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Cibacron Blue F3G-A-attached monosize poly(vinyl alcohol)-coated polystyrene microspheres for specific albumin adsorption

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ABSTRACT

Monosize polystyrene (PS) microbeads (4 μm in diameter) were produced by phase inversion polymerization of styrene in ethanol-methoxyethanol medium. They were coated with poly(vinyl alcohol) (PVAL) by adsorption and chemical cross-linking to decrease the non-specific protein adsorption. Cibacron Blue F3G-A was then attached for specific protein adsorption. The adsorption conditions were optimized to increase the amount of PVAL by changing the initial concentration of PVAL, and using different types of salts at different ionic strengths. Higher amounts of PVAL (up to 19 mg PVAL/g PS) were loaded by increasing the PVAL initial concentration and by using Na_2SO_4 at a higher ionic strength (0.2). Bovine serum albumin (BSA) adsorption and desorption on these PS-based microbeads were also investigated under different conditions. PVAL coating prevented the non-specific BSA adsorption. A higher amount of BSA (up to 60 mg BSA/g dye-attached PS/PVAL) was specifically adsorbed on dye-attached PS microbeads, especially around pH 5 and lower ionic strengths (0.01). About 90% of the adsorbed BSA was desorbed in 1 h by using 0.5 *M* NaSCN.

INTRODUCTION

The interest in and demand for proteins in biotechnology, biochemistry and medicine have contributed to an increased exploitation of affinity chromatography. Unlike other forms of protein separation, affinity chromatography relies on the phenomenon of biological recognition, which enables biopolymers to recognize specifically, and bind reversibly, their complementary ligands (e.g., enzymes) and their substrates, hormones and their receptors, antibodies and their antigens [1–3].

Unfortunately, the preparation of sorbents carrying biological ligands is usually very expensive because the ligands themselves often require extensive purification and it is difficult to immobilize them on the carrier matrix with retention of their biological activity. As an alternative to their natural biological counterparts, the reactive triazinyl dyes have been investigated as ligands for protein affinity separation [4–6]. These dyes are able to bind proteins in a remarkably specific

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manner. They are inexpensive, readily available, biologically and chemically inert and are easily coupled to support materials. Cibacron Blue F3G-A and many other reactive dyes have been coupled to a variety of supports including agarose, cellulose, polyacrylamide , Sephadex, silica and glass [7–12]. Dye-ligand chromatography has now permitted the purification of a wide range of proteins (e.g., lactate hydrogenase, alcohol dehydrogenase, hexokinase, carboxyl peptidase) [4–12].

Recently we prepared monosize polystyrene (PS) microbeads [13,14], and in this work we attempted to use these microbeads as a carrier matrix for affinity purification of proteins. In order to prevent non-specific interactions between the hydrophobic polystyrene surface and protein molecules, and also to attach the ligand (*i.e.,* Cibacron Blue F3G-A) to the carrier matrix, these microbeads were coated with a hydrophilic layer, namely poly(vinyl alcohol) (PVAL). We selected albumin as a potential model protein. We studied both non-specific albumin adsorption on the polystyrene microbeads and also specific albumin adsorption on the dye-attached PS/PVAL microbeads. This paper describes the methods of preparation of PS/PVAL microbeads and dye attachment, and presents the results of albumin adsorption and desorption studies.

EXPERIMENTAL

Production of monosize PS microbeads

Monosize PS microbeads were produced by phase inversion polymerization of styrene. Details of the polymerization procedure were given elsewhere and are summarized below [15].

Styrene was obtained from Yarpet and was first treated with NaOH solution to remove inhibitor. The solvents, 2-methoxyethanol (BDH) and ethanol (Merck) were used without further purification. The initiator was 2,2′-azobisisobutyronitrile (AIBN) (BDH). The steric stabilizer, polyacrylic acid, was prepared by solution polymerization of acrylic acid (BDH) in 1,4-dioxane (BDH) as described previously [15]. All of the ingredients were dissolved in the solvent mixture. This single phase was polymerized at 75°C for 16 h, and then at 80°C for 8 h. The stirring rate was 250 rpm. The phase inversion polymerization recipe and conditions to obtain 4-μm monosize PS microbeads are given in Table I.

