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Preparation of poly(ε-lysine) adsorbents and application to selective removal of lipopolysaccharides

Chuichi Hirayama[a,*], Masayo Sakata[a], Mariko Nakamura[a], Hirotaka Ihara[b], Masashi Kunitake[a], Masami Todokoro[c]

[a]*Department of Applied Chemistry & Biochemistry, Faculty of Engineering, Kumamoto University, 2-39-1 Kurokami, Kumamoto 860-8555, Japan*

[b]*Graduate school of Science & Technology, Kumamoto University, 2-39-1 Kurokami, Kumamoto 860-8555, Japan*

[c]*Chisso Co. Ltd., 1-1 Noguchi-Cho, Minamata-Shi, Kumamoto 867-8501, Japan*

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Abstract

To remove endotoxins (lipopolysaccharides; LPS) from cell products used as drugs, water-insoluble poly(ε-lysine) (PL) particles were prepared by cross-linking with PL originating from *Streptomyces albulus* and chloromethyloxirane (CMO). The apparent pK_a (p$K_{a,app}$) and the anion-exchange capacity of the particles were easily adjusted by changing the PL ratio and the CMO ratio. The higher the p$K_{a,app}$, the greater the LPS-adsorption capacity of the particles. On the other hand, when the PL ratio (in the particles) increased to 75 unit-mol% or higher, the adsorption of bovine serum albumin by the particles also increased, but decreased with increasing ionic strength of the buffer to $\mu=0.2$ or higher. The adsorption of γ-globulin increased with decreasing PL ratio to 65 unit-mol% or lower. As a result, when the PL ratio was 70 unit-mol% and the p$K_{a,app}$ was 6.7, the PL/CMO particles selectively removed LPS from various protein solutions that were naturally contaminated with LPS, at pH 6.0 and $\mu=0.05$.

Keywords: Lipopolysaccharides; Endotoxins; Poly(ε-lysine)

1. Introduction

The removal of lipopolysaccharides (LPS) from drugs and fluids before injection is very important, because of its potent biological activities causing pyrogenic and shock reactions in mammals [1,2]. LPS, constituents of the cell wall of gram-negative bacteria, are potential contaminants of physiological fluids and aqueous solutions and are very stable, resisting extreme temperatures and pH values. For the removal of LPS from solutions of cell products, such as proteins, adsorption methods have proved to be the most effective [3].

We previously reported [4,5] that aminated poly(γ-methyl L-glutamate) (PMLG-NH_2) particles (Fig. 1) can adsorb LPS over a wide range of ionic strength, $\mu=0.05$ to 0.8. Furthermore, we have found that the interaction of some hydrophobic proteins with the PMLG-NH_2 particles decreases extremely when the pore size of the particles is adjusted to be less than the molecular mass of the protein [6]. However, the adsorbing capacity of the particles, which have small pore size, was high for both LPS and acidic protein, such as bovine serum

*Corresponding author.

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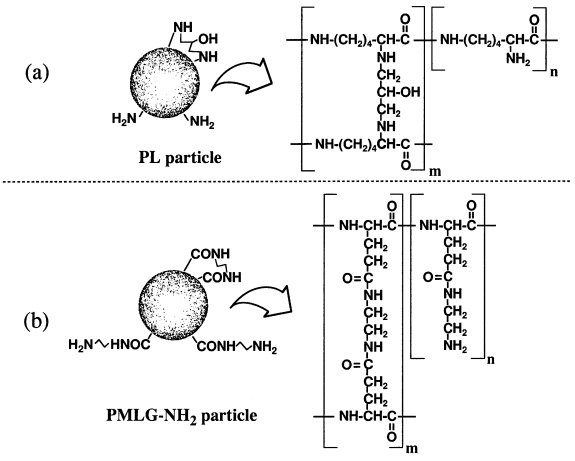

Fig. 1. Schematic illustrations of crosslinked poly(ε-lysine) (PL) (a) and aminated poly(γ-methyl L-glutamate) (PMLG-NH_2) particles (b).

albumin (BSA) at as low an ionic strength as $\mu = 0.05$ [7].

