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Separation of biotin labeled proteins from their unlabeled counterparts using immobilized platinum affinity chromatography

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Abstract

A stationary phase selective for biotin labeled proteins has been developed by immobilizing platinum(II) ions to a polyacrylamide gel. Bovine serum albumin (BSA) and biotin labeled BSA (BSA-Biotin) have been applied individually to a packed column containing the modified gel. At pH 4.8 using column superficial velocities of 1.0 and 0.25 cm/min respectively, 40% and 73% of the applied BSA-Biotin were bound to the activated gel while no unconjugated BSA was bound. A 1.0 *M* imidazole–HCl solution at pH 7 was successfully used to elute bound BSA-Biotin, indicating that binding to immobilized Pt(II) is reversible.

1. Introduction

Avidin (M_r 16 000) and streptavidin (M_r 14 000) are small proteins which form extremely strong non-covalent bonds with biotin (dissociation constant 10^{-15} *M*) [1]. Avidin is a glycoprotein derived from egg white with an isoelectric point of 10, whereas streptavidin, which is obtained from Streptomyces avidinni, is carbohydrate-free and has an isoelectric point of about 5.5 [1]. (Strept)avidin has been shown to contain biotin binding sites deeply recessed within the protein, and four tryptophanyl residues per binding site have been implicated in hydrophobic interaction with biotin [1]. The (strept)avidin-biotin system is appealing because the binding of biotin to (strept)avidin is complete and rapid, and does not require special reaction conditions. Furthermore, the biological activity of most proteins is preserved after biotinylation, and the ability of each (strept)avidin moiety to bind four biotin molecules amplifies the desired result [1].

Since 1980, an explosion in the number of applications utilizing avidin-biotin technology has occurred. Some applications are listed by Wilchek and Bayer [1] in a recent review. An extensive literature is available on avidin-biotin technology, and several excellent reviews have been published [1–6]. The high affinity of avidin for biotin is advantageous for initial capture of biotinylated species from solution, but a disadvantage for elution and recovery [1]. Even under harsh elution conditions, such as 6.0 *M* guanidine chloride at pH 1.5, poor recovery of the biotinylated molecules is sometimes observed. When elution is successful, the activity of the biotinylated species is often irreversibly reduced. Biotin itself can sometimes be used to

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release biotinylated compounds from an avidin affinity column, although poor recoveries are generally noted, even when extremes of pH and long equilibration times are used.

Several researchers have used modified forms of avidin in order to improve recoveries. Avidin immobilized to a sepharose column can be converted to a monomeric form by denaturing the avidin tetramer with guanidinium chloride, leaving a single subunit immobilized to the sepharose. The dissociation constant for immobilized monomer-biotin is about 10^{-7} M. However, there are still some high affinity binding sites which must be blocked with biotin before using the column for affinity chromatography [1]. In general, elution of the retained product is accomplished by removing the target from the binder, or cleaving the biotin–binder bond, with the (strept)avidin–biotin bond left intact. This has led to low recoveries or protein denaturation [1].

It would be desirable to develop a universal affinity chromatography column based on the (strept)avidin-biotin system which could be recharged with a different biotinylated binder depending on the target protein desired. Such a system has been studied by Hofmann et al. [7] who attached 2-iminobiotin instead of biotin to each binder. The advantage of this system is that 2-iminobiotin binding to avidin is a strong function of pH, and varies from $3 \cdot 10^{-8}$ at pH 9 to $1.2 \cdot 10^{-6}$ at pH 6. Therefore, when the column is operated at pH 9 or above, the 2-iminobiotinylated binder is more firmly attached to the column [1]. The column can be charged with a new iminobiotinylated binder after first washing off the original binder using a buffer with $pH < 6$. Although this system appears very attractive, it has several drawbacks. First, many proteins are irreversibly denatured at high pH, therefore, the system cannot be used for these types of proteins. Second, many iminobiotinyl derivatives have affinities for biotin too low to be useful.

Another approach to develop a reversible biotin affinity column is through the use of antibodies to biotin which bind biotin with a dissociation constant of approximately 10^{-9} *M* [1]. However, most antibodies have a molecular mass at least twice that of avidin, and only contain two binding sites per molecule. Therefore, the capacity of a column which uses immobilized antibodies for affinity chromatography of biotinylated proteins is generally much less than a similar column which employs avidin. In addition, the Fc portions of antibodies consist of oligosaccharide residues that may be responsible for non-specific binding. Moreover, some macrophages and lymphocytes recognize the Fc portion of the antibody and bind to the antibody. Although the lower dissociation constant (10^{-9}) allows more efficient removal of the antibody, it comes at the expense of less efficient and less selective capture of biotinylated species during sample application to the column [1]. Other biotin-binding proteins have been discovered which exhibit dissociation constants from 10^{-7} to 10^{-12}. The use of these proteins in affinity chromatography is currently being explored [1].

Based on previous research with chromatography of amino acids [8], we have shown that immobilized Pt(II) has a very high affinity and selectivity for methionine. This behavior differs sharply from amino acid chromatography using immobilized transition metal ions normally used in immobilized metal affinity chromatography (IMAC) [9]. Because the Pt(II)–methionine interaction is primarily due to the thioether functional group on methionine and follows Pearson's hard–soft acid base theory [10], we theorized that immobilized Pt(II) could be used to bind biotin since it also exhibits a thioether functional group. Use of metal ions for biotin binding is advantageous due to lower cost, ease in maintaining column sterility as well as column regeneration [9], and the potential for higher packing densities and minimization of non-specific protein–protein interactions. However, metal ions could introduce electrostatic interactions that could induce non-specific binding or alter protein binding kinetics as well as unwanted binding due to electrostatic attractions unless care is taken to minimize these effects through the use of buffer salts.

Although IMAC with Cu(II) has been used to separate biotinylated proteins and nucleic acids,

complexation with avidin, not biotin, resulted in retention of the conjugate on the column [11]. In a separate study, up to 15 m*M* biotin was tested as an eluent for immobilized photosystem proteins I and II sorbed to immobilized Cu(II). No significant elution of the protein was noted, indicating that biotin could not effectively compete with the protein for Cu(II) binding sites [12] again suggesting that copper ions do not coordinate strongly to biotin.

However, we have shown through amino acid chromatography with both immobilized Pt(II) and Ag(I), and by analogy, that soft metal ions have a high specificity for molecules containing thioether functional groups. Moreover, since methionine residues are usually found in the interior of proteins due to their hydrophobic nature, biotin present on the protein surface would likely be the sole driving force for biotinylated protein binding. We thus decided to test conditions where biotin conjugated BSA (BSA-Biotin) would bind to a column containing immobilized Pt(II) while BSA would not. In this way, immobilized Pt(II) would be rigorously tested for its suitability as a substitute for (strept)avidin in biotin affinity columns. Conditions under which BSA-Biotin can be eluted from the column without loss of Pt(II) were also briefly explored in the course of this work.

2. Experimental

2.1. Chemicals

Bio-Gel P200 polyacrylamide gel (Bio-Rad Laboratories, Richmond, CA, USA) extra fine, 100–200 mesh (wet) M_r 200 000 exclusion limit. Albumin, bovine (Sigma Chemical, St. Louis, MO, USA) fraction V, 99%, globulin free, prepared by cold alcohol precipitation. Albumin, bovine-biotin labeled. 95% protein (Sigma) balanced with citrate buffer salts, prepared from a companion lot of the BSA described above, which was processed at the same time as BSA and contains 10.6 mol biotin/mol BSA . Glutaraldehyde (Aldrich Chemical, Milwaukee, WI, USA) 25 wt.% in water. K_2PtCl_4, $PtCl_4^{2-}$ or Pt(II) potassium tetrachloroplatinate(II) 98% (Aldrich) was used for immobilization. Protein Assay Kit (P65656) (Sigma) for quantitative microdetermination of total protein. Thiourea (J.T. Baker Chemical, Phillipsburg, NJ, USA) used in gel activation was reagent grade.