The PS microbeads were first cleaned with doubly distilled water by using the serum replacement technique and then treated with an anion- and cation-exchange resin mixture (H^+ and OH^- type, Amberlite) (BDH) to remove the stabilizer and initiator molecules physically attached to the surfaces of the microbeads.

Coating of PS microbeads with PVAL

Monosize PS microbeads were coated with PVAL by a two-step procedure. In the first step, PVAL (average M_r 14000, 100% hydrolysed) (Aldrich) was deposited on the surface of PS microbeads by a simple adsorption process carried out in an aqueous medium. In order to establish the optimum adsorption conditions, PVAL adsorption experiments were performed in the presence of three different salts, NaCl, $CaCl_2$ and Na_2SO_4. These adsorption studies were repeated at three different ionic strengths (0.05, 0.1 and 0.2). The initial PVAL concentration was also changed between 10 and 700 mg/l. In a typical adsorption experiment, first a suitable amount of PVAL was dissolved in 100 ml of water and the ionic strength of solution was adjusted by adding salt, then 3 g of dried PS microbeads were added. The solution was stirred for 2 h (*i.e.,* the equilibrium time, determined in preliminary studies) with a magnetic stirrer at 200 rpm at 25°C. At the end of the equilibrium period, microbeads were separated from the solution by centrifugation. The PVAL adsorbed

TABLE I

POLYMERIZATION RECIPE AND CONDITIONS FOR THE PRODUCTION OF MONOSIZE POLYSTYRENE MICROBEADS WITI-I A DIAMETER OF 4 μm

AIBN	0.75 g
Styrene	35 ml
Ethanol	100 ml
2-Methoxyethanol	100 ml
Polyacrylic acid	3.5 g
Temperature and time	75°C for 16 h and 80°C for 8 h
Stirring rate	250 rpm

on the PS microbeads was determined by measuring the initial and final concentrations of PVAL within the adsorption medium, according to the KI-I, method, spectrophotometrically at 690 nm [16].

In the second step, PVAL molecules adsorbed on the PS microbeads were chemically cross-linked to give a stable PVAL coating on the microbeads. After adsorption of PVAL from a suitable solution, the final acid concentration of the medium was adjusted to 0.1 *M* by adding HCl. A 10-mg amount of terephthaldehyde (Sigma) was dissolved in 10 ml of water and this solution was added to the previous medium. The batch was first stirred for 48 h at 500 rpm at 25°C. The temperature was then increased to 80°C and cross-linking was completed in 4 h in a sealed reactor with a stirring rate of 400 rpm. The microbeads were filtered and washed several times with distilled water. The PVAL-coated microbeads were stored under distilled water.

Characterization of microbeads

The size and size distribution of PS microbeads were measured by using an optical microscope (Nikon, Alphaphot YS). The PS microbeads were filtered and dried in a vacuum oven and then optical micrographs were taken. The presence of PVAL on the surface of the PS microbeads was confirmed by IR spectrophotometry (Hitachi Model 230 instrument). PS and PS/PVAL microbeads were filtered and washed several times with distilled water and dried in a vacuum oven. The IR spectra of the dried microbeads in a KBr (IR grade, Merck) disc were obtained. The IR spectrum of a KBr disc containing no dried polymeric microbeads was also recorded and no hydroxyl peak which might originate from the moisture of KBr was detected in this spectrum.

