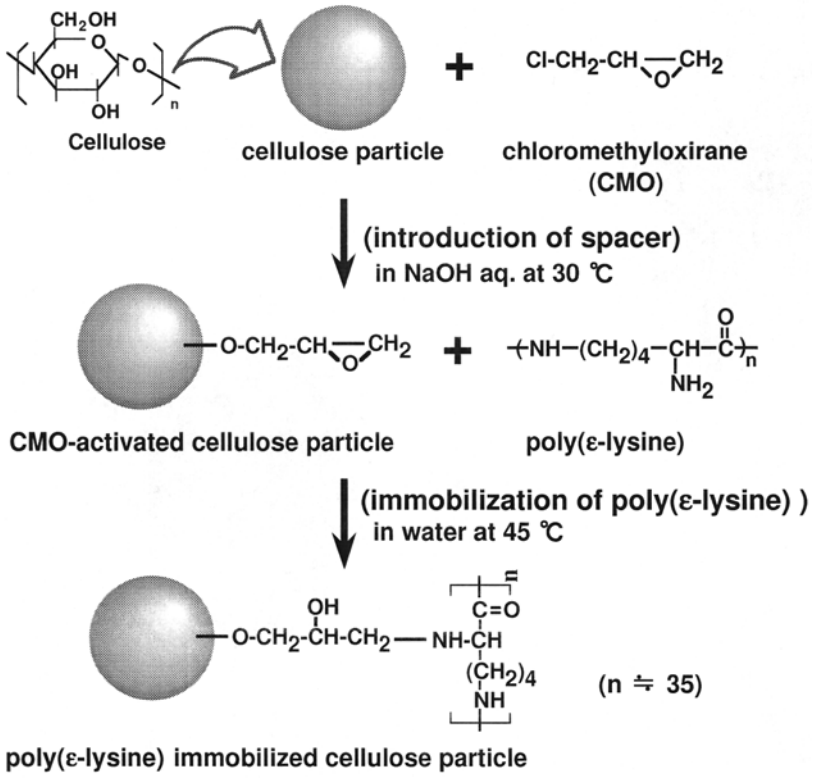

Figure 1. Preparation of poly(ε-lysine) immobilized cellulose particles.

Adsorption of LPS

The adsorption of purified LPS was measured using a batchwise method as follows: the adsorbent was washed and equilibrated with one of the various buffers. As 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) were used. The ionic strength of the buffer was adjusted by changing the sodium chloride concentration and LPS dissolved in the various buffers. A 0.1- to 0.3 mL portion of wet adsorbent was suspended in 2 to 4 mL of LPS solution. The suspension was shaken for 2 h at 25°C and then filtered through a Millipore filter (cellulose acetate, 0.8 μm) to remove the adsorbent from the suspension. The LPS content of the filtrate was then determined. The LPS-adsorbing capacity of the adsorbent and the apparent dissociation constant ($K_{d,app}$) of LPS to the adsorbent were estimated by

adsorption isotherm as previously described (3,8). The adsorption of protein and removal of LPS in protein solution were estimated by the same method as used for the LPS-binding assay.

RESULTS AND DISCUSSION

Effect of Adsorbent Pore Size on Adsorption of LPS

In the removal of LPS from a dilute LPS solution by adsorption, the LPS-removing activity depends on the dissociation constant (K_d) between LPS and the adsorbent more than on the adsorbing capacity. Table 1 shows the effect of the adsorbent pore size (M_{lim}) on the LPS-adsorbing capacity and the apparent K_d ($K_{d,app}$) (3,8). Purified LPS originating from *E. coli* O111:B4 was used for the LPS-containing samples. M_{lim} was reduced as the molecular mass exclusion of polysaccharide, by aqueous size exclusion chromatography as previously described (13). The M_{lim} values of the PL-cellulose adsorbents were adjusted by using a differing range of M_{lim} values for the matrix in the immobilization. We previously reported (8,9) that the LPS-removing activity for cationic adsorbent increased with increases in their amino-group contents when M_{lim} values were adjusted to 2.0×10^3.