2.2. Equipment

A Pharmacia Augmented FPLC system (Pharmacia, Uppsala, Sweden) was used for all chromatography experiments described here. A DU-70 Spectrophotometer (Beckman Instruments, Fullerton, CA, USA) was used for determination of total protein concentration via the Lowry Microprotein Assay Kit. A Varian Model 1100 (Varian, USA) was used in order to determine the amount of platinum immobilized on the activated gel.

2.3. Determination of total protein

Total protein concentrations were determined using Peterson's modification of the micro-Lowry method [13,14] using a Protein Assay Kit purchased from Sigma. Assays were performed by following the kit instructions exactly as specified for protein determination with protein precipitation. Essentially, the procedure required dilution of the sample with distilled water, followed by addition of trichloroacetic acid and sodium deoxycholate to precipitate the protein. After centrifuging each sample, the supernatant was decanted and blotted way from the protein pellet. The pellet was redissolved using the Lowry reagent solution, to which was added Folin and Ciocolteu's phenol reagent. After a sufficient incubation period, the absorbance was read at 750 nm. Standards with known BSA concentrations were prepared and analyzed identically along with the samples and used to construct a calibration curve.

2.4. Determination of Pt(II) concentration in solution by atomic absorption

All platinum analyses were carried out using an atomic absorption spectrometer. A Varian

Model 1100 was used for all measurements using an air–acetylene flame. The lamp current, slot width, and detection wavelength were 10 mA, 0.2 nm and 265.9 nm, respectively. All Pt(II) standard solutions were prepared fresh from K_2PtCl_4 in distilled water, unless otherwise indicated. In some cases, a small amount of nitric acid was added to each sample and standard prior to analysis. Fresh standards were analyzed to generate a calibration curve prior to sample analysis.

Pt(II) was sorbed to the resin by gentle shaking at room temperature for several hours. After sorption, the resin was allowed to settle and an aliquot of the supernatant was removed and analyzed directly by atomic absorption. The Pt(II) concentration in the supernatant was read from a calibration curve relating absorbance to Pt(II) concentration. The amount of Pt(II) sorbed to the resin was determined indirectly from decrease in Pt(II) concentration in the supernatant after sorption. In some cases, a small amount of HNO_3 was added to the sample and controls prior to analysis to improve the stability of the aqueous Pt(II).

2.5. Preparation of biogel polyacrylamide resins with immobilized Pt(II)

Biogel P200–Glut–Thio resin was prepared by Kim [15] as follows: First, 25% glutaraldehyde was polymerized at 70°C for 36 h in a heated water bath. Then 50 ml of the polymerized glutaraldehyde solution was added to 50 ml of D.I. water, and 1.85 g of Biogel P200 resin and shaken gently at 50°C for 24 h. Resin swelling and glutaraldehyde activation were allowed to proceed simultaneously. Upon completion of glutaraldehyde activation, the resin was washed batchwise 5 times with 70 ml of distilled water.

Thiourea activation was accomplished by adding 40 ml of a 1.0 *M* thiourea solution to 50 ml of the glutaraldehyde activated resin. The resin slurry was shaken gently for 24 h at 50°C. After completion of the thiourea sorption step, the resin was washed 20 times batchwise with 70 ml of distilled water, followed by column washing at 1.0 ml/min with 720 ml of distilled water. Each batchwise wash was accomplished by decanting the supernatant, adding the specified amount of distilled water, gently shaking the resin for several seconds to suspend it in the fresh water, then allowing the resin to settle for one h.

The Biogel P200–Glut–Thio–Pt(II) resin was prepared by activation of BP200–Glut–Thio described above with Pt(II). A 16.0-ml volume of 0.0126 *M* K_2PtCl_4 solution was added to 20.0 ml of Biogel P200–Glut–Thio resin and rotated gently for 1.2 h. The resin was allowed to settle for approximately 1 h, and the volume after sorption was determined to be 24 ml. The resin was then column washed at 0.15 ml/min with 700 ml of distilled water. The 700 ml of rinse solution was evaporated down to 26.6 ml, and then analyzed by atomic absorption to determine the concentration of Pt(II) in the rinse. The Pt(II) loading was determined to be 0.008 mmol Pt(II) per ml of wet resin.

2.6. Column liquid chromatography: effects of mobile phase composition

A glass column (5.45 × 0.5 cm) packed to a final bed volume of 1.1 ml was used at room temperature for all experiments. Mobile phase buffers were all prepared at a concentration of 0.05 *M* at pH 2.5, 4.8, and 7.0 using sodium phosphate (in NaH_2PO_4–H_3PO_4 form), sodium acetate, and sodium phosphate, respectively. NaCl was used to set the chloride concentration in the imidazole-free mobile phases. When both chloride ion and imidazole were present in the mobile phase buffer, the buffer was prepared by adding concentrated hydrochloric acid and imidazole solid to distilled water first. Then sodium hydroxide and the buffer salt were added to adjust the pH to the desired value. Individual stock solutions of BSA and BSA-Biotin were prepared by weighing each protein into a separate vial and diluting to a final concentration of $1.49 \cdot 10^{-4}$ *M* using a dissolution buffer. The stock solution was further diluted by a factor of 15 to a final concentration of $1.0 \cdot 10^{-5}$ *M* using the mobile phase prior to column application. The protein dissolution buffer consisted of 0.05 *M* sodium citrate–0.145 *M* NaCl, pH 7.0. BSA-

Biotin from the manufacturer had approximately 5% citrate salts. No citrate was present in the lyophilized BSA reagent. As citrate is known to chelate strongly to metal ions, excess citrate was added to the dissolution buffer in order to equalize any effect of citrate on the elution of each protein. Due to the small size of the citrate molecule, it was eluted after the protein.

For all experiments described below, protein applications to the column were performed pairwise sequentially, with BSA applied individually to the column first immediately followed by BSA-Biotin.

In order to investigate the variation in retention with mobile phase flow rate, a glass column (5.3 × 0.5 cm) was packed with 1.0 ml of Biogel P200–Glut–Thio–Pt(II) resin loaded with 0.008 mmol Pt(II)/ml wet resin and connected to the FPLC system. The same column and packing were used for all runs which used the Pt(II) activated resin. All control runs (no immobilized Pt(II)) were also performed using the same freshly packed Biogel P200–Glut–Thio resin (1.2 ml bed volume). Each column was washed with 10 bed volumes of a 0.05 *M* sodium acetate mobile phase (pH 4.8) prior to the initial injection of protein.

Each sample application to the column consisted of a 0.02-ml injection of protein. Three flow rates were studied in the following order: 0.05, 0.1, and 0.2 ml/min. BSA was always applied to the column first, followed by BSA-Biotin. Three pairs of BSA/BSA-Biotin injections (6 total) were applied initially to the column at 0.1 ml/min to test the stability of the column. Thereafter, a single injection of BSA was followed by a single injection of BSA-Biotin at each of the other two flow rates. Detection was accomplished at 280 nm and 0.02 AUFS. The column was equilibrated with at least 2 bed volumes of mobile phase prior to each injection.

The effect of pH, chloride concentration, and imidazole concentration on BSA and BSA-Biotin binding to Biogel P200–Glut–Thio–Pt(II) was studied in three experimental series, each performed using a separate column. In the first series, the effect of pH was studied. A glass column (5.6 × 0.5 cm) was packed with 1.1 ml of Biogel P200–Glut–Thio–Pt(II) resin loaded with 0.008 mmol Pt(II)/ml wet resin. The same column and packing were used for all runs in this series. Injections of BSA and BSA-Biotin were made sequentially at pH 7, 4.8 and 2.5. Sodium phosphate (0.05 *M*) was used to prepare buffers at pH 2.5 and 7, and sodium acetate (0.05 *M*) for pH 4.8.

