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Phosphorylated bacterial cellulose for adsorption of proteins

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ABSTRACT

Bacterial cellulose (BC) and chemically modified BC are attractive adsorptive materials for biomacromolecules due to their fine network structure. In the present study, the adsorption behavior of proteins on phosphorylated bacterial cellulose (PBC), which has much larger specific surface area than phosphorylated plant cellulose (PPC), was investigated. The proteins were quantitatively adsorbed on PBC at pHs lower than their isoelectric points. The adsorption capacities for lysozyme using PBCs with varying degrees of phosphorylation were determined from adsorption isotherms. The adsorption capacity for the protein increased as percentage phosphorylation increased. The adsorption capacity of PBC was much higher than that of PPC, even though their phosphorylation percentages were similar. However, the adsorption capacities of PBC and PPC were similar for the smaller cationic materials such as tryptophan methyl ester and trivalent lanthanum. From the results of adsorption experiments, PBC was found to be an attractive adsorbent with a large adsorption capacity for proteins.

1. Introduction

Cellulose synthesized by bacteria such as *Acetobacter xylinum* is referred to as bacterial cellulose (abbreviated as BC) (Klemm, Heublein, Fink, & Bohn, 2005; Yamanaka et al., 1989; Yoshinaga, Tonouchi, & Watanabe, 1997). BC has been attracting attention as a raw material for preparation of advanced materials, due to its advantageous properties that are imparted by the fine network structure. In the dry state BC shows high tensile strength, and BC sheet is used as a sensitive diaphragm for stereo headphones. In recent years, optically transparent composites (Nogi & Yano, 2008; Yano et al., 2005) and sensitive detection systems for biomolecules (Tabuchi & Baba, 2005; Tabuchi, Kobayashi, Fujimoto, & Baba, 2005) have been developed using BC as a starting material. For instance, a polymer solution containing BC shows a high separation factor for DNA fragments.

BC is also attractive for preparing adsorptive materials for various species, due to its microfibrous structure. The oxoanionic species of Sb(III) is adsorbed on BC under basic conditions (Suetsugu, Oshima, Ohe, & Baba, 2007). Various chemically modified BCs have been developed recently for adsorption of metal ions. Carboxymethylated BC was synthesized by *A. xylinum* by adding water-soluble carboxymethylcellulose (CMC) to the culture medium, and its ability to remove metal ions was investigated (Chen, Zou, et al., 2009). Diethylenetriamine BC and amidoximated BC have also been prepared for adsorptive removal of Cu(II) and Pb(II), and the adsorption kinetics were studied (Chen, Shen, Yu, & Wang, 2009; Shen et al., 2009). From the results of adsorption experiments, it was suggested that the chemically modified BCs are suitable as adsorbents for metal ions. The microfibrous network of BC is also suitable as a matrix for preparing nanoparticles (Barud et al., 2008; Maneerung, Tokura, & Rujiravanit, 2008; Zhang & Qi, 2005). CdS nanoparticles have been synthesized and stabilized in situ on BC nanofibers (Li et al., 2009). In a previous study, we prepared phosphorylated bacterial cellulose (abbreviated as PBC) as an adsorbent for transition metal ions and lanthanide metal ions (Oshima, Kondo, Ohto, Inoue, & Baba, 2008). Phosphorylation of BC was found to proceed more efficiently than phosphorylation of plant cellulose (abbreviated as PC) under the same conditions. Transition metal ions and lanthanide ions were quantitatively adsorbed on PBC. However, the adsorption selectivity for transition metal ions was based simply on the characteristics of the phosphoric acid group, as in the case of conventional cellulose phosphate.

The fine network structure of bacterial cellulose is expected to hold a large amount of biomacromolecules such as proteins, due to its large surface area. BC itself has been reported to exhibit a larger adsorption capacity for cellobiose dehydrogenase, a protein showing affinity to cellulose, compared with wood pulp cellulose (Ougiya et al., 1998). Also, chemically modified BC is an adsorptive material that exhibits a large adsorption capacity for proteins. Adsorption of lysozyme on PBC was examined in a preliminary study (Oshima, Taguchi, Fujiwara, Ohe, & Baba, 2007). It was found that PBC exhibited a large adsorption capacity for lysozyme compared with phosphorylated plant cellulose (abbreviated as PPC); however, the parameters for protein adsorption using PBC are still not

