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Selective removal of DNA from protein solution with copolymer particles derived from *N*,*N*-dimethylaminopropylacrylamide

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Abstract

To remove nucleic acids from cellular products as drugs, cross-linked *N*,*N*-dimethylaminopropylacrylamide (DMP) particles with cationic functional groups were prepared. The particle's hydrophobicity and its anion-exchange capacity were easily adjusted by changing the cross-linking agent and the DMP ratio in the cross-linking, respectively. When divinylbenzene (DVB) was used as a cross-linking agent and the DMP ratio (in the cross-linking) was adjusted to 90 mol%, the particles (DMP–DVB, 90:10) showed the highest adsorbing activity of DNA (salmon spermary). Its adsorption capacity was 54 mg/ml adsorbent. On the other hand, the adsorption of bovine serum albumin (BSA) to the DMP–DVB extremely increased with increase in the adsorbent's pore size (molecular mass exclusions; M_{lim}) from 2×10^3 to 1×10^4, but decreased with increase in the buffer's ionic strength (μ) to 0.2 or stronger. As a result, when the DMP–DVB (80:20) with M_{lim} 2×10^3 was used as adsorbent by a column method at pH, 7.2 and $\mu = 0.17$, it only selectively removed DNA from a BSA solution, including 1000 μg/ml of BSA and 10 μg/ml of DNA. The adsorbent decreased the concentration of DNA in the BSA solution to <10 ng/ml, and the recovery rate of BSA was more 98%.

Keywords: Adsorbents; Ion-exchange capacity; Hydrophobicity; Pore size; DNA; Albumin; Proteins; Dimethylaminopropylacrylamide

1. Introduction

In the industry of recombinant DNA technology, the cellular products, such as protein and protective antigen, are always contaminated with DNA originating from host cell. Therefore, such contaminants have to be removed from biopharmaceutical product solutions used for intravenous administration, because of concerns about the possibility of nonspecific pathophysiological reactions in mammals [1]. In 1997, a World Health Organization consultative group recommended [2] that the safety risk was negligible or non-existent in products that contained less than 10 ng per dose of cellular DNA.

To remove DNA from protein solutions, the selective adsorption method has proven to be most effective. Already it has been reported that various cationic polymer adsorbents, such as chitosan particles [3,4] and aminated poly(γ-methyl L-glutamate) (PMLG) spherical porous particles [5], were very useful as adsorbents for anionic bio-related polymers, such as lipopolysaccharides (LPs) and DNA. However, it has been reported [6] that the commercial DNA adsorbent based on chitosan cannot selectively remove DNA from acidic protein solutions, such as bovine serum albumin (BSA), because of their high adsorption for both DNA and BSA. We have already found that the pore-sized controlled and aminated PMLG particles showed superior DNA selectivity than the chitosan adsorbent [6]. However, a great disadvantage of this type of adsorbent is the low chemical stability of an ester bond originating from the side chain of PMLG [7]. Thus, their regeneration at high and low pH is ruled out.

In this work, as novel DNA adsorbents, we developed cross-linked *N*,*N*-dimethylaminopropylacrylamide (DMP) spherical copolymers, which have *N*-allylacrylamide (AA), *N*,*N*′-methylene-*bis*-acrylamide (MBAA), or divinylbenzene (DVB) as a cross-linking agent (structures of the monomers: Fig. 1). The DMP monomer has cationic properties originating from tertiary amino-groups, and has higher pK_a (10.35) than chitosan (pK_a: 6.2). We already reported

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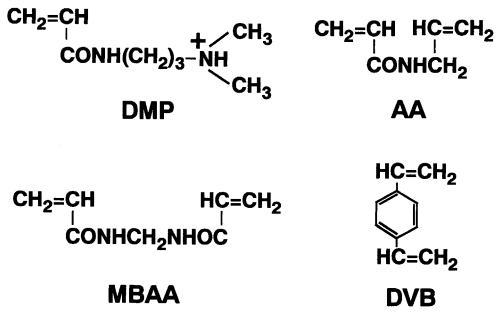

Fig. 1. Structures of the monomers used.