Each protein application to the column consisted of a 0.02-ml injection of $1.0 \cdot 10^{-5}$ *M* BSA followed by an individual injection of BSA-Biotin at each pH. The flow rate was fixed at 0.1 ml/min. Detection was accomplished at 280 nm with 0.02 AUFS. At least 5 column volumes of mobile phase were used to equilibrate the column at each pH before making injections. The resin was equilibrated with at least 2 bed volumes of mobile phase prior to each injection.

In the second series, both the pH and concentration of sodium chloride were varied. A glass column (3.1 × 0.5 cm) was packed with 0.61 ml of Biogel P200–Glut–Thio–Pt(II) resin loaded with 0.008 mmol Pt(II)/ml wet resin. The same column and packing were used for all runs in this series. All experiments were carried out at 0.1 ml/min with detection at 280 nm/0.02 AUFS. Table 1 shows (in order) the mobile phases used with this column. The column was equilibrated with approximately 10 bed volumes prior to initial sample application for a given mobile phase. Thereafter, the column was washed with at least 2 bed volumes between sample applications. BSA and BSA-Biotin were individually applied to the column for each mobile phase. Injections consisted of 0.02 ml of $1.0 \cdot 10^{-5}$ *M* protein.

In the third series, chloride and imidazole concentrations were varied together. A glass column (2.7 × 0.5 cm) was packed with 0.53 ml of Biogel P200–Glut–Thio–Pt(II) resin loaded with 0.008 mmol Pt(II)/ml wet resin. The same column and packing were used for all runs in this series. All experiments were carried out using a 0.05 *M* sodium acetate mobile phase at pH 4.8. The imidazole concentration was fixed at the same value in the mobile phase as the chloride concentration for all experiments. For each mobile phase with a specific imidazole/chloride

Table 1
Mobile phases used to study the effects of pH and added salt on BSA and BSA-biotin retention

Mobile phase (in order used)	Sodium chloride, *M*	pH	Buffer, 0.05 *M*
1	0.1	7.0	Sodium phosphate
2	0.1	4.8	Sodium acetate
3	0.1	2.5	Sodium phosphate
4	0	7.0	Sodium phosphate
5	0	4.8	Sodium acetate
6	0	2.5	Sodium phosphate
7	1	7.0	Sodium phosphate
8	1	4.8	Sodium acetate

concentration, a single injection of BSA was followed by a single injection of BSA-Biotin. Mobile phases containing 1.0, 0.1, 0.01, and 0 *M* imidazole/chloride were each used in order from high to low imidazole/chloride concentration as indicated. For each mobile phase containing imidazole, a control run was performed by applying the protein-free dilution buffer (diluted by same factor in mobile phase as the protein samples) to the column. The "control" chromatographic response curve was subtracted from the sample curve for each mobile phase prior to plotting and integration, which reduced the impact of a secondary negative peak observed at $(V_e)/(V_L) = 1$. The secondary peak resulted from dilution of imidazole in the mobile phase by the dilution buffer. Subtraction of the control curve for BSA did not completely eliminate the negative peak, especially at high imidazole concentrations [16].

Approximately 10 bed volumes were used to equilibrate the column prior to making the initial protein injection using each mobile phase. When the mobile phase was devoid of imidazole/chloride, over 20 bed volumes were used to equilibrate in order to aid removal of any imidazole retained on the column from previous runs.

Each protein application to the column consisted of a 0.02-ml injection of $1.0 \cdot 10^{-5}$ *M* BSA or BSA-Biotin. The flow rate was fixed at 0.1 ml/min. Detection was accomplished at 280 nm with 0.02 AUFS. The resin was equilibrated with at least 2 bed volumes of mobile phase prior to each injection.

2.7. Column liquid chromatography: binding and elution with imidazole

A glass column (5.45 × 0.5 cm) was packed once to a final bed volume of 1.1 ml with Biogel P200–Glut–Thio–Pt(II) resin and used for all runs discussed in this section. Prior to the initial application of protein, the column was equilibrated by washing with 10 bed volumes of 0.05 *M* sodium acetate, pH 4.8.

Two trials were performed using the same column. Each trial consisted of application of a BSA-Biotin sample using 0.05 *M* sodium acetate, pH 4.8 (application buffer), followed by elution with 1.0 *M* imidazole–HCl, pH 7 (elution buffer), and restoration using 0.05 *M* sodium acetate (pH 4.8). The first trial consisted of application of 0.15 ml of $1.0 \cdot 10^{-5}$ *M* BSA-Biotin into the column. The flow rate was 0.05 ml/min, and 2.2 bed volumes of the application buffer were passed through the column after injection. Detection was made at 280 nm, 0.05 AUFS. Fractions measuring 1 ml were collected as soon as the BSA-Biotin peak appeared. Immediately after eluting the initial peak representing unbound protein, the elution buffer was used to recover bound protein from the column. A total of 3.2 bed volumes of this buffer were passed through the column, followed by 28 bed volumes of 0.05 *M* sodium acetate (pH 4.8) to restore the column.

The second trial consisted of an identical BSA-Biotin application and subsequent imidazole elution repeated using the restored column.

Essentially the same procedure just described was used, except that only 1.5 bed volumes of the application buffer were passed through the column after protein injection.

The amount of BSA-Biotin eluted from the column was determined by analysis of fractions using the Protein Assay Kit. Sample absorbance values were observed to drift (increase) significantly. In order to minimize the effect of absorbance drift on concentration measurement, absorbance values were measured twice for each sample according to the following procedure: The first absorbance value for each sample was obtained by reading samples sequentially. The second absorbance value for each sample was obtained by immediately reading samples in the reverse order, and the two absorbance values for each sample were averaged. This procedure greatly reduced the effect of absorbance drift.

Because the column was initially equilibrated with sodium acetate, the first fraction obtained during imidazole/HCl elution contained both imidazole and acetate buffer components. In order to estimate the relative concentration of both buffers in this fraction, the imidazole breakthrough curve was integrated over the time period during which the first fraction was collected [16].

3. Discussion

Under mobile conditions where BSA and BSA-Biotin bind to the column there is no change in peak retention volume or shape, but the peak area decreases in proportion to the amount bound. This behavior can be modeled using the following differential equation:

$$\epsilon dV_t \frac{\partial C_m}{\partial t} + F dC_m + \epsilon dV t_o C_m = 0 \qquad (1)$$

where the term $\epsilon dV_t K_o C_m$ represents irreversible removal of protein from the mobile phase. Using the following initial and boundary conditions:

$$C_m(0,z) = 0 \qquad C_m(t,0) = C_{m_o}\delta(t)$$

Eq. 1 is solved to obtain:

$$\frac{C_m}{C_{m_o}} = \delta\left(t - \frac{z}{u}\right) \exp\left(\frac{-K_o z}{u}\right) \qquad (2)$$

If quasi-equilibrium is assumed, the rate of solute removal from the mobile phase (N_m) can be assumed to equal the rate of transport through the film (N_f). Furthermore, the rate at which material binds to the gel surface (N_r) is assumed to equal the rate at which material enters the pore through the film. Therefore, the following relations hold [17]:

$$N_m = N_f = N_r$$

$$N_f = k_f(C_m - C_p)$$

$$N_r = k_r$$

$$N_m = K_o C_m$$

$$\frac{1}{K_o} = \frac{1}{k_f} + \frac{1}{k_r} \qquad (3)$$

C_s is assumed to be zero due to the large excess of Pt(II) binding sites on the resin. K_o can then be viewed as an overall mass transfer coefficient for solute removal from the mobile phase.