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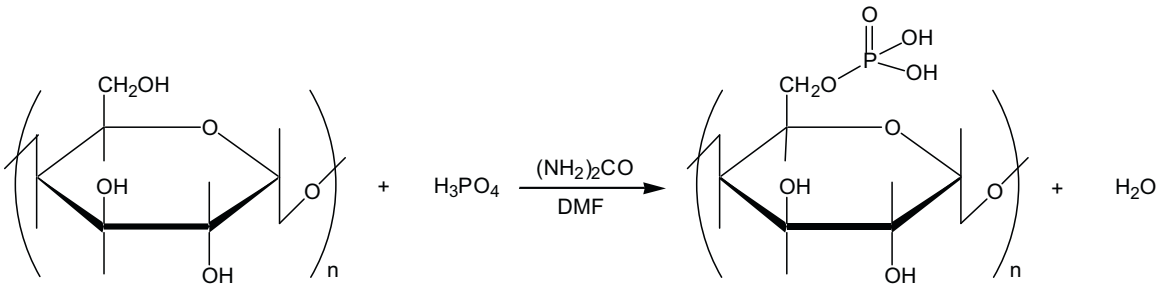

Fig. 1. The preparation scheme for PBC (and PPC).

clear in detail. Quaternary ammonium bacterial cellulose (QABC) was recently prepared from BC for adsorption of proteins (Niide et al., 2010), and it was found that hemoglobin was adsorbed on QABC under basic conditions via electrostatic interaction. Remarkably, the adsorption capacity for hemoglobin on QABC was higher than on quaternary ammonium plant cellulose prepared under the same conditions.

In the present study, the adsorption behavior of PBC for proteins was investigated to clarify the effect of the unique microfibrous structure on the adsorption. PBCs and PPCs were prepared under different conditions so as to have different degrees of phosphorylation. Adsorption experiments for proteins using PBC and PPC adsorbents were carried out, and the effect of the fibrous structure of the cellulosic adsorbents on protein adsorption was studied. BC was expected to be more favorable than PC as a polymer support for protein adsorption due to the larger surface area of BC.

2. Experimental

2.1. Materials

The BC starting material was prepared from "nata de coco" by grinding, washing with distilled water, and lyophilization. Cellulose powder as PC originating from a plant source was purchased from AdvantecToyo Kaisha, Ltd., Japan. The cellulose powder is made from high purity cotton cellulose to which an acid treatment is applied to remove ash. The following protein reagents for the adsorption experiments were used as received: hemoglobin from bovine blood (Wako Pure Chemical Industries, Osaka, Japan), myoglobin from equine skeletal muscle, albumin from chicken egg white (Sigma–Aldrich Co., St. Louis, MO) and lysozyme (Lyso) from chicken egg white (Nacalai Tesque Inc., Kyoto, Japan). All other reagents were reagent grade and were used as received.

2.2. Preparation of phosphorylated bacterial cellulose

PBC was prepared from BC according to the previously described procedure shown in Fig. 1 (Granja et al., 2006; Oshima et al., 2008). Preparation of PBC was examined under various conditions, to prepare materials with different degrees of phosphorylation. After lyophilization and grinding to about 200 μm, PBC was obtained as white powder. PPC was prepared from PC in a similar manner. The degree of phosphorylation of BC was determined as follows: 40 mg of PBC was soaked in aqueous 1.5 mol dm^{-3} sulfuric acid and the mixture distilled at 90 °C for 24 h to eliminate the phosphoric acid group by hydrolysis. After cooling, the mixture was filtered and the filtrate diluted with distilled water. The concentration of phosphoric acid in the solution prepared from the filtrate was determined using an ICP/AES spectrometer (Shimadzu ICPS-7100). The residue from the filtration was treated again using aqueous 1.5 mol dm^{-3} sulfuric acid to ensure complete elimination of phosphoric acid from PBC. The concentration of phosphoric acid in the second filtrate was negligible, confirming complete elimination of phosphoric acid from PBC. The percentage substitution with phosphoric acid groups of the C-6 primary hydroxyl group of BC (phosphorylation [%]) was calculated from the molar number of phosphoric acid molecules eliminated based on the mass balance.

PBC and PPC were observed using a scanning electron microscope (HITACHI S-4100). The specific surface areas of PBC and PPC were determined by the N_2-BET method using a volumetric adsorption measurement instrument (BEL Japan BELSORP mini).