[8] that the cross-linked DMP porous particles showed high adsorption of LPs having anionic regions (phosphoric acid groups). Nucleic acids (DNA and RNA) are also anionic bio-related polymers with phosphoric acid groups. Thus, it is expected that the cross-linked DMP particles also show high DNA-binding activity. The particles, being composed of –CONH– bonds originating from DMP, are also expected to keep their entire structure in solutions of high and low pH.

This paper describes the effects of the cross-linking content and pore size of the cross-linked DMP particles on the adsorption of purified DNA or protein by the particles, and then provides a method for the chromatographic removal of DNA from a protein solution, which are contaminated with DNA, using the cross-linked DMP adsorbents.

2. Experimental

2.1. Materials

DMP monomer (Kohjin, Tokyo, Japan) and AA monomer (Kohjin) were purified by vacuum distillation at 131 °C/1 mmHg and 115 °C/0.7 mmHg (1 mmHg = 133.322 Pa), respectively. DVB monomer (Wako, Osaka, Japan) was purified by extraction with 0.1 M sodium hydroxide. MBAA monomer was purchased from Sigma. The purified DNA (from salmon spermary, M_r: 3×10^5) was purchased from Wako. BSA [M_r: 6.9×10^4; isoelectric point (p*I*), 4.9], and γ-globulin (from human serum, M_r: 1.6×10^5; p*I*, 7.4) were purchased from Sigma. The fluorescent dye 4′,6-diamidino-2-phenylindole dihydrochloride hydrate [9] for fluorometric analysis was purchased from Nacalai Tesque (Japan). The chitosan particles (Kurimuver II) [3,4] were purchased from Kurita Water Industries, Tokyo and used as a standard adsorbent.

2.2. Preparation of cross-linked DMP adsorbents

DMP–AA spherical particles were prepared by suspension cross-linking as follows: DMP monomer, AA monomer, 30% (v/v) of 1-hexanol as a diluent, and 2% (w/w) azo-*bis*-*iso*-butyronitrile (AIBN) as an initiator were mixed at room temperature. The mixture was added to a 25% (w/w) anhydrous sodium sulfate solution containing 1% sodium carboxymethylcellulose. The suspension was stirred and heated at 80 °C for 12 h. The DMP–AA copolymer particles obtained were washed successively with cold and hot water, methanol and ethanol.

DMP–MBAA particles were prepared by the method used for DMP–AA particles using DMP monomer, MBAA monomer, 20% (v/v) chloroform as a diluent, and 2% (w/w) AIBN.

DMP–DVB particles were prepared by the method used for DMP–AA particles using DMP monomer, DVB monomer, 20% (v/v) diethylbenzene as a diluent, and 2% (w/w) AIBN.

These particles with diameters of 44–105 μm were used as adsorbents.

2.3. Determination of pore size, hydrophobicity, and anion-exchange capacity

The pore size of the matrix in the adsorbent was estimated as the molecular mass exclusions (M_{lim}) by aqueous size-exclusion chromatography. The M_{lim} value was determined as the molecular mass of polysaccharide by extrapolating the linear part of the curve as described previously [10].

The hydrophobicity of the adsorbent was estimated from elution behavior of 1-alcohol obtained by an aqueous chromatography similar to determination of the pore size. 1-Methanol, 1-ethanol, 1-propanol, 1-butanol, 1-pentanol, and hexanol were used as permeable substances.

The anion-exchange capacity of the adsorbent was quantified by pH titration and by elemental analyses.

2.4. DNA and protein assay

The DNA concentration in the sample solution was determined by fluorometric analysis [9] with a spectrofluorophotometer FP-6500 (Jasco) using the fluorescent dye 4′,6-diamidino-2-phenylindole dihydrochloride hydrate. The protein concentration was measured at 280 nm with a UVIDEC-660 spectrophotometer (Jasco).

2.5. Adsorption of DNA and other cellular products

The adsorption of nucleic acids was measured by a batchwise method as follows: the adsorbent was washed and equilibrated with various 0.02 M phosphate buffers (pH, 7) with different ionic strengths. The ionic strength of the buffer was adjusted by changing the content of sodium chloride. A 0.1–0.2 ml portion of wet adsorbent was suspended in 2–4 ml of DNA 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 DNA content of the filtrate was determined. The apparent dissociation constant ($K_{d,app}$) between the DNA and the adsorbent was estimated by adsorption isotherm as previously described [6]. The adsorption

of cellular products other than DNA was investigated by a method similar to DNA-binding assay.