The functionality of k_f can be elucidated using the correlation of Wilson and Geankoplis [18]:

$$k_f = Au^{1/3} \qquad (4)$$

for $0.0016 < Re < 55$ and $165 < Sc < 70\,600$, where

$$A = 1.09\left(\frac{d_p}{\epsilon D}\right)^{2/3}$$

If k_r is assumed to be constant, then the derivative of K_o with respect to the interstitial velocity can be calculated as:

$$\frac{\partial K_o}{\partial u} = \frac{A}{3u^{2/3}\left[\frac{Au^{1/3}}{k_r} + 1\right]} \qquad (5)$$

The Schmidt number was calculated to be 16 000 for the protein chromatography experiments assuming a diffusivity of $6.1 \cdot 10^{-7}$ cm^2/s for BSA [19]. The Reynolds number was calculated to be 0.006 and 0.024 respectively for 0.05 and 0.2 ml/min of aqueous solution flow through a 0.5 cm I.D. column. These values suggest that

Table 2
Application of BSA and BSA-biotin to Biogel P200–Glut–Thio–Pt(II) at pH 4.8 with various imidazole/chloride concentrations in the mobile phase [16]

Imidazole or Chloride, M	Protein	V_r/V_t	% Bound	K_o
1.0	BSA	0.58	0 ± 7	0
	BSA-biotin	0.60	7 ± 6	0.015 ± 0.013
0.1	BSA	0.58	0 ± 7	0.001 ± 0.013
	BSA-biotin	0.60	16 ± 6	0.033 ± 0.013
0.01	BSA	0.55	4 ± 6	0.008 ± 0.013
	BSA-biotin	0.60	18 ± 6	0.039 ± 0.013
0	BSA	0.51	0 ± 7	0
	BSA-Biotin	0.53	42 ± 4	0.107 ± 0.013

the correlation of Wilson and Geankoplis is suitable to predict the film mass transfer coefficient, k_f.

Results obtained by chromatography of BSA and BSA-Biotin are summarized in Figs. 1–8 and Tables 2 and 3. The data have been normalized by plotting K_o, the overall mass transfer coefficient, so that data taken on columns of different lengths can be compared. Increasing values of K_o translate to increased protein binding to the column. For a 5.3×0.5 cm column operated at 0.1 ml/min, 20% and 80% binding to the column corresponds to K_o values of 0.045 and 0.323 min^{-1}, respectively.

BSA-Biotin bound significantly to Biogel P200–Glut–Thio–Pt(II) using a 0.05 M sodium phosphate mobile phase at pH 4.8, and binding increased with decreasing flow rate (Table 3).

Table 3
Application of BSA and BSA-Biotin to Biogel P200–Glut–Thio and Biogel P200–Glut–Thio–Pt(II) resins

Flow rate (cm/min)	Packing (%)	Protein (%)	V_r/V_t	Bound (%)	Est. error	K_o (min^{-1})	Est. error
0.2	Biogel P200–Glut–Thio	BSA(2)	0.40	0	±6.7	0.000	±0.012
		BSA(2)-Biotin	0.42	0	±6.7	0.000	±0.012
	Biogel P200–Glut–Thio–Pt(II)	BSA(2)	0.39	0	±6.7	0.001	±0.013
		BSA(2)-Biotin	0.39	40	±4.0	0.103	±0.013
0.1	Biogel P200–Glut–Thio	BSA(2)	0.42	1	±6.6	0.001	±0.006
		BSA(2)-Biotin	0.45	0	±6.7	0.001	±0.006
	Biogel P200–Glut–Thio–Pt(II)	BSA(2)	0.43	40	±4.0	0.051	±0.007
	(First pair of injections)	BSA(2)-Biotin	0.41	82	±1.2	0.172	±0.007
	Biogel P200–Glut–Thio–Pt(II)	BSA(2)	0.42	0	±6.7	0.001	±0.007
	(Second pair of injections)	BSA(2)-Biotin	0.42	62	±2.6	0.096	±0.007
	Biogel P200–Glut–Thio–Pt(II)	BSA(2)	0.41	0	±6.8	0.001	±0.007
	(Third pair of injections)	BSA(2)-Biotin	0.40	58	±2.8	0.087	±0.007
0.05	Biogel P200–Glut–Thio	BSA(2)	0.44	0	±6.7	0.000	±0.003
		BSA(2)-Biotin	0.47	0	±6.7	0.000	±0.003
	Biogel P200–Glut–Thio–Pt(II)	BSA(2)	0.43	4	±6.4	0.002	±0.003
		BSA(2)-Biotin	0.41	73	±1.8	0.065	±0.003

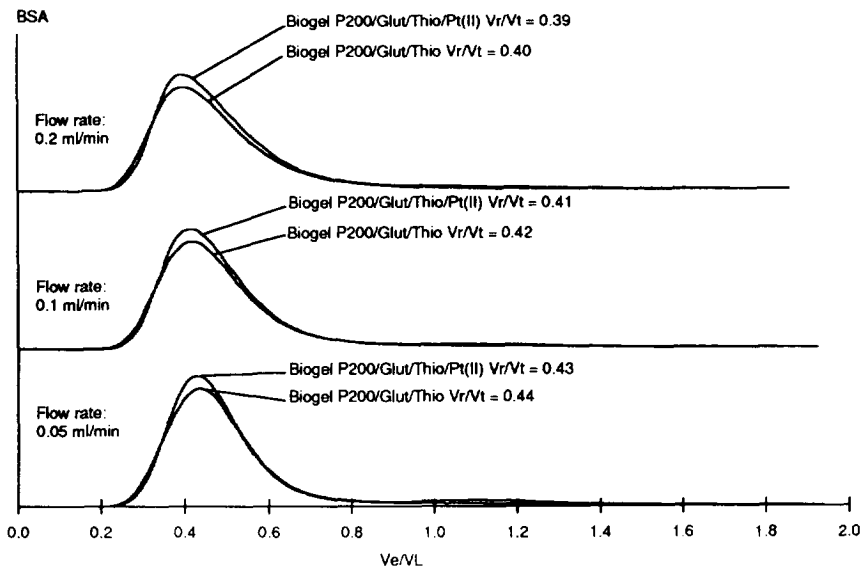

Fig. 1. Effect of flow rate on BSA binding to Biogel P200–Glut–Thio–Pt(II) and Biogel P200–Glut–Thio resins at pH 4.8.

However, as shown in Table 3, no BSA binding occurred to the gel containing immobilized Pt(II) at any of the three flow rates studied once the column was conditioned. Column conditioning can be accomplished either by addition of NaCl or by injecting BSA. BSA-Biotin binding to the resin appeared to be very strong, but exhibited slow kinetics. Approximately the same $(V_r)/(V_t)$ value was determined for BSA and BSA-Biotin at each flow rate on the activated gel, even when 73% of the BSA-Biotin was bound by the column (Figs. 1 and 2).

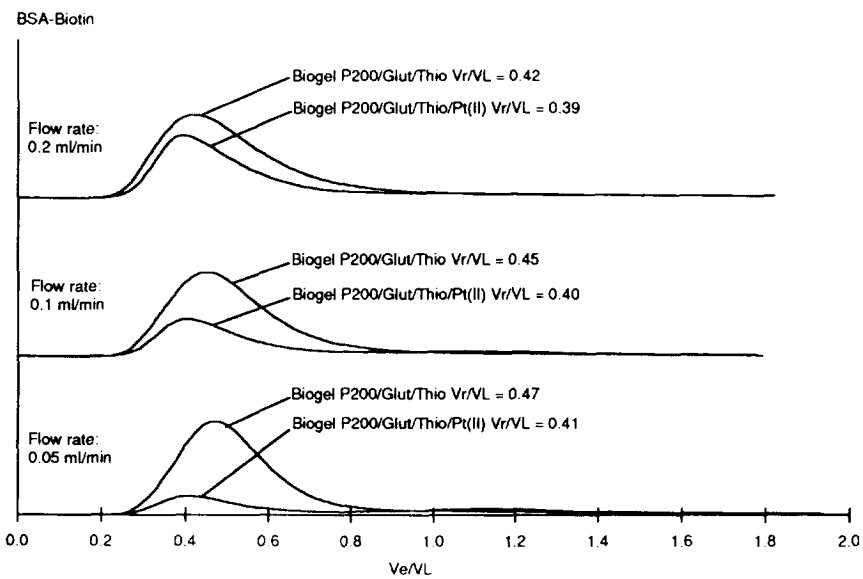

Fig. 2. Effect of flow rate on BSA-Biotin binding to Biogel P200–Glut–Thio and Biogel P200–Glut–Thio–Pt(II) resins at pH 4.8.