Some adsorbents for LPS, polymyxin-immobilized Sepharose [8,9] and histidine-immobilized Sepharose [3,10], also cannot selectively adsorb LPS from an acidic protein solution containing LPS at low ionic strength [10]. As the ionic strength increased, the adsorption of BSA by these adsorbents decreased, but this was accompanied by a decrease in the adsorption of LPS [7,10].

In this work, we developed novel adsorbents (Fig. 1) from poly(ε-lysine) (PL) and chloromethyloxirane (CMO) by cross-linking. The apparent pK_a (p$K_{a,app}$) (7.6) [11,12] of PL originating from *Streptomyces albulus* is lower than that of PMLG-NH_2 (8.2) [13]. It seems that this favorable property of the PL makes it possible to decrease the ionic interaction between BSA and the novel adsorbent. We also considered that the ionic interaction of the adsorbent with LPS is stronger than that with BSA (p*I*: 4.9), because the LPS has a lower pK_a than BSA.

In this report, we describe the synthesis of cross-linked PL adsorbents. We then report the effects of the content of PL (in the adsorbent), pH and ionic strength on the selective removal of LPS from various protein solutions by the cross-linked PL adsorbents.

2. Experimental

2.1. Materials

A 25 wt-% PL aqueous solution, produced by *Streptomyces albulus*, was obtained from Chisso (Tokyo, Japan). The number-average molecular weight ($\overline{M}_n$) of the PL was about 4.0×10^3 (degree of polymerization, 25) [14]. The $\overline{M}_n$ was analysed by size-exclusion chromatography using poly(styrene sulfonic acid, sodium salt) standards [14]. A 10 wt-% of PMLG (degree of polymerization, 1000; $\overline{M}_n$, 1.4×10^5) solution was kindly supplied by Ajinomoto (Tokyo, Japan). CMO and diaminoethane were 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 Ind. (Osaka, Japan). Purified LPS from *E. coli* O111:B4 was purchased from Difco Laboratories (Detroit, MI, USA). Albumin (from bovine serum; $\overline{M}_n$, 6.9×10^4; p*I*, 4.9), insulin (from bovine pancreas; $\overline{M}_n$, 3.6×10^4; p*I*,: 5.3), myoglobin (from horse heart; $\overline{M}_n$, 1.8×10^4; p*I*, 6.8), γ-globulin (from human serum; $\overline{M}_n$, 1.6×10^5; p*I*, 7.4) and cytochrome *c* (from horse heart; $\overline{M}_n$, 1.3×10^4; p*I*, 10.6) were purchased from Sigma (St. Louis, MO, USA). LPS-free water was kindly supplied by Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). All other chemicals were of analytical reagent grade.

2.2. Preparation of adsorbents

A 25 wt% PL solution and CMO (as a cross-linking agent) were mixed at 0°C. The mixture was stirred at 0°C for 30 min and then at 25°C for 24 h. The PL/CMO polymers obtained were broken into particles using a crusher, and washed successively with acetone, chloroform, methanol, ethanol and LPS-free water. The PL/CMO particles with diameters of 44 to 105 μm were used as the adsorbent. PMLG-NH_2 spherical particles (diameters: 44–105 μm) were prepared by the suspension–evaporation method [15] and aminolysis [3,4] with diaminoethane, as described previously. The anion-exchange capacity (AEC) and the p$K_{a,app}$ of adsorbents were quantified by pH titration [6].

2.3. LPS assay

LPS was assayed by the Limulus test, which involves a 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 the reagent for the reaction.

2.4. Protein assay

The protein concentration in a sample solution was determined by the absorbance at 280 nm (proteins other than cytochrome *c*) or at 410 nm (cytochrome *c*) with a Spectrophotometer UV-160 (Shimadzu), as previously described [10].