2.6. Removal of DNA from a protein solution

The removal of DNA was measured by a frontal column method as followed: A 1.7 ml portion of wet adsorbent was packed into a sterilized stainless column (10 cm × 0.46 cm, i.d.). The column was washed with 20 ml of 2.0 M sodium chloride and then equilibrated with 0.02 M phosphate buffer (pH, 7.2; $\mu = 0.17$). A 120 ml portion of protein solution (1000 μg/ml) containing DNA (10 μg/ml, purified DNA from salmon spermary) was passed through the column at a flow rate of 0.2 ml/min at room temperature. The column was then washed with 30 ml of the buffer. Fractions of 6 ml were collected and the concentrations of protein and DNA in each fraction were measured. The column was reused after washing with 20 ml of 0.2 M sodium hydroxide, 2.0 M sodium chloride, ultra-pure water, and the buffer.

3. Results and discussion

3.1. Effects of adsorbent's properties on adsorption of DNA

In order to achieve the selective adsorption of DNA, it is important to decrease the interaction between the adsorbent and other cellular products, such as protein. We had already found [6] that the adsorbing activity of net-negatively charged polymers, such as BSA and DNA, by the aminated PMLG adsorbent increased drastically with increasing pore size in the adsorbent's matrix to sizes over the molecular mass of the polymers. On the other hand, it was clear that when the pore size of aminated PMLG was adjusted to M_{lim} below 10^4, the adsorbent showed good DNA-selectivity without adsorption of the protein. This result suggests that although adsorption of the protein caused mainly by entry of the protein's molecules into the pore of the adsorbent, the DNA was adsorbed not only into the pore of the adsorbent but also on its surface. Therefore, various cross-linked DMP (DMP–AA, DMP–MBAA, and DMP–DVB) particles with small pore sizes of M_{lim} 1×10^3 to 8×10^3, anion-exchange capacities of 3.7–5.2 meq./g, and swelling degrees (in water) of 4.3–7.9 were prepared as adsorbents (Table 1).

The adsorbing activities of the various adsorbents for DNA were examined by a batchwise method. The purified DNA (from salmon spermary) was used as a standard DNA sample. When the effect of cross-linking agent of adsorbent on the DNA-adsorbing activity was examined under conditions of DMP ratio of 80 mol% and cross-linking agent's ratio of 20 mol%, the adsorbent with DVB as a cross-linking agent showed highest adsorbing activity (Table 1). The adsorbing capacities of DMP–AA (80:20), DMP–MBAA (80:20), and DMP–DVB (80:20) were 0.5, 2, and 11 mg per ml of wet adsorbent, respectively. In the DMP–DVB adsorbents, while the DMP ratio increased from 50 to 90 mol%, the DNA-adsorbing capacity increased from 2 to 54 mg/ml and the $K_{d,app}$ of DNA to the adsorbent decreased remarkably from 5.5×10^{-8} to 4.5×10^{-11} M (as M_r of the DNA: 3×10^5), at ionic strength $\mu = 0.05$ and pH, 7.0. The $K_{d,app}$ value shows DNA-removing activity of the adsorbent which arise when the DNA removal from a dilute DNA solution is examined, and the adsorbing capacity shows its DNA-adsorbing content which arise when the DNA adsorption from a thick DNA solution is examined. In the removal of DNA from a dilute DNA solution by adsorption, the DNA-removing activity depends more on the $K_{d,app}$ of DNA than on the adsorbing capacity. The smaller the $K_{d,app}$ value, the stronger the DNA-removing activity of the adsorbent. Therefore, it was found that the DMP–DVB (90:10) with the smallest $K_{d,app}$ value has highest DNA-removing activity in all adsorbents. It was also found that the removing activities of DMP–DVB (80:20) and (70:30) are higher