Gel permeation chromatography capacity is usually independent of flow rate [20,21]. Molecules fill their permeation volumes rapidly while traversing the column [22,23]. In many cases, local equilibrium can be assumed between protein molecules inside and outside the pores [24]. Published data suggest that for high-pressure size exclusion chromatography, retention volumes of solutes ranging from M_r 411 000 to M_r 41 are essentially independent of flow rate for bead sizes from 10 to 42 mm, and flow rates from 0.1 to 12.5 ml/min [25]. Although the hydrated Biogel P200 beads (90–180 mm) used in the BSA and BSA-Biotin chromatography experiments are considerably larger than the beads used in the experiment just described, the very slow flow rates probably ensured complete filling of the permeation volume by the proteins.

Rate theory has been applied to size exclusion chromatography in which pore diffusion and mobile phase lateral diffusion were included, but axial diffusion neglected [26]. Numerical solution of the governing differential equations showed gaussian chromatographic response peaks for near-equilibrium distribution of the solute into a porous stationary phase [26]. Furthermore, when flow rates were increased to create a condition of non-equilibrium distribution of solute into the stationary phase, response peaks were broader and significantly skewed in the direction of lower retention volume, suggesting that dispersion of SEC peaks is rate limited [26].

For the chromatograms shown in Figs. 1 and 2, there is no evidence of increased skewness or broadening of either BSA-Biotin or BSA peaks relative to each other or with increasing flow rate. In light of these observations, it appears that slow affinity binding of the BSA-Biotin to the immobilized Pt(II) causes a reduction in peak area, without changing the retention volume. Eq. (1) given above incorporates irreversible protein removal from the mobile phase proportional to the mobile phase protein concentration, and neglects diffusional limitations within the column. The analytical solution to this equation clearly predicts reduced peak areas with no change in retention volume for the protein chromatographic response peaks.

Binding of the BSA-Biotin to the resin probably requires proper orientation of the protein, and the actual binding of the biotin moiety to Pt(II) may itself exhibit slow kinetics. Furthermore, a protein molecule may bind to the column at several different points because each BSA molecule contains several biotinyl, imidazoyl and/or other moieties which may have an affinity for Pt(II). These factors together suggest that the interaction of BSA-Biotin with immobilized Pt(II) is slow, and strongest binding is achieved when multiple biotin moieties on a BSA-biotin conjugate molecule interacts on a 1:1 basis with Pt(II) ions on the resin.

If transport of protein to the bead surface was primarily limited by reaction at the bead surface, K_o would not be expected to depend on flow rate. However, Fig. 3 clearly shows that K_o decreases with decreasing flow rate, which is consistent with limited transport through a film surrounding each gel bead. If pore diffusional limitations were significant, K_o would be expected to increase with decreasing flow rate. If values of A and k_r are chosen to minimize the sum of squared deviations for K_o when calculated according to Eq. (1) above after substitution for k_f using Eq. (3), convergence to specific values of A and k_r does not result, and the sum

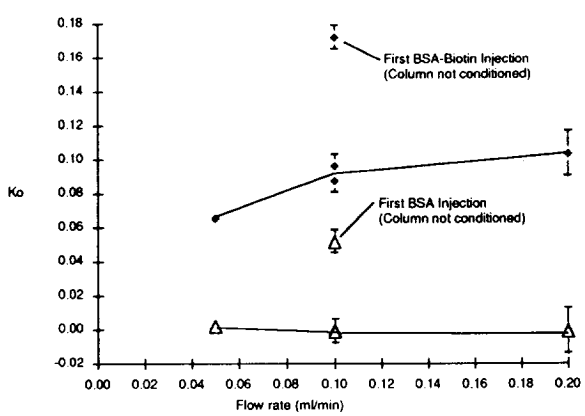

Fig. 3. Effect of previous column treatment and flow rate on BSA and BSA-Biotin binding at pH 4.8 (no added salt). Resin, Biogel P200–Glut–Thio–Pt(II); bed volume, 1.0 ml; mobile phase, 0.05 M sodium acetate; detection at 280 nm, AU 0.02. Injection, 0.02 ml of $1.0 \cdot 10^{-5}$ M BSA (△) or BSA-Biotin (◆).

of squares decreases as k_r is increased for optimal values of A. This behavior suggests that film transport is entirely responsible for mass transport limitations. However, such an interpretation is not consistent with the column conditioning data observed earlier, where the amount of protein bound for each successive BSA-Biotin application decreased. Furthermore, partial exclusion of the protein from the gel pores due to film limitations should result in skewed peaks and/or retention volume differences for protein applications to the Biogel P200–Glut–Thio gel compared to identical applications to Biogel P200–Glut–Thio–Pt(II), which has a high negative surface charge density. Retention volumes and chromatographic response peak shapes were nearly identical for BSA application to Biogel P200–Glut–Thio and Biogel P200–Glut–Thio–Pt(II).

If Eq. (3) is rewritten as

$$K_o = \text{Constant} + \frac{1}{\frac{1}{k_f} + \frac{1}{k_r}}$$

then the slope $(\partial K_o)/(\partial u)$ can be calculated at each flow rate using the experimental data [fitted to a function of the form $K_o = k_1 + k_2 \ln (u)$] and compared with the value of $(\partial K_o)/(\partial u)$ obtained from Eq. 5. In this case, more realistic values for A and k_r can now be determined as 0.43 and 0.33 respectively, suggesting that the mass transfer resistances for film transport and sorption are approximately of the same order of magnitude. In this case, film transport limitations can be used to rationalize the decrease in K_o observed with decreasing flow rate. Differences in k_r can be used to rationalize differences in K_o observed for BSA versus BSA-Biotin binding at a specified flow rate and differences in binding when different mobile phases are used, as well as to explain different binding behavior of the Biogel P200–Glut–Thio–Pt(II) resin compared to the Biogel P200–Glut–Thio resin.

For total permeation, a $(V_r)/(V_L)$ value for the packed column of approximately 1.1 was measured using imidazole and a conservative $(V_r)/(V_L)$ value for total exclusion was estimated to be 0.1. Typically, $(V_r)/(V_L)$ values of 0.5 were obtained for BSA and BSA-Biotin applications to Biogel P200–Glut–Thio–Pt(II). A linear relationship between molecular weight and $(V_r)/(V_L)$ for M_r 10 000 to 200 000 (as specified by the manufacturer for the native gel) was used to estimate the molecular weight of BSA as 120 000. Since BSA is known to have a molecular weight of approximately 70 000, the retention data suggest that BSA applied to Biogel P200–Glut–Thio or Biogel P200–Glut–Thio–Pt(II) is only able to permeate 50% of the internal gel volume normally permeated when the protein is applied to unactivated Biogel P200 gel. Activation of the gel using polymerized glutaraldehyde and thiourea probably result in a reduction in the accessible volume of the gel.