2.3. Adsorption of proteins using phosphorylated bacterial cellulose

Adsorption experiments for lysozyme were carried out using a batchwise method as follows: an aqueous solution was prepared by dissolving 10 μmol dm^{-3} of lysozyme in 100 mmol dm^{-3} sodium phosphate buffer solution. A portion (15 cm^3) of the aqueous solution and 20 mg of an adsorbent (PBC or other) were mixed in a stoppered glass tube and shaken at 120 rpm in a thermostat-regulated shaker at 30 °C. After 20 h adsorption equilibrium was attained and the mixture was filtered. The concentration of residual lysozyme in the filtrate was determined by UV–VIS spectrophotometry (JASCO U-best v560) to determine the percentage adsorption (Adsorption [%]) and amount adsorbed (q [mmol g^{-1}]) according to the following equations:

$$\text{Adsorption} = \frac{C_0 - C_e}{C_0} \times 100 \quad [\%]$$

$$q = \frac{C_0 - C_e}{W} \times V \quad [\mu\text{mol g}^{-1}]$$

where C_0 and C_e are the protein concentrations before and after adsorption in μmol dm^{-3}, W is the dry mass of adsorbent in g, and V is the volume of solution in dm^3. Adsorption experiments for other proteins were carried out in a similar manner, using a portion (15 cm^3) of an aqueous solution of protein and 20 mg of the adsorbent. Adsorption isotherms of lysozyme on the adsorbents were obtained in similar batchwise experiments at 30 °C, using aqueous solutions containing varying concentrations of lysozyme and adsorbents.

3. Results and discussion

3.1. Characterization of phosphorylated bacterial cellulose

Reaction conditions for preparing PBC were reported in our previous study (Oshima et al., 2008). The degree of phosphorylation of PBC was controlled by changing the amount of urea and phosphoric acid in the reaction mixture. In the present study, PBCs and PPCs with varying degrees of phosphorylation were prepared under different conditions to study the adsorption properties of proteins. In the scanning electron micrographs of typical PBC and PPC shown in

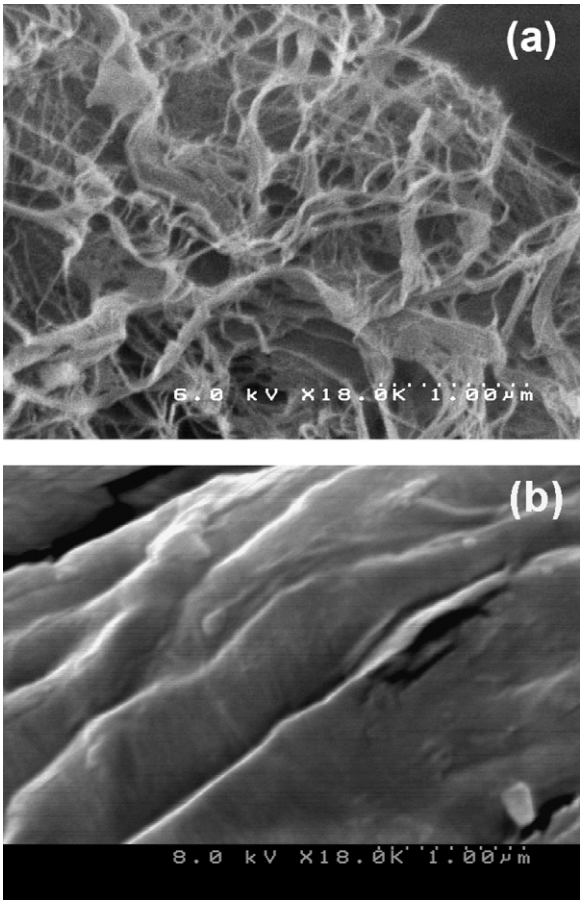

Fig. 2. Scanning electron micrographs of (a) PBC, and (b) PPC; at 18,000× magnification.