Dye attachment to PSIPVAL microbeads

A 300-mg amount of Cibacron Blue F3GA (Polyscience) was dissolved in 10 ml of water. This dye solution was added to the aqueous microbeads latex prepared by dispersing 3.0 g of PS/PVAL microbeads in 90 ml of distilled water, then 4 g of NaOH were added. The medium was heated at 80°C in a sealed reactor for 4 h at a stirring rate of 400 rpm. The microbeads were filtered and washed with distilled water and methanol several times until all the unbound dye was removed. The microbeads carrying the dye were then redispersed in 30 ml of distilled water.

BSA adsorption and desorption studies

Bovine serum albumin (BSA, lyophilized, Fraction V; Sigma) was selected as a model protein. BSA adsorption on the PS, PS/PVAL and dye-attached PS/PVAL microbeads was studied. Adsorption studies were performed at different pH values. The pH of the adsorption medium was changed between 4 and 8 by using different buffer systems (CH_3COONa–CH_3COOH for pH 4-6, K_2HPO_4–KH_2PO_4 for pH 7 and NH_3–NH_4Cl for pH 8). Adsorption experiments were repeated at two different ionic strengths (0.01 and 0.1, adjusted by using NaCl). The initial BSA concentration was varied between 0.5 and 7.0 mg/ml. In a typical adsorption experiment, BSA was dissolved in 25 ml of buffer solution containing NaCl, 0.2 g of microbeads was added and the adsorption experiments were conducted for 2 h at 25°C at a stirring rate of 100 rpm. At the end of equilibrium period, the microbeads were separated from the solution by centrifugation. The albumin adsorption capacity was determined by measuring the initial and final concentrations of BSA within the adsorption medium spectrophotometrically at 280 nm [17-19].

BSA desorption experiments were performed in a buffer solution containing 0.5 *M* NaSCN at pH 8.0. The BSA-adsorbed microbeads were placed in the desorption medium and stirred for 1 h at 25°C. The final BSA concentration was determined spectrophotometrically at 280 nm. The desorption ratio was calculated from the amount of BSA adsorbed on the microbeads and the final BSA concentration in the desorption medium.

RESULTS AND DISCUSSION

Characteristics of PS microbeads

As shown in Fig. 1, monosize (R.S.D. <1%) PS microbeads with a diameter of 4 μm were

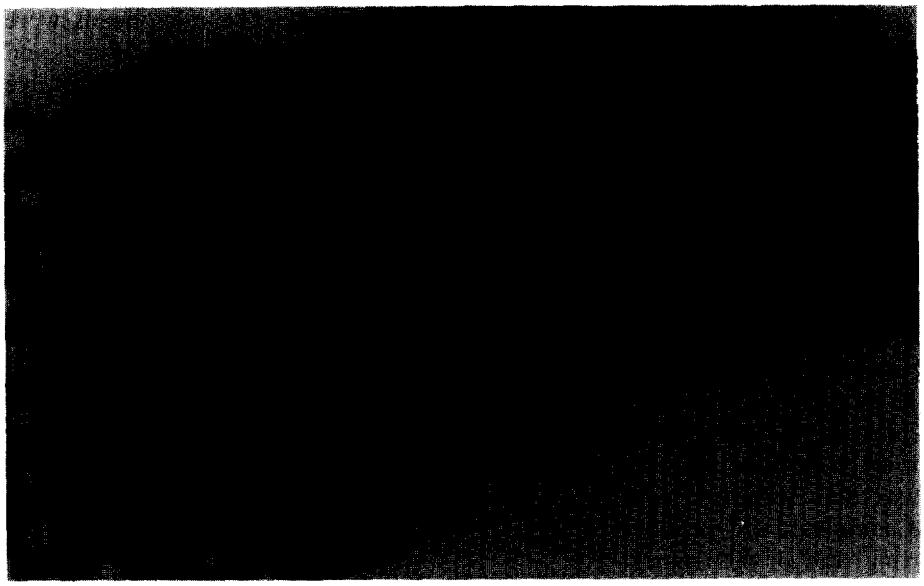


Fig. 1. Optical micrograph of monosize PS microbeads (4 μm in diameter).

obtained at the polymerization conditions given in Table I.