Table 4 shows the mobile phases used with each column. As indicated in Figs. 4 and 5, protein injections were made at pH 4.8 on all of the individually packed columns A, B, C, and D. Column C initially showed significantly higher BSA and BSA-Biotin binding values the first time these proteins were injected at 0.1 ml/min, but stabilized at lower values for subsequent injections. For Column A, single BSA and BSA-Biotin injections were made at pH 7 prior to BSA injection at pH 4.8. In this case, operating the column at pH 7 appeared to attenuate the capacity of the column for BSA and BSA-Biotin at pH 4.8 compared to initial injections of BSA and BSA-Biotin injections into Column C. The first injection of BSA into Column B resulted in approximately the same binding as observed for Column A. Single injections of BSA and BSA-Biotin into Column B were made using each of the following mobile phases prior to BSA injection at pH 4.8 (no NaCl): pH 7, 4.8 and 2.5 (all with 0.1 M NaCl) and pH 7 (no salt). The injections made using the aforementioned mobile phases should have resulted in conditioning of the gel. However, at pH 4.8 (no salt), high BSA binding was observed similar to that observed for Column A. In this case, it is possible that the low pH or the high concentration of chloride ions served to regenerate the column or restructure the resin such that high BSA binding could be obtained. As a result, the resin would

Table 4
Description of various columns and mobile phases used to obtain data shown in Figs. 4–6

Column	Bed volume (ml)	Flow rate (ml/min)	Buffers (in order used)
A	1.1	0.1	0.05 *M* sodium phosphate pH 7.0 0.05 *M* sodium acetate pH 4.8 0.05 *M* sodium phosphate pH 2.5
B	0.61	0.1	0.05 *M* sodium phosphate, 0.1 *M* NaCl pH 7.0 0.05 *M* sodium acetate, 0.1 *M* NaCl pH 4.8 0.05 *M* sodium phosphate, 0.1 *M* NaCl pH 2.5 0.05 *M* sodium phosphate pH 7.0 0.05 *M* sodium phosphate pH 4.8 0.05 *M* sodium phosphate pH 2.5 0.05 *M* sodium phosphate, 1.0 *M* NaCl pH 7.0 0.05 *M* sodium phosphate, 1.0 *M* NaCl pH 4.8
C	1.0	0.1	0.05 *M* sodium phosphate pH 4.8
D	0.53	0.1	0.05 *M* sodium phosphate, 1.0 *M* Cl^-, 1.0 *M* imidazole, pH 4.8 0.05 *M* sodium phosphate, 1.0 *M* Cl^-, 1.0 *M* imidazole, pH 4.8 0.05 *M* sodium phosphate, 0.1 *M* Cl^-, 0.1 *M* imidazole, pH 4.8 0.05 *M* sodium phosphate, 0.01 *M* Cl^-, 0.01 *M* imidazole, pH 4.8

All mobile phase buffers were prepared at 0.05 *M* concentration. UV detection at 280 nm with 0.02 AUFS. Each column was packed with Biogel P200–Glut–Thio–Pt(II) loaded with 0.008 mmol Pt(II)/ml wet gel.

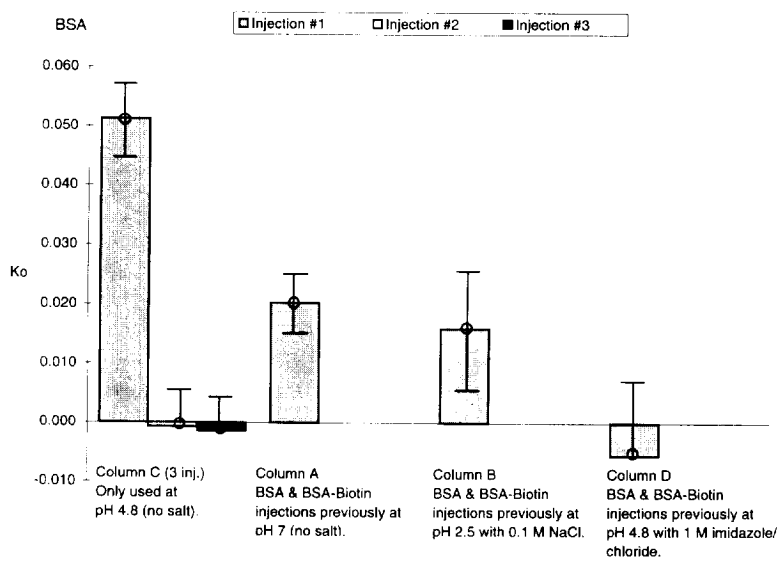

Fig. 4. Effect of previous column treatment on BSA binding at pH 4.8 (no added salt). Flow rate 0.1 ml/min; other experimental conditions as in Fig. 3.

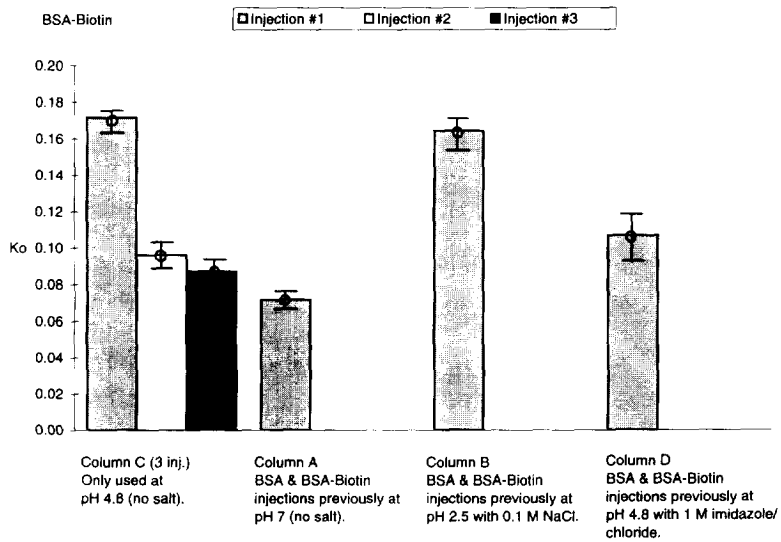

Fig. 5. Effect of previous column treatment on BSA-Biotin binding at pH 4.8 (no added salt). Experimental conditions as in Fig. 3.

behave as if it were not conditioned. BSA binding on column D was significantly reduced compared to columns A, B and C. Single BSA and BSA-Biotin injections were made into column D at pH 4.8 using imidazole/chloride concentrations of 1.0, 0.1 and 0.01 respectively, prior to making the injection of BSA at pH 4.8 with no salt or imidazole. Furthermore, some residual imidazole may not have completely washed from the strongest binding sites of this column. Neutralization of these sites would have the same effect as conditioning the column. BSA-Biotin binding at pH 4.8 can be analyzed in the same manner as for BSA described above, and the same trends were noted with regard to column conditioning and attenuation of binding at pH 7.0.

As shown in Figs. 6 and 7, BSA and BSA-Biotin binding generally increased with decreasing pH within the limits of experimental error. This result suggests that the gel surface carries a fixed negative charge. The isoelectric point for BSA is approximately 4.8. Therefore at pH 7.0, BSA and BSA-Biotin are negatively charged, and repulsion between the protein and the surface probably prevents the close distances required for binding of the biotin moiety of BSA-

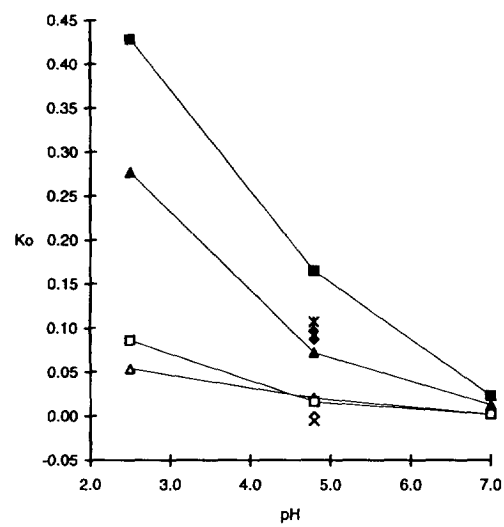

Fig. 6. Effect of pH on BSA and BSA-Biotin binding to Biogel P200–Glut–Thio–Pt(II) resin (no salt). Mobile phase, 0.05 *M* sodium phosphate (pH 2.5, 7.0)–0.05 *M* sodium acetate (pH 4.8); flow rate 0.1 ml/min; detection at 280 nm, AU 0.02. Injection, 0.02 ml of $1.0 \cdot 10^{-5}$ *M* BSA or BSA-Biotin. Symbols: ▲ = BSA-Biotin column A; ■ = BSA-Biotin column B; ◆ = BSA-Biotin column C (after conditioning); * = BSA-Biotin column D; △ = BSA column A; □ = BSA column B; ◇ = BSA column C (after conditioning); × = BSA column D