As shown in Table 1, the activity of the PL-cellulose adsorbent was strongly dependent on the M_{lim} value when the amino-group content was $0.6\,\text{meq}\,\text{g}^{-1}$: the LPS-adsorbing capacity increased from 185 to 480 μg per mL of wet adsorbent and the $K_{d,app}$ decreased from 3.8×10^{-10} to 1.1×10^{-11} M, while the M_{lim} increased from 2.0×10^3 to $>2 \times 10^6$. The smaller the $K_{d,app}$ of LPS to the adsorbent is, the stronger the LPS-removing activity of the adsorbent (3). As a result, it was found that PL-cellulose-10^6, having the largest M_{lim} of $>2 \times 10^6$, showed the greatest LPS-removing activity.

It would appear that LPS cannot enter the pores of PL-cellulose-10^3 because its molecular weight (M_w 4×10^5 to 1×10^6 as super molecular assemblies) (10) is remarkably larger than the M_{lim} of PL-cellulose-10^3. Much of the standard LPS, however, was well adsorbed even by PL-cellulose-10^3 with M_{lim} 2×10^3, as shown in Table 1. Therefore, we assume that the LPS is adsorbed not only into the pores of adsorbents but also on their surfaces. By contrast, polymyxin-Sepharose (4,5) showed the weakest LPS-removing activity ($K_{d,app}$: 5.0×10^{-9} M) of all the adsorbents, in spite of its largest M_{lim} of $>2 \times 10^6$. This weak LPS-removing activity is probably due to its smallest content of the amino group ($0.2\,\text{meq}\,\text{g}^{-1}$). The $K_{d,app}$ of PL-cellulose-10^3 and cross-linked DMP (8,9) were respectively about 1/13 and 1/18, as large as that of polymyxin-Sepharose, although each had the smallest M_{lim} of 2×10^3. The results suggest

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Table 1. Effect of Pore Size of Adsorbent on Adsorption of LPS

Name	Pore Size of Matrix ML_{im}[a]	Amino-Group Content[b] meq g^{-1}	Adsorbing Capacity[c] of LPS μg mL^{-1} adsorbent	$K_{d,app}$[c] of LPS M
PL-cellulose-10^3	2×10^3	0.6	185	3.8×10^{-10}
PL-cellulose-10^4	1×10^4	1.4	280	5.5×10^{-11}
PL-cellulose-10^6	$>2 \times 10^6$	0.6	480	1.1×10^{-11}
Cross-linked DMP[d]	2×10^3	4.1	360	2.7×10^{-10}
Polymyxin-Sepharose[e]	$>2 \times 10^6$	0.2	250	5.0×10^{-9}

[a]Value deduced as a molecular weight of polysaccharide.
[b]Content of amino groups in the adsorbent.
[c]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 (3,8). The adsorption isotherm of LPS was determined using a batchwise method with 0.1 mL of wet adsorbent and 4 mL of a LPS solution (LPS from *E. coli* O111:B4 (M_n: 1×10^6): 1 to 200 μg mL^{-1}, pH 7.0, $\mu = 0.05$). The $K_{d,app}$ was expressed in mol L^{-1} (M) of molecular weight of LPS.
[d]Spherical copolymers of *N,N*-dimethylaminopropylacrylamide (DMP) and *N,N*-buthylene-bis-methacrylamide (9).
[e]Detoxi-Gel (4,5).

that the LPS-removing activities of PL-cellulose-10^3 and cross-linked DMP are superior to that of polymixin-Sepharose.

Effect of Ionic Strength and pH on Adsorption of LPS

The effect of ionic strength on adsorption of LPS by the various adsorbents was examined at pH 7.0 and $\mu = 0.05$–1.0. As shown in Figure 2, the higher the ionic strength of the buffer, the lower the LPS-adsorbing activity of all cationic adsorbent. By contrast, the activity on Cellufine-GC15 (non-immobilized cellulose particles), which was used as the matrix of PL-cellulose-10^3, increased from 17 to 38% with the increasing of ionic strength from $\mu = 0.05$ to 1.0. Both PL-cellulose-10^6 and -10^3 always showed a greater LPS-adsorbing activity (99 to 92%) at a wide ionic strength of $\mu = 0.05$–0.8. The adsorbing activity of polymyxin-Sepharose and cross-linked DMP decreased markedly when the ionic strength was increased to 0.4 or higher.