The presence of PVAL on the surface of PS/PVAL microbeads was confirmed by IR spectrophotometry. Fig. 2 shows the IR spectra of both PS and PS/PVAL microbeads. The hydroxyl band observed at 3500 cm^{-1} indicated the presence of PVAL on the PS/PVAL surface.

The blue colour of Cibacron Blue F3G-A-attached PS/PVAL microbeads clearly indicated the presence of dye on the surfaces of the microbeads.

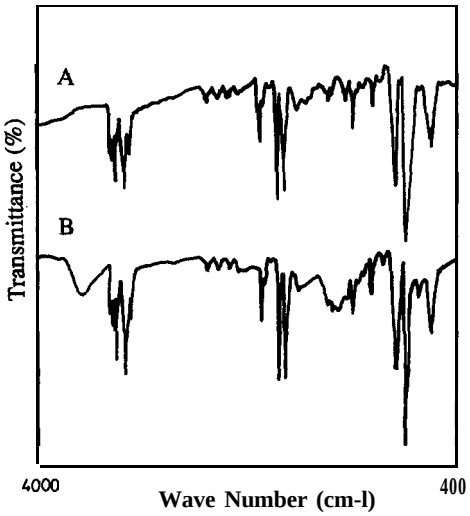


Fig. 2. IR spectra of (A) PS and (B) PS/PVAL microbeads.

PVAL adsorption/coating

The PVAL adsorption rate and capacity on PS microbeads were studied in adsorption experiments in which PVAL was adsorbed from aqueous PVAL solutions (consisting of only PVAL and distilled water) with different initial PVAL concentrations. Fig. 3A gives the adsorption rate curves. These curves indicate that the adsorption process was completed within 2 h and this value can be considered to be the equilibrium time for PVAL adsorption. The plateau values of these curves give the adsorption capacities of PS microbeads which change with the initial concentration of PVAL in the adsorption medium. The adsorption isotherm (at 25°C) in Fig. 3B was obtained by using the plateau values of these curves. As can be seen, the amount of PVAL absorbed first increased with increasing initial PVAL concentration, but reached a plateau after an initial PVAL concentration of 500 mg/l. This plateau means that PVAL adsorption on PS microbeads is typically a Langmuir-type monolayer adsorption [14].

In order to increase the amount of PVAL adsorbed on the PS microbeads, three different

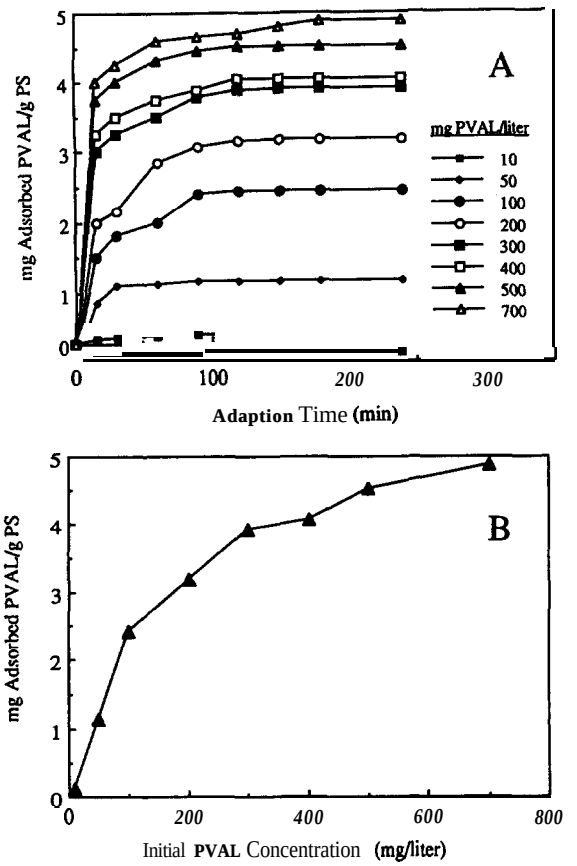


Fig. 3. PVAL adsorption on PS microbeads: (A) PVAL adsorption rate curves; and (B) PVAL adsorption isotherm. Initial PVAL concentration: ❑ = 10; ◆ = 50; ● = 100; 0 = 200; ■ =300; □ = 400; ▲ = 500; A=700 mg/l.