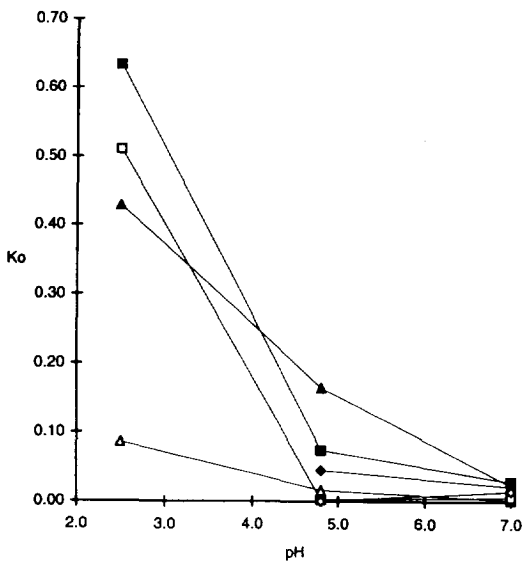

Fig. 7. Effect of pH and chloride concentration on BSA and BSA-Biotin binding to Biogel P200–Glut–Thio–Pt(II) resin. Experimental conditions as in Fig. 6. Symbols: ▲ = BSA-Biotin 0.0 *M* Cl^-; ■ = BSA-Biotin 0.1 *M* Cl^-; ◆ = BSA-Biotin 1.0 *M* Cl^-; △ = BSA 0.0 *M* Cl^-; □ = BSA 0.1 *M* Cl^-; ◇ = BSA 1.0 *M* Cl^-.

Biotin binding to immobilized Pt(II). At pH 2.5, BSA and BSA-Biotin are positively charged, and an ionic attraction exists which draws both proteins to the gel surface and may even cause partial unfolding of the protein. Unfolding may expose additional amino acid side chains which could coordination to Pt(II). Furthermore, the ionic attraction may cause reorientation of the protein relative to the gel surface. The net result is a loss of selectivity for BSA-Biotin and increased binding of both biotinylated and non-biotinylated proteins. Although it is surprising that BSA does not ion exchange on Biogel P200–Glut–Thio–Pt(II) at pH 7.0, this result is consistent with amino acid chromatography on Biogel P2–Glut–Thio–Pt(II) and Biogel P2–Glut–Thio–Ag(I) [15] where ion exchange was not seen to be a major factor, and may result because of competition by counterions in the mobile phase buffer [16].

Clearly, best column performance is expected when the mobile phase pH equals the protein isoelectric point, and experimentally, the best selectivity for BSA-Biotin was observed at pH 4.8, the isoelectric point of BSA. As biotinylation involves the side chain amino group of lysine in the formation of an amide bond, a lower isoelectric point would be expected for BSA-Biotin than for BSA, which has been confirmed by showing that BSA-Biotin precipitates from solution when the pH is lowered below pH 4.0, and remains dissolved at pH 4.8. Therefore, BSA-Biotin would be expected to have a decreased affinity for the Biogel P200–Glut–Thio–Pt(II) gel at pH 4.8 compared to BSA because it would carry a more negative charge. The fact the BSA-Biotin binding is higher than BSA binding at pH 4.8 indicates that effects other than ionic interactions are responsible for BSA-Biotin selectivity, such as coordination of the thioether sulfur of the biotin moiety to Pt(II).

Addition of salt to the mobile phase resulted in less protein binding at pH 4.8 and 7.0, and more binding at pH 2.5. The effect of salt appeared to be quite complex. Salt may affect the conformation of the protein, compete for ionic binding sites on the gel, and/or effect the electrical double layer that exists on the surface of the gel.

Over 90% of BSA-Biotin was bound to the resin at pH 2.5 for all mobile phases studied. Curiously, a tremendous increase in BSA binding was observed at pH 2.5 when 0.1 *M* sodium chloride was added to the mobile phase, causing BSA and BSA-Biotin binding to be nearly equal. At sodium chloride concentrations significantly above 0.1 *M*, BSA-Biotin precipitated from the acetate mobile phase at pH 2.5. Therefore, increased binding of BSA and BSA-Biotin with increasing salt concentration at pH 2.5 may result because chloride ions decrease the solubility of these proteins. Sodium chloride at 0.1 *M* concentration eliminates BSA binding to the resin within experimental error at pH 4.8. Under these conditions, a significant amount of BSA-Biotin was bound to the resin while no BSA was bound to the resin. Interestingly, use of 0.1 *M*

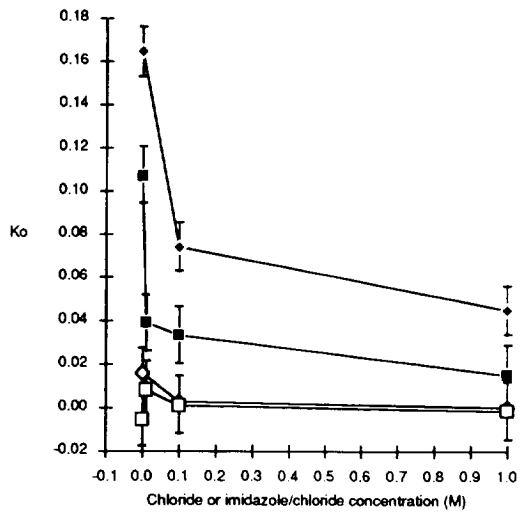

Fig. 8. Effect of imidazole/chloride concentration on BSA and BSA-Biotin binding to Biogel P200–Glut–Thio–Pt(II) resin at pH 4.8. Experimental conditions as in Fig. 6. Symbols: ◆ = BSA-Biotin, chloride; ■ = BSA-Biotin, imidazole/chloride; ◇ = BSA, chloride; □ = BSA, imidazole/chloride.

sodium chloride gave the same result as shown in Fig. 4 for column conditioning, yet no conditioning was performed.

Fig. 8 shows the effects of simultaneously changing mobile phase imidazole/chloride concentrations on the binding of BSA and BSA-Biotin to Column D. For all runs, BSA binding was less than 5%, and slightly less than observed for column B. The effect of imidazole was more pronounced for BSA-Biotin binding, resulting in approximately half the BSA-Biotin binding at each imidazole/chloride concentration on Column D compared to the corresponding chloride concentrations on Column B. Imidazole probably competes with the protein for Pt(II) binding sites on the gel surface.

3.1. Binding and elution of BSA and BSA-Biotin

BSA-Biotin was applied to the gel using a sodium phosphate buffer at pH 4.8. Elution was accomplished using a 1.0 *M* imidazole–HCl buffer at pH 7.0, resulting in 60% of the bound BSA-Biotin being recovered. After extensive washing of the same column with the sodium phosphate buffer at pH 4.8, BSA-Biotin was applied again, followed by imidazole elution. A brief summary of these results is given in Table 5. Because the amount of BSA-Biotin recovered during imidazole elution for Trial no. 2 was greater than that recovered the first time the column was used (73% versus 60%), it is possible that some uneluted BSA-Biotin from Trial no. 1 may have been eluted along with the BSA-Biotin recovered during the elution step for Trial no. 2.

The fraction of the initial application of BSA-Biotin bound to the column was slightly greater for the second injection than for the first injection. This increase can be rationalized as experimental error. In an earlier experiment (Fig. 5), subsequent application of BSA-Biotin at pH 4.8 resulted in less protein binding for the second injection. This "conditioning" effect was not seen in the experiment reported here because the loss of sample due to conditioning would be much smaller than the experimental error for such a large sample injection.