2.5. 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 and 8) and 0.02 *M* Tris (pH 9 and 10). The ionic strength of the buffer was adjusted by changing the sodium chloride concentration. The adsorption of LPS was measured by a batchwise method as follows: the adsorbent was washed and equilibrated with one of various buffers with different ionic strengths. A 0.1–0.3-ml portion of wet adsorbent was suspended in 2–4 ml of LPS solution. The suspension was shaken for 2 h at 25°C and filtered through a Millipore filter (cellulose acetate, 0.8 μm). The filter removed the adsorbent from the suspension without affecting the recovery of LPS and protein. The LPS content of the filtrate was determined. The apparent dissociation constant ($K_{d,app}$) between LPS and adsorbent was estimated using an adsorption isotherm as previously described [7,10]. The adsorption of other substances was estimated by the same method as used for the LPS binding assay. The adsorbing activities for LPS and other substances are shown as the average of five measurements.

3. Results and discussion

3.1. Preparation of LPS adsorbents

To decrease the adsorption of an acidic protein, it is necessary to decrease the pK_a of the adsorbent to less than 8.2 (p$K_{a,app}$ of PMLG-NH_2). The PL has a pK_a of 7.6 and no buffer action at pH 10 [11,12]. This suggested that the α-amino group of lysine was a free amino group, while the ε-amino group was combined to a peptide linkage. Therefore, as shown in Table 1, PL particles with p$K_{a,app}$ of 7.4 to 5.9 were prepared. The p$K_{a,app}$ and AEC were easily adjusted by changing the ratio of PL and the ratio of CMO in polymerization. While the ratio of cross-linking agent (in the PL particles) increased from 10 to 60 unit-mol%, p$K_{a,app}$ of the adsorbent decreased

Table 1
Characteristics of PL and PMLG-NH_2 adsorbents

Name	Molar ratio (mol%)		Anion exchange capacity[b]		S_d[c] (wet-ml/dry-g)	p$K_{a,app}$
	poly(ε-lysine)[a]	chloromethyloxirane	(mequiv./dry-g)	(mequiv./wet-ml)		
PL-90	90	10	5.0	0.40	12.5	7.4
PL-80	80	20	4.6	0.58	8.1	7.0
PL-75	75	25	4.2	0.67	6.4	6.8
PL-70	70	30	3.6	0.58	6.2	6.7
PL-65	65	35	3.2	0.55	5.8	6.6
PL-60	60	40	3.0	0.67	4.5	6.4
PL-50	50	50	2.8	0.72	3.9	6.1
PL-40	40	60	2.5	0.69	3.6	5.9
PMLG-NH_2	–	–	3.5	0.73	4.8	8.2

[a] Unit-mol% of poly(ε-lysine) in the adsorbent (1 unit, -NH-$(CH_2)_4$CH(NH_2)CO-; degree of polymerization, 25).
[b] Anion-exchange capacity (AEC) of the adsorbent.
[c] Degree of swelling in water.

from 7.4 to 5.9, and AEC decreased from 5.0 to 2.5 mequiv./dry-g. However, the AEC per ml of wet adsorbent was not independent of the ratio of cross-linking agent, since the degree of swelling in water (S_d) of the adsorbent decreased with an increase in the content of cross-linking agent. An increase in the content of cross-linking agent lead to an increase in the hydrophobicity of the adsorbent. We consider that an increase in the hydrophobicity lead to a decrease in the p$K_{a,app}$ of the adsorbent.

3.2. Effects of various factors on adsorption of LPS and protein.

The LPS-adsorbing activities of the adsorbents were examined by a batchwise method with various kinds of buffers. The purified LPS (from *E. coli* O111:B4) was used as a standard LPS sample. Fig. 2 shows the LPS-adsorbing activities of various PL adsorbents (PL ratio: 40–90 unit-mol%) at pH 7.0 and at an ionic strength of $\mu=0.05$. The LPS-adsorbing activity of all adsorbents increased with decreasing LPS concentration of the sample. When the LPS concentration in a sample solution was 500 μg/ml, the LPS-adsorbing activity increased remarkably, from 23 to 99%, with an increase in the PL ratio from 60 to 90 unit-mol%. When the removal of LPS from a dilute LPS solution (0.5 μg/ml) was attempted, the adsorbents with high PL ratio (70–90 unit-mol%) showed excellent adsorbing activity: they were able to decrease the concentration of LPS from 0.5 μg/ml to less than 10 pg/ml.