The effect of pH on adsorption of LPS by PL-cellulose-10^6 and -10^3, and by polymyxin-Sepharose, are shown in Figure 3. When the ionic strength of the buffer was adjusted to $\mu = 0.05$, PL-cellulose-10^6 with the largest pore size

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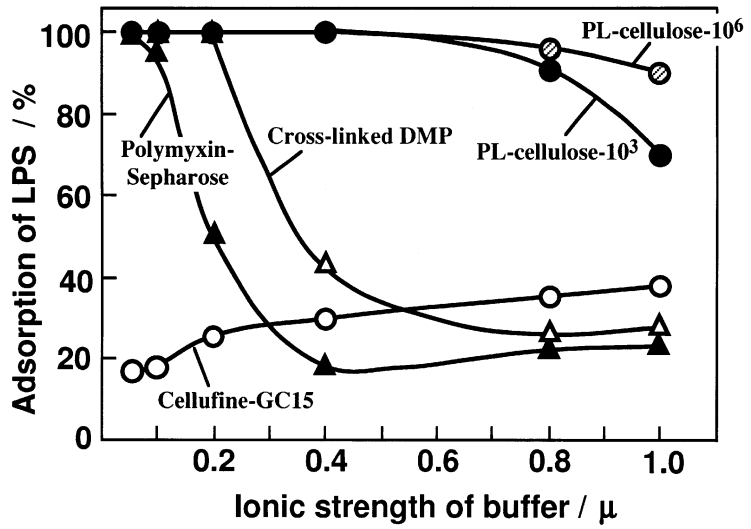

Figure 2. Effect of ionic strength on adsorption of LPS by various adsorbents. The adsorption of LPS was determined using a batchwise method with 0.2 g of the wet adsorbent and 2 mL of a LPS solution (1 μg mL^{-1}, pH 7.0, $\mu = 0.05$–1.0). Cellufine-GC15 particles: Matrix before introduction of poly(ε-lysine).

always showed the highest LPS-adsorbing activity (>98%) at a wide pH range of 4.0 to 9.0. PL-cellulose-10^3 also showed a high activity (>98%) at pH 6.0 to 9.0, although it decreased from 99 to 87% as pH range decreased from 6.0 to 4.0. On the other hand, polymyxin-Sepharose showed high adsorbing activity only at pH 7.0. PL-cellulose-10^4 and cross-linked DMP also showed high activities at pH 6.0 to 9.0, similar to that of PL-cellulose-10^3 (data not shown).

From these results (Figures 2 and 3) we assumed that the LPS-adsorbing activity of PL-cellulose was due to the simultaneous effects of cationic properties originating from ligands, and hydrophobic or other properties originating from the matrix. LPS is an amphipathic substance having both anionic (the phosphate groups) and hydrophobic regions (the lipophilic groups) (2). These characteristics also suggest that the LPS-adsorbing activity of an adsorbent is due to these simultaneous effects. The adsorbing activity of PL-cellulose, being dependent on the ionic strength of the buffer and its pH values, suggests a cationic interaction. Further, the LPS-adsorbing activity of non-immobilized cellulose particles (the matrix of PL-cellulose-10^3) increased with an increasing of the buffer's ionic strength (Figure 2). Matsumae *et al*. (7) reported that a hydrophobic bond was formed between LPS and histidine-immobilized Sepharose, under conditions of

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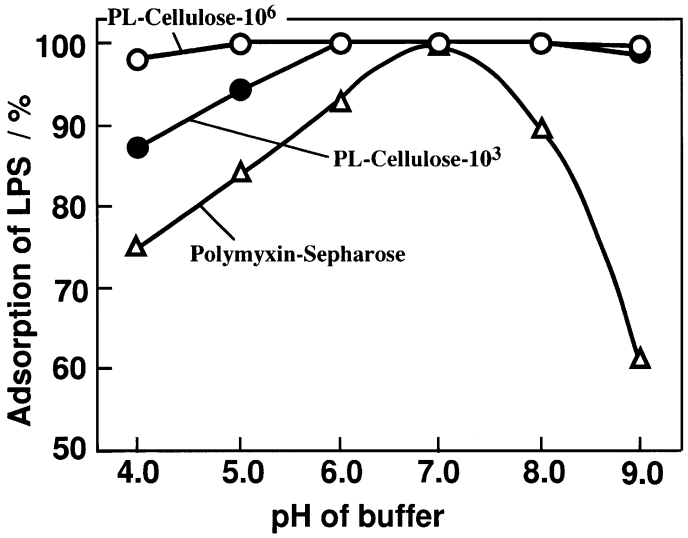