Table 1
Characteristics of cross-linked DMP adsorbents

Adsorbent	Molar ratio[a] (mol%)				Anion-exchange capacicty (meq./g)	Pore size of matrix (M_{lim}[b])	S_d[c] (wet-ml/dry-g)	Adsorption capacity of DNA[d] (mg/ml)	$K_{d,app}$ of DNA[d] (M)
	DMP	AA	MBAA	DVB					
DMP–AA (80:20)	80	20	0	0	5.1	2×10^3	7.9	0.5	9.5×10^{-8}
DMP–MBAA (80:20)	80	0	20	0	5.0	2×10^3	5.0	2	1.0×10^{-9}
DMP–DVB (90:10)	90	0	0	10	5.2	8×10^3	5.9	54	4.5×10^{-11}
DMP–DVB (80:20)	80	0	0	20	5.0	2×10^3	5.2	11	5.7×10^{-10}
DMP–DVB (70:30)	70	0	0	30	4.5	2×10^3	4.8	10	8.2×10^{-10}
DMP–DVB (50:50)	50	0	0	50	3.7	1×10^3	4.3	5	2.5×10^{-8}
Chitosan particles[e]	–	–	–	–	3.5	1×10^5	16.5	17	9.3×10^{-9}

[a] Mol% of the monomer in the cross-linking.
[b] Value deduced as a molecular mass of polysaccharide [10].
[c] Degree of swelling in water.
[d] The DNA-adsorption capacity per ml adsorbent and the $K_{d,app}$ of DNA to adsorbent were estimated by adsorption isotherm. The adsorption isotherm of DNA was determined using a batchwise method with 0.1 ml of wet adsorbent and 4 ml of a DNA solution (DNA from salmon spermary: 1–1000 μg/ml, pH, 7.0; $\mu = 0.05$). The $K_{d,app}$ was expressed in mol/l (M) of molecular weight of DNA ($M_r = 3 \times 10^5$).
[e] Kurimover-II [3,4].

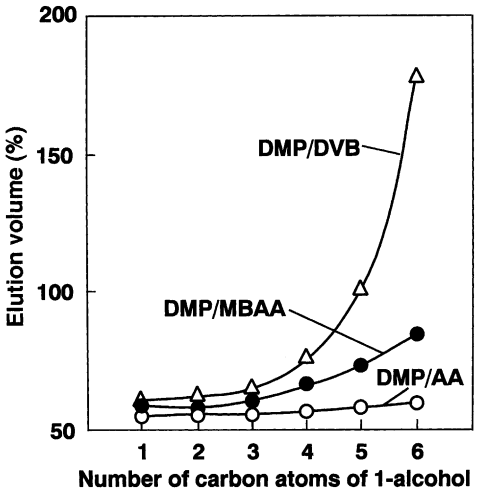

Fig. 2. Elution behaviors of 1-alcohols with various cross-linked DMP columns (DMP–AA (80:20), DMP–MBAA (80:20), and DMP–DVB (80:20). A 5 μl portion of 1-alcohol dissolved in water (2 mg/l) was injected to the column (10 cm × 0.46 cm, i.d.) at 0.5 ml/min and 25 °C. The elution volume of 1-alcohol is expressed as a percentage of the column volume. Number of carbon atoms of 1-alcohol: (1) methanol; (2) ethanol; (3) propanol; (4) butanol; (5) pentanol; (6) hexanol.

than those of chitosan particles because of their smaller $K_{d,app}$ value (5.7×10^{-10} and 8.2×10^{-10}, respectively) than the chitosan particles ($K_{d,app}$: 9.3×10^{-9}).

Fig. 2 shows the elution behaviors of 1-alcohols by an aqueous chromatography with the columns packed of DMP–AA, DMP–MBAA, and DMP–DVB particles. In aqueous chromatography, the larger the elution volume of 1-alcohol (as a permeable sample), the stronger the hydrophobic properties of the column packing [8]. The hydrophobicity of 1-alcohol increases with an increase in its carbon number from 1 (methanol) to 6 (hexanol). As shown in Fig. 2, when the M_{lim} of the column and the mol ratio of the cross-linking agent were 2×10^3 and 20 mol%, respectively, the DMP–DVB column having DVB as cross-linking agent showed always the largest elution volumes of all 1-alcohols. This result indicates that the DMP–DVB has the strongest hydrophobic properties in the three adsorbents.