For selective adsorption of LPS, it is necessary to check the interaction between the adsorbent and protein. The effects of PL ratio in the adsorbent on the adsorption of BSA, γ-globulin and cytochrome *c* were examined. As shown in Fig. 3, little cytochrome *c* was adsorbed (<2%) by all the particles. The adsorption of BSA increased remarkably, from 11 to 84%, when the PL ratio was increased from 70 to 90 unit-mol% (with an increase in p$K_{a,app}$ from 6.8 to 7.4). By contrast, the adsorption of γ-globulin increased from 2 to 22% on decreasing the PL ratio from 70 to 40 unit-mol% (with an increase in ratio of cross-linking agent from 30 to 60 mol-%). As a result, PL/CMO (70:30) (PL-70) showed the lowest adsorbing activity of the three proteins.

As the results (Figs. 2 and 3) show, the PL-70 adsorbent had the highest selectivity for LPS at pH 7.0 and an ionic strength of $\mu=0.05$.

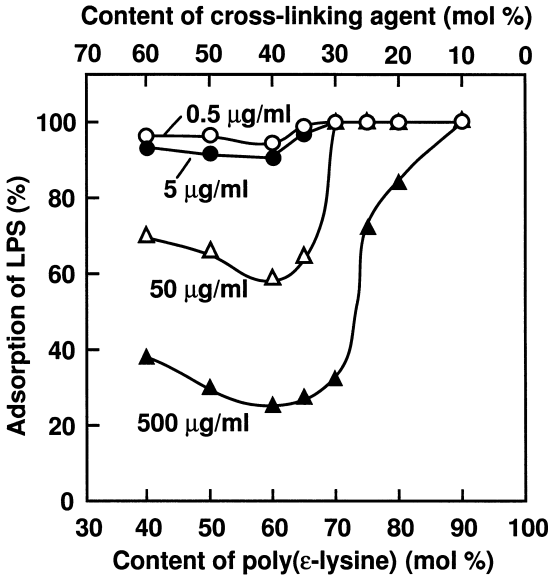

Fig. 2. Effect of molar ratio of poly(ε-lysine) in adsorbents on adsorption of LPS. The adsorption of LPS was determined by a batchwise method with 0.2 ml of wet adsorbent and 2 ml of a LPS (*E. coli* O111:B4) solution (pH 7.0, $\mu=0.05$).

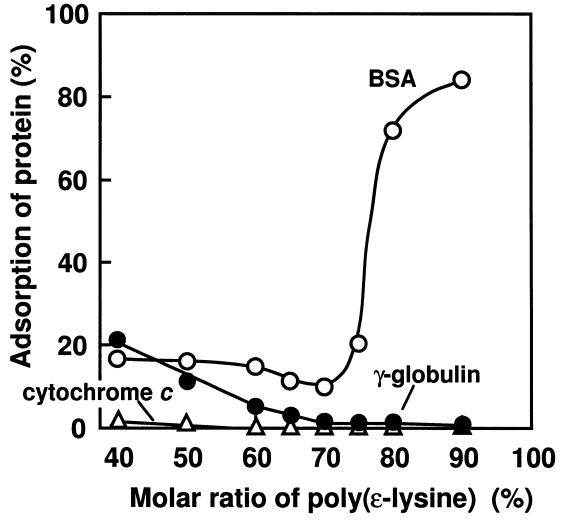

Fig. 3. Effect of the molar ratio of poly(ε-lysine) in adsorbents on adsorption of protein. The adsorption of a protein was determined by a batchwise method with 0.2 ml of wet adsorbent and 2 ml of a protein (500 μg/ml) solution (pH 7.0, $\mu=0.05$).