salts, $NaCl$, $CaCl_2$ and Na_2SO_4, were added to the adsorption medium. The ionic strength of the medium was also varied between 0.05 and 0.2 by simply changing the concentration of these salts. It should be noted that no aggregation of microbeads was observed within this ionic strength range.

Examples of PVAL adsorption isotherms are given in Fig. 4. The results indicate that the largest amount of PVAL was adsorbed from the medium containing Na_2SO_4. The amount of PVAL adsorbed on the microbeads increased with increasing ionic strength of each salt. The maximum PVAL adsorption capacity obtained in this study was 12.7 mg/m^2 (or 19.0 mg/g PS) (with an initial PVAL concentration of 700 mg/l, Na_2SO_4 as the salt and the ionic strength 0.2).

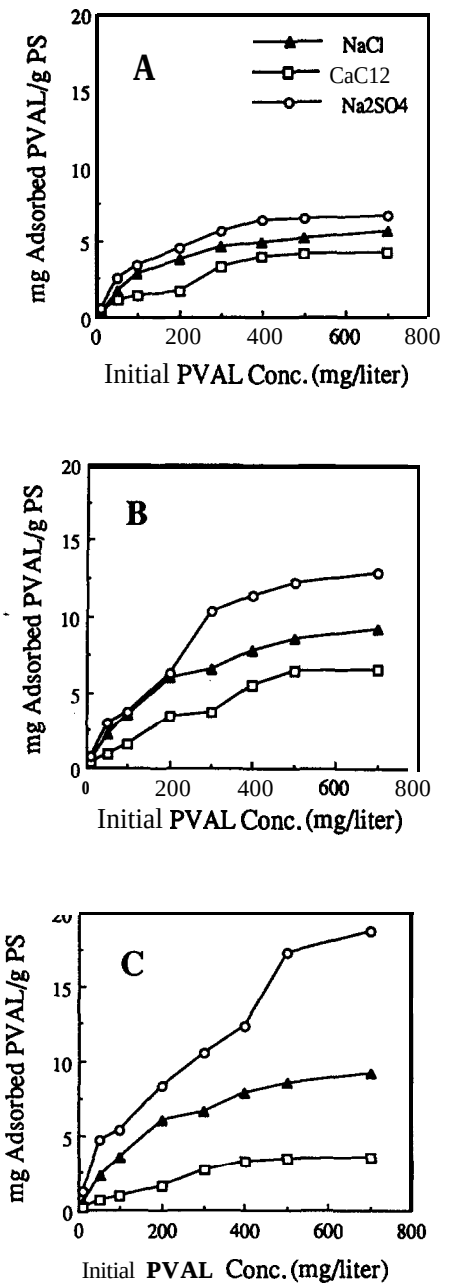


Fig. 4. PVAL adsorption isotherms obtained in the presence of three different salts at three different ionic strengths: (A) 0.05; (B) 0.1; and (C) 0.2. A = NaCl; ❑ = $CaCl_2$; 0 = Na_2SO_4.