3.2. Effects of various factors on selective adsorption of DNA

The effects of adsorbent's cross-linking agent (AA, MBAA, or DVB) and buffer's ionic strength on DNA adsorption are shown in Fig. 3. The DNA adsorption was measured by the batchwise method. The DNA-adsorbing activity of each adsorbent decreased with an increase in the ionic strength of the buffer. As a result, at a wide ionic strength of $\mu = 0.05$–0.8, the DMP–DVB has always the greatest DNA-adsorbing activity (99–57%) in the three adsorbents.

For the selective adsorption of DNA, it is necessary to check the interaction between the adsorbent and cellular

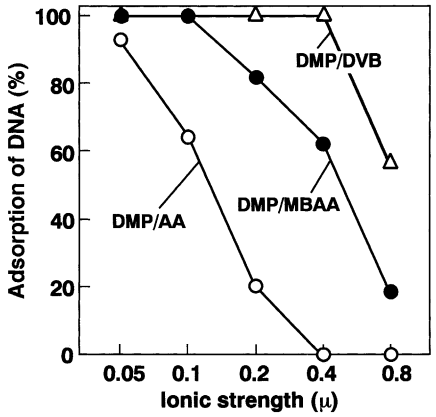

Fig. 3. Effect of the buffer's ionic strength on the adsorption of DNA by various cross-linked DMP adsorbents used in Fig. 3. The adsorption of DNA was determined by a batchwise method with 0.2 ml of wet adsorbent and 2 ml of a DNA solution (purified DNA from salmon spermary: 500 μg/ml).

products. Fig. 4 shows the effect of DMP content in the DMP–DVB adsorbent on adsorption of cellular products (DNA, BSA, and γ-globulin) at pH, 7.0 and $\mu = 0.05$. The DNA-adsorbing activity increased from 80 to 99% with an increase in DMP ratio from 50 to 80 mol%. The DMP–DVB (80:20) and (90:10) adsorbents showed excellent DNA-adsorbing activity. Each is able to decrease the concentration of DNA from 500 μg/ml to less than 10 ng/ml. On the other hand, BSA-adsorbing activity depended more strongly on adsorbent's M_{lim} than its DMP content. The adsorbing activity remarkably increased from 8 to 88% with an increase in M_{lim} from 2×10^3 to 8×10^3 (the DMP ratio from 80 to 90 mol%). We believe that an increase in the content of DMP having amino groups leads to an increase in the degree of swelling of the adsorbent in the buffer, and

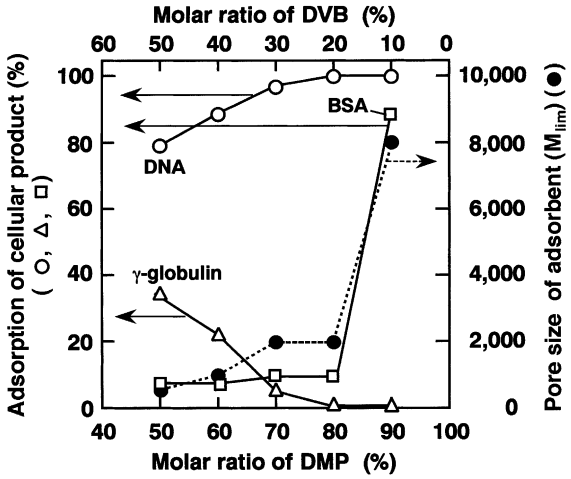

Fig. 4. Effects of adsorbent's DMP molar ratio and its pore size on the adsorption of cellular products (DNA or protein) by DMP–DVB adsorbents. The adsorption of DNA or protein was determined by a batchwise method with 0.2 ml of the wet adsorbent and 2 ml of a DNA or protein solution (500 μg/ml; pH, 7.0, ionic strength of $\mu = 0.05$).