Fig. 2 the microfibrous ribbon structure can be observed on the surface of PBC: the surface morphology was quite different from that of PPC. The ribbons of PBC were approximately 0.1 μm thick. As the microfibrous structure of PBC was similar to that of BC, the phosphorylation procedure did not influence the microfibrous structure. The specific surface areas of cellulose adsorbents determined using the N_2-BET method were 19.2 $m^2\,g^{-1}$ for PBC, 2.4 $m^2\,g^{-1}$ for PPC, 27.3 $m^2\,g^{-1}$ for BC, and 1.0 $m^2\,g^{-1}$ for PC. The specific surface area of PBC decreased compared with that of the BC starting material; however, its specific surface area was still much larger than that of PPC.

3.2. Adsorption behavior of proteins on phosphorylated bacterial cellulose

As PBC should function as an ion exchanger, the pH of the aqueous phase is one of the most important factors in protein adsorption. Adsorption profiles of albumin (M.W. 67,000 g mol^{-1}, isoelectric point (p*I*) 4.5–6), hemoglobin (M.W. 64,500 g mol^{-1}, p*I* 6.8), myoglobin (M.W. 17,000 g mol^{-1}, p*I* 6.8), and lysozyme (M.W. 14,300 g mol^{-1}, p*I* 11.1) on PBC as a function of pH are shown in Fig. 3. The proteins were adsorbed on PBC at pHs below the corresponding pIs. At such pH values the proteins are positively charged and adsorbed on the PBC anionic adsorbent. As the isoelectric points of hemoglobin and myoglobin are similar, their adsorption profiles were similar. Lysozyme was adsorbed on PBC in a wide pH region compared with other proteins, due to lysozyme having the highest isoelectric point. By contrast, albumin was adsorbed on PBC only in weakly acidic conditions, because albumin had the lowest p*I*. The

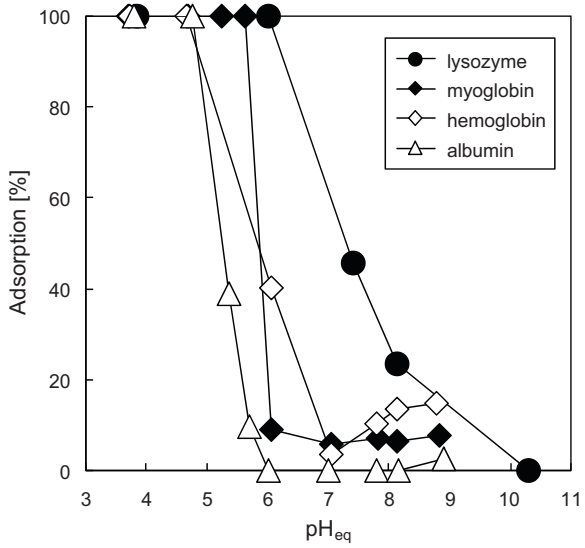

Fig. 3. Adsorption profiles of proteins on PBC as a function of pH. Adsorbent, 20 mg; volume = 15 cm^3; $[lysozyme]_{ini}$ = 0.14 g dm^{-3}, $[myoglobin]_{ini}$ = 0.10 g dm^{-3}, $[hemoglobin]_{ini}$ = 0.20 g dm^{-3}, $[albumin]_{ini}$ = 0.80 g dm^{-3}.

BC starting material did not adsorb lysozyme at all pHs that were examined (data not shown). The implication is that the proteins are adsorbed on PBC via electrostatic interaction.

Proteins which are adsorbed on PBC via electrostatic interaction can be desorbed by contacting with aqueous alkali solution, because the proteins are negatively charged and electrically repelled against phosphoric acid group of PBC. More than 90% of lysozyme adsorbed on PBC was desorbed at pH 11.8.

Fig. 4 shows the effect of sodium chloride concentration on adsorption of lysozyme on PBC at pH 4.5. As the adsorption of the protein proceeds via electrostatic interaction, adsorption was reduced by increasing the salt concentration, due to interference from sodium ion. As the solution contained 100 mmol dm^{-3} sodium phosphate buffer, the effect of addition of less than 100 mmol dm^{-3} sodium chloride on protein adsorption was small. The percentage adsorption decreased dramatically on addition of more than 100 mmol dm^{-3} sodium chloride, and lysozyme was not adsorbed at all in the presence of 400 mmol dm^{-3} NaCl.