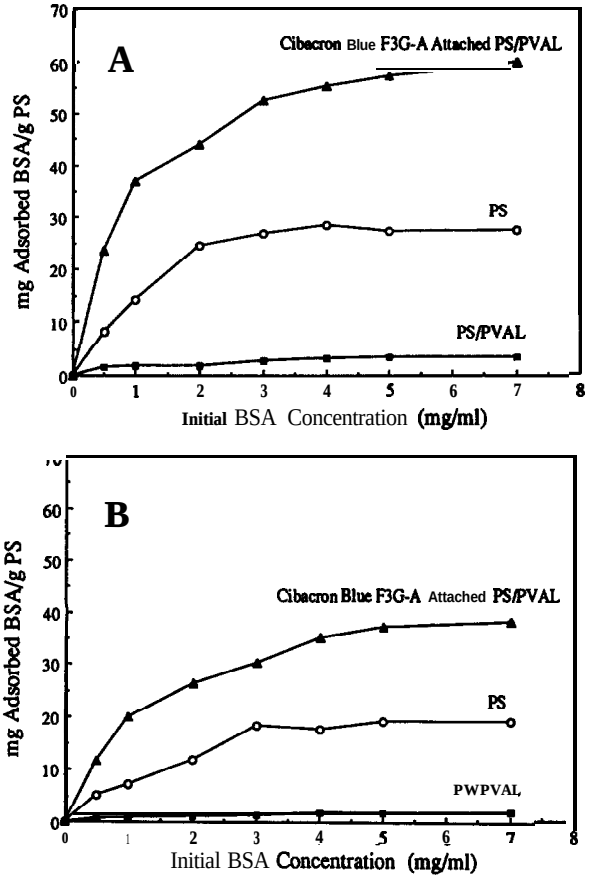
PVAL adsorption on the surface of polystyrene particles produced by conventional emulsion polymerization methods has also been studied previously by several investigators [16,20,21].

The maximum PVAL adsorption capacity obtained with such particles was *ca.* 5-6 mg/m^2. The difference in the PVAL adsorption may be explained by considering the surface charges of their PS particles and our PS microbeads. Their polymeric particles usually contained strongly acidic groups (e.g., SO_4^{2-}) on their surfaces coming from the initiator (e.g., potassium peroxydisulphate) used in their polymerization recipes. However, the PS microbeads produced in the present study exhibit only a small number of weakly acidic groups (carboxylic acid) coming from the steric stabilizer [14,15].

BSA aa'sorptionldesorption studies

Adsorption. Effects of the initial BSA concentration, ionic strength and pH on the adsorption behavior of BSA on the PS, PS/PVAL and Cibacron Blue F3G-A-attached PWPVAL microbeads were investigated in batch adsorption–equilibrium studies. Typical BSA adsorption data obtained in this group of experiments are given in Fig. 5A and B ionic strengths of 0.01 and 0.1, respectively, adjusted with NaCl. These adsorption isotherms were obtained in experiments in which the pH of the adsorption medium was 5.0 ***(i.e.,*** the isoelectric point of BSA). As expected, adsorption increased with increasing initial concentration of BSA. There was a pronounced adsorption of BSA on PS microbeads (up to 25 mg/g), possibly because of the hydrophobic interactions between albumin and PS. The PVAL coating significantly decreased the BSA adsorption, as intended. Very high BSA adsorption capacities (up to 60 mg/g) were achieved with Cibacron Blue F3G-A-attached PS/PVAL microbeads. Also, the amount of BSA adsorbed on each microbead decreased with increasing ionic strength at constant initial albumin concentration.

In order to establish the effects of pH on BSA adsorption, adsorption experiments were repeated at different pH values between 4 and 8 at two different ionic strengths (0.01 and 0.1, adjusted with NaCl). In these studies the initial concentration of BSA was 2.0 mg/ml. Fig. 6A and B give typical adsorption data obtained in this group of experiments. As can be seen, in all the cases investigated, the maximum adsorption



Fig, 5. BSA adsorption on PS-based microbeads at two different ionic strengths: (A) 0.01; (B) 0.1. pH = 5.0; salt used. NaCl.