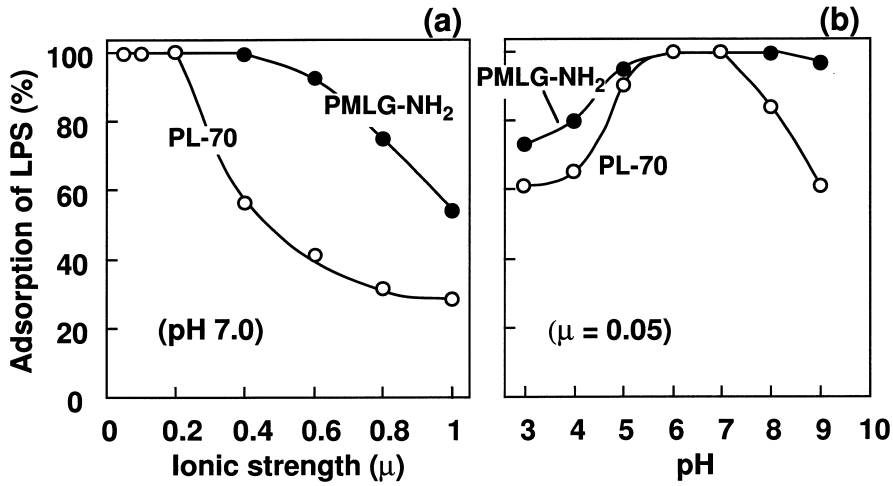

Fig. 4. Effects of ionic strength (a) and PH (b) of the buffer on adsorption of LPS by PL-70 and PMLG-NH_2 adsorbents. The adsorption of LPS was determined by a batchwise method with 0.2 ml of wet adsorbent and 2 ml of an LPS solution [LPS (*E. coli* O111:B4), 5 μg/ml].

The effects of ionic strength and pH on LPS adsorption by PL-70 were compared with that of the PMLG-NH_2 adsorbent. The results are shown in Fig. 4a and b. The higher the ionic strength of the buffer, the lower the LPS-adsorbing activity of each adsorbent, as shown in Fig. 4a. Each adsorbent showed high LPS adsorption (99%) at μ=0.05 to 0.2 and pH 7.0. At a high ionic strength of μ=0.4–1.0, PMLG-NH_2 always showed greater LPS-adsorbing activity. As shown in Fig. 4b, each adsorbent showed strong LPS-adsorbing activity in the neutral pH range, although the PMLG-NH_2 showed greater adsorption in the acidic pH range 3–5 and the basic pH range 8–9.

The effect of the ionic strength of the buffer on the adsorption of BSA and γ-globulin, was examined with the two adsorbents at μ=0.05–0.8 and pH 7.0 (Fig. 5a and b). The BSA-adsorbing activity of each adsorbent increased with decreasing ionic strength, as shown in Fig. 5a. At a wide range of ionic strengths, from μ=0.05 to 0.6, the BSA-adsorbing activity of PL-70 was always lower than that of

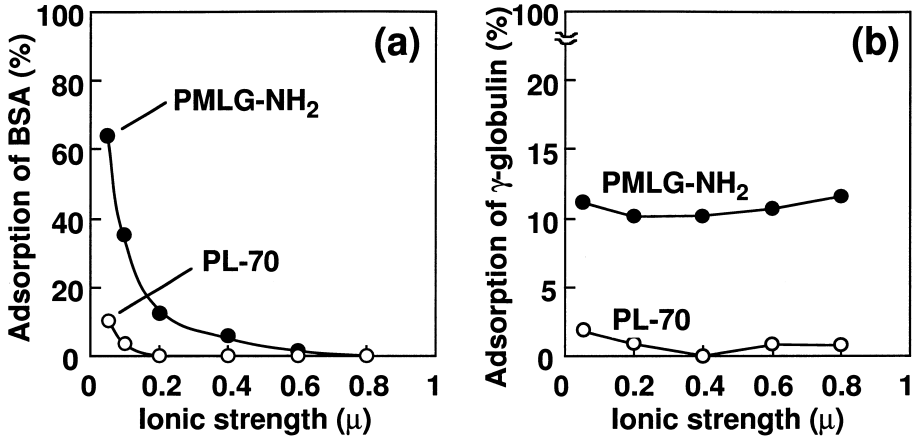

Fig. 5. Effects of ionic strength on the adsorption of BSA (a) and γ-globulin (b) by PL-70 and PMLG-NH_2 adsorbents. The adsorption of a protein was determined by a batchwise method with 0.2 ml of wet adsorbent and 2 ml of a protein solution (protein: 500 μg/ml, pH 7.0).