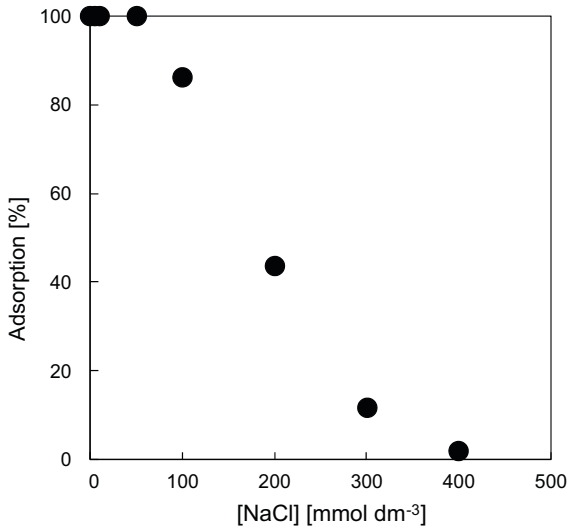

Fig. 4. Effect of concentration of sodium chloride on the adsorption of lysozyme on PBC. Adsorbent, 10 mg; volume = 15 cm^3; pH_{ini} 4.5 (100 mmol dm^{-3} sodium phosphate media), $[lysozyme]_{ini}$ = 20 μmol dm^{-3}, $[NaCl]_{ini}$ = 0–400 mmol dm^{-3}.

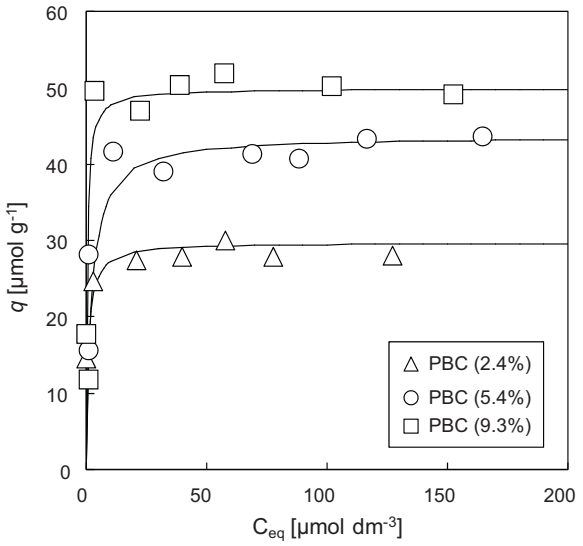

Fig. 5. Adsorption isotherms of lysozyme on PBCs at 30 °C. Parentheses show the phosphorylation percentage of each PBC adsorbent. Adsorbent, 10 mg; volume = 15 cm^3; pH 3.0.

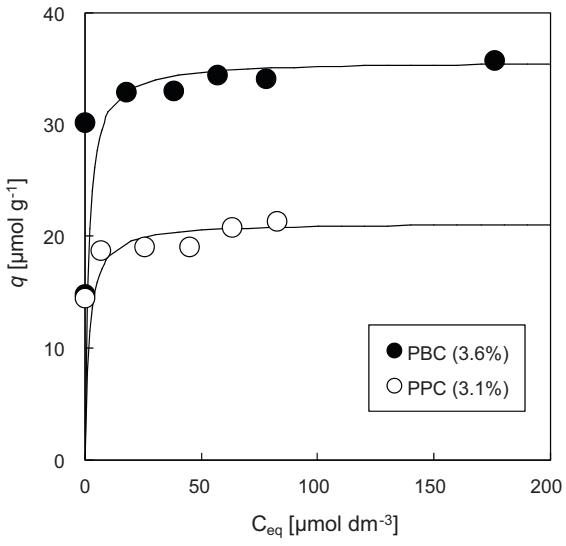

Fig. 6. Adsorption isotherms of lysozyme on PBC (3.6%) and PPC (3.1%) at 30 °C. Parentheses show the phosphorylation percentage of each adsorbent. Adsorbent, 10 mg; volume = 15 cm^3; pH 3.0.