Figure 3. Effect of pH on adsorption of LPS by various adsorbents. The adsorption of LPS was determined using a batchwise method with 0.2 g of the wet adsorbent and 2 mL of a LPS solution (1 $\mu g\,mL^{-1}$, pH 4.0–9.0, $\mu = 0.05$).

high ionic strengths. These results suggest that PL-cellulose particles also adsorb LPS by a hydrophobic property that originates from the matrix.

Adsorption Behavior of Other Cellular Products

In order to examine the selective removal of LPS, it is important to check the adsorbing activity for protein. The effects of the buffer's ionic strength and the adsorbent's M_{lim} on adsorption of BSA (Figure 4a) and γ-globulin (Figure 4b) were examined. Under conditions of ionic strengths $\mu = 0.05$–0.8 and a pH of 7.0, the adsorbing activity of each protein increased with an increase in the M_{lim}. PL-cellulose-10^3 with M_{lim} 2×10^3 adsorbed little of each protein (below 2%) under these conditions. The result showed that adsorption of the protein was caused mainly by the entry of the protein into the pores of the adsorbent. This finding indicates that both BSA (M_n: 6.9×10^4) and γ-globulin (M_n: 1.6×10^5) can penetrate readily into the pores of $_{lim}$ $> 2 \times 10^6$, but cannot penetrate into the pores of M_w 2×10^3.

We previously reported (9) that the adsorption of protein by cross-linked DMP adsorbents was induced by both cationic and hydrophobic properties.

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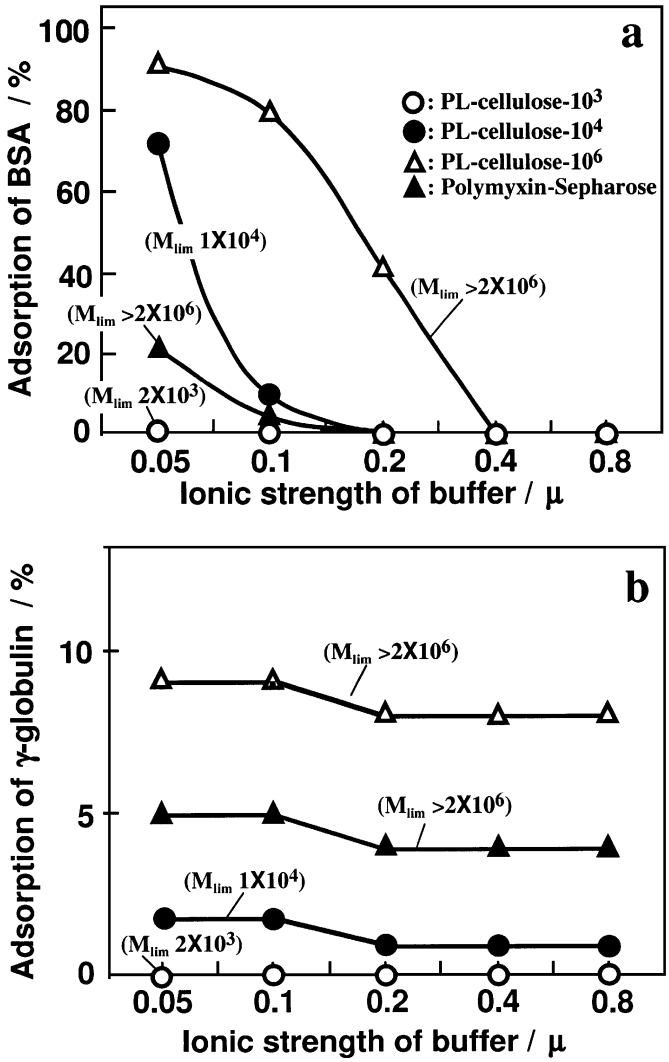

Figure 4. Effects of the buffer's ionic strength and the adsorbent's pore size (M_{im}) on adsorption of BSA (a) and γ-globulin (b) by various adsorbents. The adsorption of protein was determined using a batchwise method with 0.2 g of the wet adsorbent and 2 mL of a protein solution (500 $\mu g\,mL^{-1}$, pH 7.0, $\mu = 0.05$).