PMLG-NH_2. When the ionic strength was increased to $\mu=0.2$ or higher, the BSA adsorption of PL-70 was $<$ 1%. As shown in Fig. 5b, the adsorption of γ-globulin by PL-70 was $<$2% at all ionic strengths. By contrast, the PMLG-NH_2 retained about 10% γ-globulin adsorption at all ionic strengths.

From these results (Figs. 2–5), we assumed that the adsorbing-activity of PL-70 for LPS and protein was induced by cooperation of cationic and weakly hydrophobic properties. LPS is an amphipathic substance, having both anionic (phosphate groups) and hydrophobic (lipophilic groups) regions [17]. These characteristics also suggest that the LPS-adsorbing activity of adsorbents was due to the simultaneous effects of their cationic properties and hydrophobic properties. We have previously reported that the PMLG particles show an inherent tendency towards hydrophobicity [18], and the adsorbing-activity of PMLG particles for LPS resulted from their hydrophobic properties [4,5]. The introduction of amino groups into the PMLG particles induced an ionic interaction between LPS and the adsorbent [4,5]. As shown in Fig. 4a and b, the adsorption of LPS by PL-70 is dependent on the ionic strength and pH and the charge of LPS is anionic at pH values greater than its pK_a ($<$3). This suggests that ionic interaction occurs between the PL adsorbent and LPS.

On the other hand, LPS adsorption by the PL adsorbent was increased with an increase in the content of cross-linking agent (in the adsorbent) from 40 to 60 mol-%, as shown in Fig. 2. This result suggests that LPS binds to the PL adsorbent through its hydrophobic properties.

The charge of protein is anionic at pH values over the p*I* and is cationic at pH values under it. Therefore, the ionic interaction of the adsorbent with cytochrome *c* (p*I* 10.6) and γ-globulin (p*I* 7.4) is not induced at pH 7.0 (lower than the p*I* value). On the other hand, the charge of BSA is anionic at pH values greater than its p*I* (4.9), and the adsorption of BSA by PL-70 and PMLG-NH_2 is dependent on the ionic strength, as shown in Fig. 5a. This suggests that there is an ionic interaction between each adsorbent and BSA. By contrast, γ-globulin is a neutral and weakly hydrophobic protein, and its adsorption by PMLG-NH_2 was independent of ionic strength, as shown in Fig. 5b. The adsorption by the PL adsorbent increased with an increase in the hydrophobicity (content of cross-linking agent) of the adsorbent (Fig. 3). These findings suggest the participation of hydrophobic binding. LPS was bound more strongly by PMLG-NH_2 than by PL-70 at a high pH (8 and 9), as shown in Fig. 4b. This difference is attributable to the fact that the pK_a of the aminated PMLG adsorbent (pK_a, 8.2) is higher than that of the PL-70 adsorbent (pK_a: 6.7). For a wide range of ionic strengths ($\mu=0.05$–0.8), the γ-globulin-adsorbing activity of PMLG-NH_2 was always higher than that of the PL -70 adsorbent (Fig. 5b). This difference is attributable to the fact that the hydrophobicity of the PMLG-NH_2 adsorbent is greater than that of the PL-70 adsorbent.

These results (Figs. 2–5) suggest that PL-70 can adsorb LPS selectively without affecting protein recovery, although LPS-adsorbing activity is lower than that of PMLG-NH_2.

3.3. Effect of dissociation constant between LPS and adsorbent on removal of LPS

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 the LPS and adsorbent. The adsorption capacity and the apparent dissociation constant ($K_{d,app}$) of PL-70 were compared with those of PMLG-NH_2. Fig. 6a shows the LPS-adsorption isotherms of the two adsorbents. Fig. 6b and c show the Scatchard plots [6,10] derived from the isotherms