3.3. Adsorption isotherm of lysozyme on phosphorylated bacterial cellulose

Adsorption isotherms for lysozyme on PBC at varying equilibrium concentrations are shown in Fig. 5. The three PBC adsorbents used in this experiment had different phosphorylation percentage, namely 2.4, 5.4 and 9.3%. The amount of lysozyme adsorbed increased by increasing the equilibrium concentration and approached constant values. In addition, the amount of adsorption increased with increase of the phosphorylation percentage of the PBC adsorbent. The equilibrium experimental data were correlated with the Langmuir isotherm model to determine the maximum adsorption capacities of lysozyme on PBCs (q_{max}). The Langmuir model assumes monolayer adsorption as expressed by the following mathematical expression:

$$\frac{C_e}{q} = \frac{C_e}{q_{max}} + \frac{1}{q_{max}K}$$

where q denotes the amount of lysozyme adsorbed on PBC [μmol g^{-1}], C_e is the equilibrium concentration of lysozyme in the aqueous solution [μmol dm^{-3}], and K is the adsorption equilibrium constant [dm^3 μmol^{-1}]. The theoretical adsorption isotherms cal-

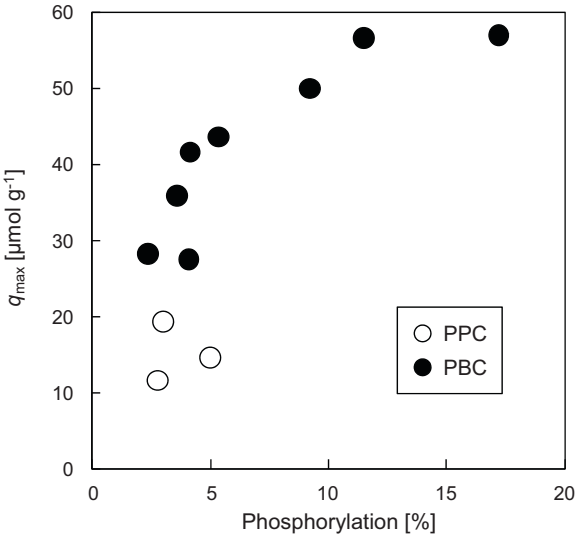

Fig. 7. Relationship between the phosphorylation percentages of PBCs and PPCs and the adsorption capacity of lysozyme on PBCs and PPCs.

Table 1
Reaction conditions for preparing PBCs and PPCs, phosphorylation percentages, and maximum adsorption capacities of lysozyme on PBC and PPC adsorbents. Parentheses in No. show the phosphorylation percentage of each adsorbent.

No.	Conditions for phosphorylation						Phosphorylation [%]	Amount of phosphoric acid group [mmol g^{-1}]	q_{max} for lysozyme [μmol g^{-1}]
	Starting material	Cellulose [g]	Urea [g]	DMF [cm^3]	Phosphoric acid [cm^3]	Reaction time [h]			
PBC (4.1%)	BC	1.0	100	500	200	8	4.1%	0.25	41.7
PBC (3.6%)	BC	1.0	75	300	15	4	3.6%	0.22	35.7
PBC (5.4%)	BC	0.2	20	500	4.0	6	5.4%	0.32	43.7
PBC (2.4%)	BC	1.0	45	500	20	8	2.4%	0.15	28.1
PBC (11.5%)	BC	2.0	90	250	40	21	11.5%	0.67	56.5
PBC (4.1%-2)	BC	2.0	90	200	40	3	4.1%	0.25	27.3
PBC (9.3%)	BC	0.4	33	50	8.0	8	9.3%	0.55	50.0
PBC (17.2%)	BC	1.0	45	100	20	8	17.2%	0.98	56.8
PPC (3.1%)	PC	1.0	45	100	20	8	3.1%	0.19	19.2
PPC (2.8%)	PC	1.0	45	100	40	10	2.8%	0.17	11.7
PPC (5.1%)	PC	2.0	90	150	40	21	5.1%	0.30	14.5