Table 5
Application of BSA-Biotin to Biogel P200–Glut–Thio–Pt(II) and subsequent elution with 1.0 *M* imidazole at pH 7.0

	Trial no. 1	Trial no. 2
Percentage of initial BSA-Biotin application bound to column	37 ± 3	38 ± 3
Percentage of bound BSA-Biotin eluted with imidazole	60 ± 6	73 ± 7

4. Conclusions

Binding of BSA-Biotin to Biogel P200 containing immobilized platinum ions is best conducted at pH 4.8, and greater BSA-Biotin binding is obtained with the column described in this paper at lower flow rates. In order to achieve binding of BSA-Biotin without concomitant binding of BSA, the column required use of an application buffer with >0.1 M NaCl or preconditioning with an injection of BSA in order to remove the sites with high charge densities. BSA and BSA-Biotin binding generally increased with decreasing pH due to the fixed negative charge of the gel surface carrying. The immobilized Pt(II) column binds BSA-Biotin and no unconjugated BSA at pH 4.8, which is the isoelectric point of BSA. Addition of salt to the mobile phase resulted in less protein binding at pH 4.8 and 7.0, and more binding at pH 2.5. The effect of salt appeared to be quite complex. Salt may affect the conformation of the protein, compete for ionic binding sites on the gel, and/or affect the electrical double layer that exists on the surface of the gel. Elution of BSA-Biotin was accomplished using a 1.0 M imidazole–HCl buffer at pH 7.0, whereby 60% of the bound BSA-Biotin was recovered.

Binding of BSA-Biotin to Biogel P200 containing immobilized platinum ions appeared to exhibit slow kinetics. Film transport partially limits protein binding to the activated gel. Therefore, steps taken to increase the film transport coefficient could increase column performance. Increasing the superficial velocity, as well as disrupting the flow pattern through the packed bed would be desirable. Furthermore, nonspherical packings would be preferable to the spherical gel used in these experiments. In addition, proteins with large diffusivities would be expected to transport faster through the film, and exhibit higher binding to the column.

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Symbols

A_c	Cross sectional area of chromatography column (cm^2)
AUFS	Absorption Unit Full Scale
B	Fraction of initial sample application that bound to the column, which equals $1-E$
C_m	Bulk concentration of solute in the mobile phase (mmol/ml)
C_p	Solute concentration within the pores of the gel (mmol/ml)
C_s	Concentration of sorbed solute on the gel (mmol/ml)
D	Diffusivity (cm^2/s)
d_p	Diameter of particle (cm)
E	Fraction of the initial sample application that was eluted from column, which equals $1-B$
F	Flow rate (ml/min)
k'	Capacity factor for a retained species applied to the gel, calculated as $(t_r - t_L)/(t_L)$ or $(V_r - V_L)/V_L)$
k_b	Binding constant defined as q/c
k_d	Distribution coefficient defined as the concentration of ligand bound to the immobilized metal ions divided by the concentration of free ligand in solution
k_f	Mass transfer coefficient representing transport through a stagnant film surrounding each gel bead (min^{-1})
K_o	Overall mass transfer coefficient (min^{-1})
k_r	Constant of proportionality for a first order reaction representing complexation of a ligand to the an immobilized metal ion at the gel surface (min^{-1})
L	Column length (cm)
q	Amount of material sorbed to column (mmol/ml hydrated solid volume)
Re	Reynolds number

Sc Schmidt number

t_L Measured value of t_r for a small molecule that totally permeates the gel, but does not interact with the gel (i.e., moves with the solvent front), in min

t_o Measured value of t_r for a species that is totally excluded from gel pores, and does not interact with the gel (min)

t_r Time at which the maximum point is reached for a chromatographic response peak (min)

t'_r $(t_r - t_L)/(t_L)$

u Superficial velocity defined as $(F)/(\epsilon A_c)$ (cm/min)

V_e Volume of eluent applied to the column (ml)

V_i Intraparticle pore volume in a packed column (ml)

V_L Total hydrated volume of the gel available for permeation by a liquid, equal to $V_i + V_o$, (ml)

V_o Interparticle void volume in a packed column (ml)

V_r Volume of eluent applied to the column at which the maximum point is reached for a chromatographic response peak (ml)

V_s Volume of solid packing within the column, equal to $V_t - V_L$ (ml)

V_t Total volume of the packed bed, including void and solid packing volumes (ml)

z Distance measured from the inlet port of the column (cm)

ϵ Void fraction of the column, equal to $(V_L)/(V_t)$

ν Kinematic viscosity (cm^2/s)

References

[1] M. Wilchek and E.A. Bayer (Editors), *Avidin-biotin Technology*, Academic Press, San Diego, CA, 1990.

[2] E.A. Bayer and M. Wilchek, *Methods Biochem. Anal.*, 26 (1980) 1–45.

[3] M. Wilchek and E.A. Bayer, *Anal. Biochem.*, 171 (1988) 1–32.

[4] M. Wilchek and E.A. Bayer, *Trends Biochem. Sci.*, 3 (1989) 408.

[5] E.P. Diamandis and T.K. Christopoulos, *Clin. Chem.*, 37 (1991) 625.

[6] E.A. Bayer and M. Wilchek, *J. Chromatogr.*, 510 (1990) 3.

[7] K. Hoffman, G. Titus, J.A. Montibeller and F.M. Finn, *Biochemistry*, 21 (1982) 978.

[8] D.H. Kim, A.A. García, D. R. Miles and R. Makam, *Reactive Polymers*, in press.

[9] J. Porath, *Protein Expression Purif.*, 3 (1992) 263.

[10] R.G. Pearson, *J. Am. Chem. Soc.*, 85 (1963) 3533.

[11] B. Rigas, A.A. Welcher, D.C. Ward and S.M. Weissman, *Proc. Natl. Acad. Sci. USA*, 83 (1986) 9591.

[12] S. Ritter, J. Komenda, E. Setlikova, I. Setlik and W. Welte, *J. Chromatogr.*, 625 (1992) 21.

[13] G.L. Peterson, *Anal. Biochem.*, 83 (1977) 346.

[14] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, *J. Biol. Chem.*, 193 (1951) 265.

[15] D.H. Kim, *PhD Thesis*, Arizona State University, Tempe, AZ, 1994.

[16] D.R. Miles, *PhD Thesis*, Arizona State University, Tempe, AZ, 1994.

[17] A.L. Hines and R.N. Maddox, *Mass Transfer: Fundamentals and Applications*, Prentice Hall, Englewood Cliffs, NJ, 1985, pp. 151–159.

[18] E.J. Wilson and C.J. Geankoplis, *Ind. Eng. Chem. Fundam.*, 5 (1966) 9.

[19] C. Lentner (Editor), *Geigy Scientific Tables*, Vol. 3, Ciba-Geigy, Basle, 1984, p. 136.

[20] W.W. Yau, C.P. Malone and H.L. Suchan, *Sep. Sci.*, 5 (1970) 259.

[21] W.W. Yau, J.J. Kirkland and D.D. Bly, *Modern Size-Exclusion Liquid Chromatography: Practice of Gel Permeation and Gel Filtration Chromatography*, John Wiley and Sons, New York, 1979, p. 29.

[22] W.W. Yau, H.L. Suchan and C.P. Malone, *J. Polym. Sci. Part A-2*, 6 (1968) 1349.

[23] J.J. Hermans, *J. Polym. Sci. Part A-2*, 6 (1968) 1217.

[24] W.W. Yau, J.J. Kirkland and D.D. Bly, *Modern Size-Exclusion Liquid Chromatography: Practice of Gel Permeation and Gel Filtration Chromatography*, John Wiley and Sons, New York, 1979, p. 28.

[25] J.N. Little, J.L. Waters, K.J. Bombaugh and W.J. Pauplis, *J. Polym. Sci. Part A-2*, 7 (1969) 1775.

[26] A.C. Ouano and J.A. Barker, *Sep. Sci.*, 8 (1973) 673.