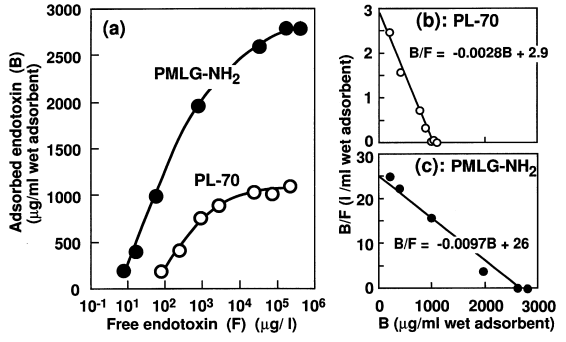

Fig. 6. LPS-adsorption isotherms (a) and Scatchard plots (b, c). The adsorption isotherms for PL-70 and PMLG-NH_2 were determined by a batchwise method with 0.1 ml of wet adsorbent and 4 ml of an LPS solution [LPS from *E. coli* O111:B4 (M_n, 1×10^6): 5×10^3 to 5×10^5 ng/ml, pH 7.0, $\mu=0.05$]. The Scatchard plots (b and c) [5,6] were derived from (a). The $K_{d,app}$ was expressed in mol/l of molecular mass of LPS.

of the PL-70 and PMLG-NH_2 adsorbents. Based on these plots, the $K_{d,app}$ (between adsorbent and LPS) and adsorbing capacities per milliliter of the adsorbents were calculated to be 3.7×10^{-10} *M* and 1 mg in PL-70, and 1.0×10^{-10} *M* and 2.7 mg in PMLG-NH_2, when the aggregation weight of LPS (*E. coli* O111:B4) was estimated as one million. PMLG-NH_2 showed the larger LPS-adsorption capacity and smaller $K_{d,app}$ value. The LPS-adsorption capacity of PMLG-NH_2 ($pK_{a,app}$, 8.2) was about 2.7 times that of PL-70 ($pK_{a,app}$, 6.7), although the AEC value (3.5 mequiv./g) was similar to that of PL-70 (3.6 mequiv./g). This result indicated that the higher the $pK_{a,app}$ value of the adsorbent, the stronger its adsorbing activity.

Minobe et al. [10] found that the LPS-adsorbing capacity and $K_{d,app}$ values of histidine-immobilized Sepharose were 0.53 mg/ml of adsorbent and 1.57×10^{-9} *M*, at pH 7.0 and $\mu=0.02$. These results indicate that the LPS-removing activity of the PL-70 adsorbent is stronger than the activity of the histidine-immobilized Sepharose.

3.4. Removal of LPS from various protein solutions

For the selective adsorption of LPS, it is necessary to control not only the pK_a of the adsorbent but also the pH of the buffer. The most favorable pH for the selective removal of LPS from a protein solution is below its p*I* and above the pK_a of the phosphate residues of LPS. It is not easy to remove LPS from an acidic protein solution such as BSA, because the LPS-removing activity also decreases at pH values lower than its p*I* (4.9), as shown in Fig. 4b. The effect of buffer pH on the selective adsorption of LPS from a BSA solution was examined with the PL-70 and the PMLG-NH_2 adsorbents. A BSA solution (500 μg/ml) to which was added purified LPS (100 ng/ml) was used as a sample solution at $\mu=0.05$ and various pH values. The results are shown in Fig. 7a and b. PL-70 selectively adsorbed LPS from the BSA solution only at pH 6.0 (Fig. 7a). In contrast, PMLG-NH_2 showed a high level of LPS adsorption (88–99%) over a wide range of pH values (pH 4.0–9.0), but this was accompanied by the adsorption of BSA (11–95%) at all pH values, as shown in Fig. 7b. The PMLG-NH_2 adsorbent therefore cannot selectively adsorbed LPS from a BSA–LPS solution at any pH.

This result suggests that the BSA-adsorbing activity of cationic adsorbent decreases remarkably when the $pK_{a,app}$ of the adsorbent decreases from 8.2 (PMLG-NH_2) to 6.7 (PL-70). As shown in Fig. 7a, the PL-70 adsorbent binds LPS more strongly than BSA over a wide range of pH values (4.0–9.0),