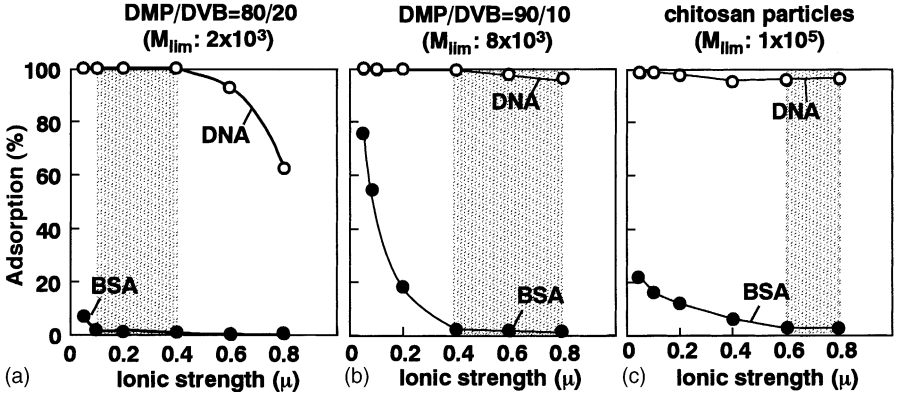

Fig. 5. Effect of a buffer's ionic strength on selective adsorption of DNA from a BSA solution (500 μg/ml; pH, 7.0, and ionic strength of $\mu = 0.05$–0.8) containing DNA (10 μg/ml) by (a) by DMP–DVB (80:20), (b) (90:10), and (c) chitosan adsorbents. The selective adsorption of DNA was determined by a batchwise method with 0.2 ml of the wet adsorbent and 2 ml of a BSA-containing solution.

that this in turn leads to an increase in M_{lim}. By contrast, the adsorption of γ-globulin increased from 2 to 34% with a decrease in the DMP ratio from 80 to 50 mol% (with an increase in the ratio of DVB from 20 to 50 mol%). Little basic proteins, such as cytochrome *c* and lysozyme, were adsorbed (<2%) by any of the DMP–DVB adsorbents [11].

The effect of the ionic strength of the buffer on the selective adsorption of DNA from a BSA-containing solution was examined by a batchwise method with various adsorbents. As shown in Fig. 5a, DMP–DVB (80:20) selectively adsorbed DNA from a BSA solution at $\mu = 0.1$–0.4, without the adsorption of BSA (<2%). DMP–DVB (90:10) and chitosan adsorbents were able to adsorb DNA selectively at $\mu = 0.4$–0.8 (Fig. 5b) and at $\mu = 0.6$–0.8 (Fig. 5c), respectively.

From these results (Figs. 2–5 and Table 1), we assumed that the adsorbing activity of the cross-linked DMP for cellular products was due to the simultaneous effects of their cationic properties and hydrophobic or other properties. The cationic adsorption is remarkably dependent on the ionic strength of the buffer, but the hydrophobic adsorption is independent of the ionic strength [6,12]. Nucleic acids (DNA and RNA) are polynucleosides having anionic regions (phosphate groups), pentoses, purine bases, and pyrimidines, and thus the charge of DNA is anionic at pH values greater than its pK_a (<2). The charge of BSA is also anionic at pH values >4.9 (its p*I*). The adsorption of DNA and BSA increased with increasing anion-exchange capacity (DMP content) of the adsorbent (Table 1 and Fig. 4). It is also dependent on the ionic strength of the buffer (Figs. 3 and 5). This suggests cross-linked DMP adsorbents adsorb DNA and BSA by ionic interaction. On the other hand, the ionic interaction of the adsorbent with γ-globulin (p*I*, 7.4) is not induced at pH, 7.0 since the charge of the protein is cationic at a pH under its p*I* value. γ-globulin is a weakly hydrophobic protein, and its adsorption by DMP–DVB adsorbent increased with an increase in hydrophobicity (content of DVB) of the adsorbent (Fig. 4). We also reported previously [6] that a hydrophobic bond was formed between DNA and non-aminated PMLG under the conditions of a wide ionic strength of $\mu = 0.05$–4.0. As shown in Figs. 2 and 3, the stronger the hydrophobicity of the adsorbent, the stronger its DNA-adsorbing activity. The DMP–DVB particles and the chitosan particles showed a high DNA adsorption at a wide ionic strength of $\mu = 0.05$–0.8 (Fig. 5). These finding suggest the DMP–DVB adsorb DNA and γ-globulin by hydrophobic binding.