of BSA was observed around its isoelectric point of 5.0. This is as expected because, as it has been shown that proteins have no net charge at their isoelectric points, the maximum extent of adsorption from aqueous solutions is therefore observed at the isoelectric point [22,23]. Significantly lower adsorption capacities were obtained with all microbeads at acidic and alkaline pH. This is also as expected, because it is known that, below or above the isoelectric points, proteins are charged positively or negatively, respectively. They are more hydrated, which increases their stability and solubility in an aqueous phase ***(i.e.,*** lower adsorption). It should be noted that there was no adsorption of BSA on PWPVAL microbeads at pH 7.0 and 8.0 at both ionic strengths of NaCl. This is very important

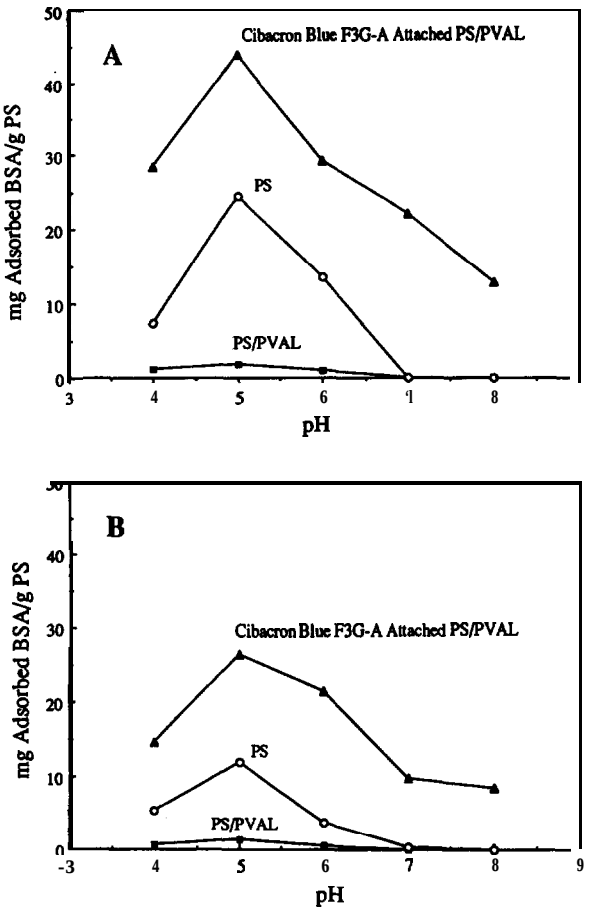


Fig. 6. Effect of **pH** on BSA adsorption on PS-based microbeads at two different ionic strengths: (A) 0.01; (B) 0.1. Initial BSA concentration, 2.0 **mg/ml; salt** used, **NaCl.**

because it means that there will be no non-specific protein adsorption if one works under those conditions.

Desorption. The desorption of the adsorbed BSA from the Cibacron Blue **F3G-A-attached PS/PVAL** microbeads was also studied in a batch experimental set-up. The dye-attached **micro-**beads loaded with different amounts of BSA were **placed** within the desorption medium containing 0.5 ***M*** NaSCN at **pH** 8.0 and the amount of BSA released in 1 h was determined. The desorption ratio was then calculated by using the following expression:

$$\text{desorption ratio} = \frac{\text{amount of BSA related to the desorption medium}}{\text{amount of BSA adsorbed on the microbeads}} \cdot 100$$

TABLE II

BSA DESORPTION RATIOS

Desorption medium, 0.5 ***M*** NaSCN; **pH,** 8.0; temperature, 5°C.

Initial BSA concentration **(mg/ml)**	Desorption ratio **(%)**	
	I[a]	II[b]
0.5	85	89
1.0	76	85
2.0	84	86
3.0	90	91
4.0	88	85
5.0	85	92

[a] Albumin adsorption conditions: ionic strength of **NaCl,** 0.01; **pH,** 5.0.

[b] Albumin adsorption conditions: ionic strength of **NaCl,** 0.1; **pH,** 5.0.

Table II gives typical desorption data obtained in this group of experiments. A significant proportion (up to 92%) of the BSA adsorbed on the microbeads could be recovered with satisfactory desorption ratio values.

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