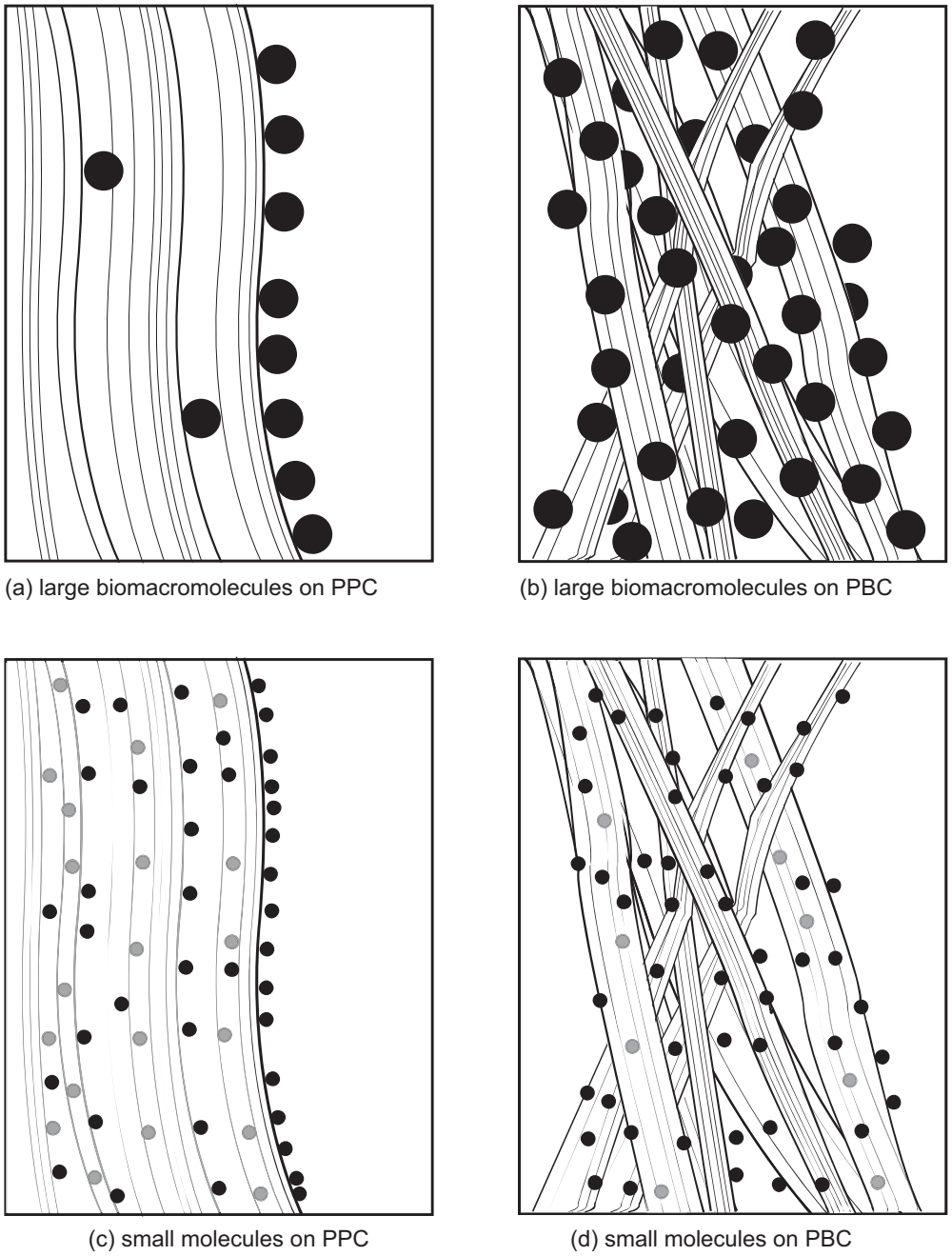

Fig. 8. Conceptual illustration of adsorption of large biomacromolecules such as proteins as well as small molecules on PBC and PPC.

culated from the Langmuir isotherm model are depicted in Fig. 5. As the calculated data agreed with the experimental values, the adsorption of lysozyme proceeds according to monolayer adsorption via electrostatic interaction. The q_{max} values of lysozyme on PBCs with percentage phosphorylation 2.4, 5.4 and 9.3% were evaluated as 28.1, 43.7 and 50.0 [μmol g^{-1}], respectively.

Fig. 6 shows adsorption isotherms of lysozyme on PBC (3.6%) and PPC (3.1%), as well as those on the BC and PC starting materials. The amount of lysozyme adsorbed on PBC was much higher than on PPC, although the phosphorylation percentages were similar. However, lysozyme was not adsorbed on BC and PC, even though the concentration of lysozyme was increased.