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PL-cellulose adsorbents have the same characteristics. BSA is negatively charged at pH values greater than its pI (4.9), and its adsorption by an adsorbent was remarkably dependent on the ionic strength, as shown in Figure 4a. These findings suggest ionic interaction between BSA and the adsorbent. On the other hand, γ-globulin is neutral (pI: 7.4) and a weakly hydrophobic protein, so its adsorption is retained even at the high ionic strength of $\mu = 0.8$, as shown in Figure 4b. These findings suggest the participation of hydrophobic binding.

At a low ionic strength of $\mu = 0.05$ to 0.1 (Figure 4b), the BSA-adsorbing activity (22 to 5%) of polymyxin-Sepharose was lower than that of PL-cellulose-10^4 (72 to 9%), although its pore size ($M_{lim} > 2 \times 10^6$) is larger than that of PL-cellulose-10^4 (M_{lim}:1×10^4). This lower BSA-adsorbing activity of polymyxin-Sepharose is attributable to its amino-group content being smaller than that of PL-cellulose-10^4, as shown in Table 1.

These findings (Table 1, Figures 4a and b) show that the PL-cellulose adsorbents bind more strongly with LPS than BSA or γ-globulin. This is because LPS has a lower pK_a and a higher hydrophobicity than each protein. Presumably, the strong LPS-adsorbing activities of the PL-cellulose adsorbents are related to ionic interaction between the cationic region of the adsorbents and the anionic region (phosphoric acid group) of LPS, and to the weak hydrophobic interaction between the alkyl chain region of the adsorbent and the lipid region of LPS.

Selective Removal of LPS from Protein-Containing Solutions

For selective removal of LPS from a protein solution, it is also necessary to adjust the buffer conditions, such as ionic strength and pH. The effects of ionic strength on selective adsorption of LPS from a BSA-containing solution with various adsorbents were examined (results are shown in Figures 5a, b, and c). When a BSA solution, including $500\,\mu g\,mL^{-1}$ of BSA and $100\,ng\,mL^{-1}$ of standard LPS, was used as a sample solution, PL-cellulose-10^3 selectively adsorbed LPS in the solution at an ionic strength of $\mu = 0.05$ to 0.4 and at a pH of pH 7.0, without adsorption of BSA (Figure 5a). PL-cellulose-10^6 selectively adsorbed LPS only at $\mu = 0.4$ (Figure 5b). By contrast, polymyxin-Sepharose showed adsorbing activities for both LPS and BSA at a low ionic strength of $\mu = 0.05$ to 0.1, and the adsorbing activities decreased with an increase in the ionic strength (Figure 4c). The adsorbent, therefore, could not selectively adsorb LPS from the BSA solution at any ionic strength.

The LPS-removing activity of PL-cellulose-10^3 was compared with that of PL-cellulose-10^6 and the results are shown in Table 2. Various protein solutions, which were naturally contaminated with LPS at concentrations from 1500 to 32,000 $pg\,mL^{-1}$, were used as samples. It is essential to reduce LPS to at least a concentration lower than $100\,pg\,mL^{-1}$ from fluids used for intravenous injection,