Furthermore, as shown in Fig. 5a-c, each adsorbent bind more strongly with DNA than protein. This is because the pK_a of the phosphate residues of DNA is lower than the p*I* of BSA, and probably because the DNA was adsorbed by the adsorbent, through its multipoint-attachment onto the aminogroups of the adsorbent surface. At an ionic strength of $\mu = 0.05$, the DMP–DVB (90:10) and chitosan adsorbents adsorbed also BSA (77 and 23%, respectively) although each DNA-adsoving activity was strong (Fig. 5b and c). We suggest that the undesirable binding between BSA and each adsorbent probably results from the entry of BSA into the large pores (DMP–DVB, 90:10: M_{lim} 8×10^3, chitosan particles: M_{lim} 1×10^5) of the adsorbent's matrix. BSA was bound more strongly by DMP–DVB (90:10) than by chitosan particles although chitosan particles have a larger pore size. This is because the pK_a of the DMP (pK_a: 10.35) is higher than that of chitosan (p*K*: 6.2), and the adsorbing activity for net-negative charged protein, such a BSA, increases with increasing pK_a of the adsorbent. As a result, only DMP–DVB (80:20) with a small pore size of M_{lim} 2×10^3 can selectively adsorb DNA under physiological conditions (ionic strength, II $\mu = 0.1$–0.4; pH, 7.0), without the adsorption of protein (Fig. 5a).

3.3. Removal of DNA from protein solution by column chromatography

The effectiveness of DMP–DVB particles as a DNA adsorbent increased when a column chromatography process was used. The selective removal of DNA from a BSA solution was investigated using DMP–DVB (80:20) column packing

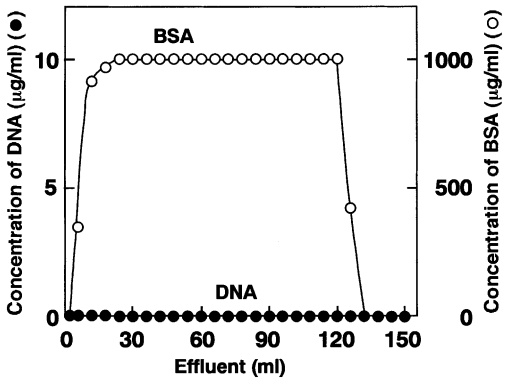

Fig. 6. Removal of DNA from a BSA solution containing DNA with the DMP–DVB (80:20) column packing. A BSA solution (1000 μg/ml, 120 ml) in 0.02 M phosphate buffer (pH, 7.2; $\mu = 0.17$) containing DNA (from salmon spermary, 10 μg/ml) was passed through a column (10 cm × 0.46 cm, i.d.) at 0.2 ml/min. The column was then washed with 30 ml of the buffer, and the concentrations of DNA and BSA in the fractions were measured.

under physiological conditions (pH, 7.2, $\mu = 0.17$). A BSA solution (1000 μg/ml) to which purified DNA (10 μg/ml) had been added was used. The result is shown in Fig. 6. The column exhibited a high DNA-removing activity without affecting the recovery of the BSA. The concentrations of DNA in the treated BSA solution decreased to less than 10 ng/ml and the recovery ratio of the BSA was 98%.

For practical applications, ease of regeneration is very important. We reported previously that the cross-linked DMP particles can be completely regenerated by frontal chromatography with 0.2 M sodium hydroxide, followed by 2.0 M sodium chloride [7]. Their stable structures, resisting extreme pH values, are due to their –CONH– bonds.

4. Conclusion

The present results suggest that DMP–DVB (80:20) spherical particles can remove any DNA from drugs and fluids used for injection, at neutral pH and ionic strength of $\mu = 0.1$–0.4. The high DNA-adsorbing activity of the DMP–DVB particles is possibly due to (1) strong cationic properties by the higher pK_a of DMP (10.35) than chitosan (6.2), (2) suitable hydrophobic properties which arise when the DVB content (in the particles) is adjusted to 20 mol%, and (3) multipoint-attachment of DNA onto the DMP–DVB chains on the particle's surface. On the other hand, their high DNA selectivity (no interaction with proteins) is due to (1) the size-exclusion effects on protein molecules through their small pore sizes (M_{lim} 2×10^3), and (2) the effect of the decrease in ionic interaction between the particles and the protein which arise when the buffer's ionic strength (μ) is adjusted to 0.1–0.4.

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