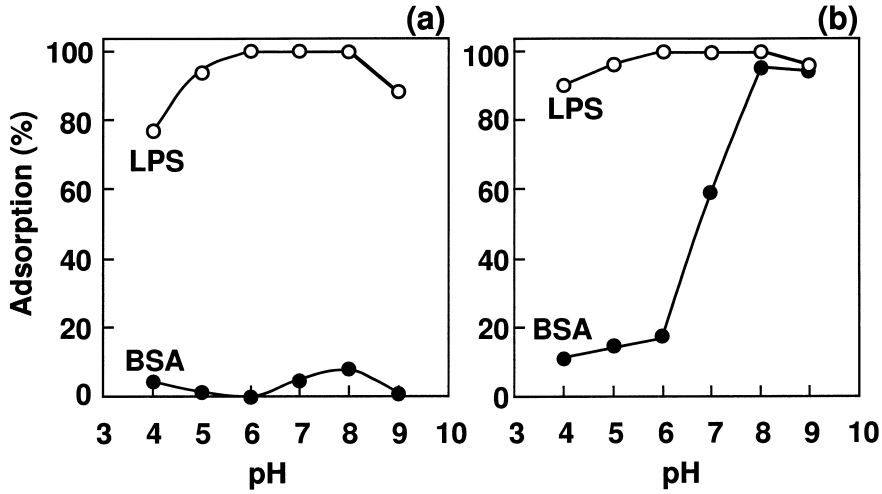

Fig. 7. Effect of pH on the selective adsorption of LPS from a BSA solution containing LPS [BSA, 500 μg/ml; LPS (*E. coli* O111:B4), 100 ng/ml; $\mu=0.05$] by PL-70 (a) and PMLG-NH_2 (b). The selective adsorption of LPS was determined by a batchwise method with 0.2 ml of wet adsorbent and 2 ml of a sample solution.

Table 2
Removal of LPS from a protein solution by PL-70 and PMLG-NH_2 adsorbents[a]

Sample solution			PL-70 adsorbent		PMLG-NH_2 adsorbent	
Compound	p*I*	Concentration of LPS before treatment (pg/ml)	Concentration of LPS after treatment (pg/ml)	Recovery of protein after treatment (%)	Concentration of LPS after treatment (pg/ml)	Recovery of protein after treatment (%)
BSA	4.9	34 000	85	99	30	56
Insulin	5.3	8000	95	97	22	60
Myoglobin	7.4	1200	<10	99	<10	97
γ-Globulin	8.4	4500	53	98	8	88
Cytochrome *c*	10.6	800	<10	98	<10	94

[a] The removal of LPS was determined by a batchwise method with 0.3 ml of wet adsorbent and 2 ml of a protein solution containing natural LPS (protein, 1 mg/ml, pH 6.0, $\mu=0.05$).

because the pK_a of the phosphate residues of LPS is lower than the p*I* of BSA, and probably because the adsorbent can adsorb LPS not only through the cationic properties of its lysine residues but also through the weak hydrophobic properties of its CMO.

The activity to remove natural LPS of the PL-70 adsorbent from various protein solutions was compared with that of PMLG-NH_2 adsorbent at pH 6.0 and $\mu=0.05$ (Table 2). Various protein solutions, which were naturally contaminated with LPS at concentrations ranging from 800 to 34 000 pg/ml, were used as samples. It is essential to eliminate LPS to at least a concentration lower than 100 pg/ml from fluids used for intravenous injection [10], since it elicits pyrogenic and shock reactions in mammals [1]. As shown in Table 2, each adsorbent was able to remove LPS from all protein solutions to a level below 100 pg/ml, although the removing activity of PMLG-NH_2 was always higher than that of PL-70. The removing activity of PMLG-NH_2, however, was also high for acidic proteins, such as BSA (p*I* 4.9) and insulin (p*I* 5.3). The recoveries of the proteins were 56 and 60%, respectively. In contrast, the PL-70 adsorbent showed high recoveries (97 to 99%) of all proteins (p*I* 4.9–10.6) as well as efficient removal of LPS at pH 6.0 and a low ionic strength of $\mu=0.05$.

4. Conclusion

The results demonstrated that the cross-linked PL adsorbent can be used for the removal of natural LPS from drugs and fluids for injection. We consider that the cross-linked PL adsorbent can remove any LPS from biologically active substances produced not only by traditional methods but also by recombinant DNA technology. This ability of cross-linked PL adsorbent for selective adsorption of LPS is due to the cationic properties of PL and hydrophobic properties of chloromethyloxirane, which arise when the p$K_{a,app}$ value is adjusted to 6.7.

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