The reaction conditions for preparing PBCs and PPCs, the phosphorylation percentages, and the maximum adsorption capacities of lysozyme on the PBC and PPC adsorbents, calculated with the Langmuir model from the experimentally determined adsorption isotherms using each adsorbent, are summarized in Table 1. From the q_{max} values of lysozyme on PBC (17.2%) and PPC (3.1%) in Table 1, it is apparent that PBC shows much larger adsorption capacity for lysozyme than does PPC prepared under the same conditions. This result can be simply explained by the difference of the phosphorylation percentages: the degree of phosphorylation of BC was higher than that of PC under the same conditions. However, PBCs showed larger q_{max} values for lysozyme compared with PPCs which had larger phosphoric acid group contents. Thus the q_{max} values of PBC (4.1%), PBC (3.6%), PBC (2.4%), and PBC (4.1%-2) were higher than that of PPC (5.1%). The relationship between the phosphorylation percentages of PBCs and PPCs and the adsorption capacity of

Table 2
Maximum adsorption capacities of lysozyme, tryptophan methyl ester (Trp-OMe), and lanthanum (La(III)) on PBC (3.6%) and PPC (3.1%).

Adsorbent	Amount of phosphoric acid group [mmol g^{-1}]	Material	Lysozyme	Trp-OMe	La(III)
		M.W. (A.W.)	14,300	219	(139)
PBC (3.6%)	0.22	q_{max} [mmol g^{-1}]	35.7×10^{-3}	0.371	0.193
PPC (3.1%)	0.19		21.4×10^{-3}	0.367	0.170
$q_{max\,(PBC\,(3.6\%))}/q_{max\,(PPC\,(3.1\%))}$			1.67	1.01	1.14

lysozyme on PBCs and PPCs are shown graphically in Fig. 7. The q_{max} value using PBCs increased with increasing percentage phosphorylation. It is apparent that the q_{max} values of PBCs were much higher than those of PPCs. The adsorption capacities of PBCs for the protein were more than twice those of PPCs with similar phosphorylation percentages.

The adsorption behavior of various cationic materials, with varying molecular weight, on PBC and PPC were investigated to further study the adsorption properties. Table 2 shows the maximum adsorption capacities of lysozyme, tryptophan methyl ester (Trp-OMe), and lanthanum (La(III)) on PBC (3.6%) and PPC (3.1%). The adsorbates differed in their molecular weight (thus in molecular size). The ratios of q_{max} for PBC to q_{max} for PPC for adsorbates ($q_{max\,(PBC\,(3.6\%))}/q_{max\,(PPC\,(3.1\%))}$) are also shown in Table 2. The q_{max} value for lysozyme on PBC was much larger than on PPC. By contrast, the q_{max} values of Trp-OMe and La(III) on PBC and PPC were similar. Thus PBC exhibited higher adsorption capacity especially for relatively large biomacromolecules.

Fig. 8 shows a conceptual illustration of adsorption of large biomacromolecules such as proteins, as well as small molecules, on PBC and PPC. As small adsorbates can access internal adsorption sites, the amount of adsorption on PBC and PPC is proportional to the percentage phosphorylation. By contrast, large adsorbates cannot access internal adsorption sites, and adsorption of large adsorbates such as proteins proceeds only at the surface of the adsorbent. As the specific surface area of PBC is much larger than that of PPC, the number of adsorption sites for the large adsorbate on PBC is larger than on PPC. As a result, PBC exhibits larger adsorption capacity for large adsorbates compared with PPC, notwithstanding similar phosphorylation percentages. These results are consistent with the tendencies observed in previous studies: The adsorption capacities of the relatively large molecules cellobiose dehydrogenase (M.W. = 89,000 g mol^{-1}) and xyloglucan (average M.W. = 980,000 g mol^{-1}) on BC were found to be much larger than those on microfibrillated cellulose and bleached hardwood kraft pulp (Ougiya et al., 1998). Similarly, quaternary ammonium bacterial cellulose showed a higher adsorption capacity for hemoglobin compared with quaternary ammonium plant cellulose (Niide et al., 2010).

4. Conclusions

Phosphorylated bacterial cellulose, which has a microfibrous structure, was found to be effective as an adsorbent for proteins. The specific surface area of phosphorylated bacterial cellulose is much larger than that of phosphorylated plant cellulose. Proteins are adsorbed on phosphorylated bacterial cellulose via electrostatic interaction at pHs lower than their isoelectric points. Due to its larger surface area, phosphorylated bacterial cellulose shows larger adsorption capacity for proteins compared with phosphorylated plant cellulose. Thus bacterial cellulose is attractive as a platform for preparation of adsorbents, for concentration of biomacromolecules in bioanalytical chemistry.

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