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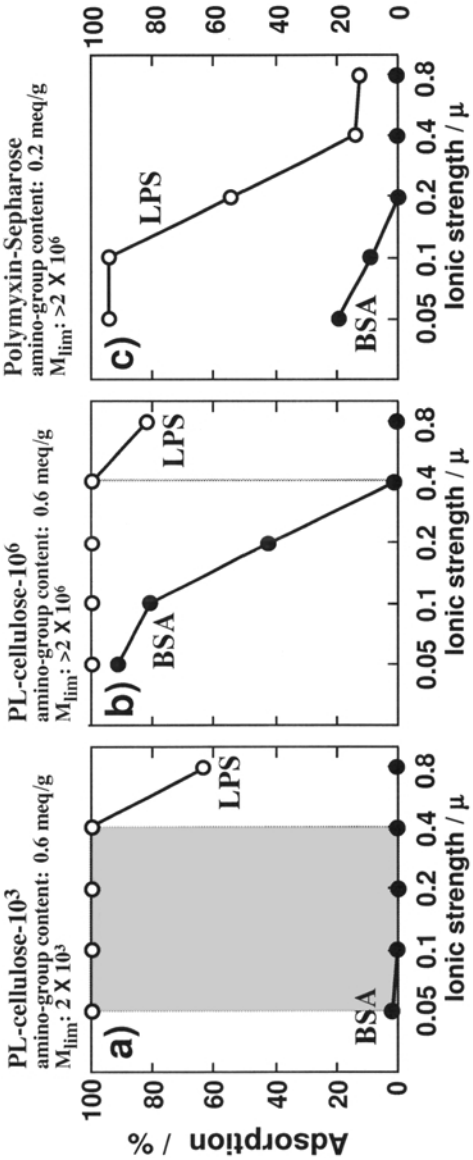

Figure 5. Effect of ionic strength on selective adsorption of LPS from a BSA solution containing LPS by the various adsorbents. The selective adsorption of LPS was determined using a batchwise method with 0.2 g of the wet adsorbent and 2 mL of a sample solution (BSA: 500 μg mL^{-1}, LPS (*E. coli* O111:B4): 100 ng mL^{-1}, pH 7.0, and ionic strength of $\mu = 0.05$-0.8).

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Table 2. Selective Removal of LPS from a Protein Solution by PL-Cellulose Adsorbents

Sample Solution Compound	pI	PL-cellulose-10^3 Adsorbent ($\mu = 0.05$, pH 7.0) Conc. of LPS Before Treatment pg mL^{-1}	Conc. of LPS After Treatment pg mL^{-1}	Rec. of Protein After Treatment %	PL-cellulose-10^6 Adsorbent ($\mu = 0.40$, pH 7.0) Conc. of LPS After Treatment pg mL^{-1}	Rec. of Protein After Treatment %
BSA	4.9	32,000	45	99	<10	97
γ-Globulin	7.4	5600	20	99	<10	97
Cytochrome C	10.6	1500	15	99	<10	98

The removal of LPS was determined using a batchwise method with 0.3 mL of wet adsorbent and 2 mL of a protein solution (1 mg mL^{-1}) containing natural LPS.

so as not to elicit pyrogenic reactions in mammals (3). As shown in Table 2, when PL-cellulose-10^3 and PL-cellulose-10^6 were used as adsorbent at ionic strengths of $\mu = 0.05$ and 0.4, respectively, each adsorbent was able to remove LPS from all protein to a level below 100 pg mL^{-1}. PL-cellulose-10^6, having a larger pore size, always showed higher removing activities: the residual concentrations of LPS after treatment were less than 10 pg mL^{-1} in all cases. On the other hand, PL-cellulose-10^3, having a smaller pore size, showed higher recoveries (99%) of all proteins.

In conclusion, the present results suggest that PL-cellulose-10^3 (M_{lim}: 2×10^3) and PL-cellulose-10^6 ($M_{lim} > 2 \times 10^6$) particles can reduce the concentrations of LPS to 100 pg mL^{-1} or lower, in drugs and fluids used for intravenous injection, at a neutral pH and ionic strengths of $\mu = 0.05$ and 0.4, respectively. These processes did not affect the recovery of even acidic proteins such as BSA. This high LPS-selectivity of PL-cellulose particles is possibly due to the cationic properties of poly(ε-lysine) and suitable hydrophobic properties of the matrix, which arise when M_{lim} values and amino-group contents are adjusted to 2×10^3 or $>2 \times 10^6$ and 0.6 meq g^{-1}, respectively.

ACKNOWLEDGMENTS

We are grateful to Chisso Co., Ltd. for providing poly(ε-lysine) and various cellulose particles. This work was supported in part by a Grant-in Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan.

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Reveived August 18, 2001
Accepted September 17, 2001
Manuscript 5644

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