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# Spermine Sepharose as a clustered-charge anion exchange adsorbent

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ABSTRACT

We previously showed that the affinity and capacity of ion exchange adsorbents of a given total charge density are improved by immobilization of the charges in pre-ordered clusters, rather than individually in random locations. This previous work used pentalysinamide and pentaargininamide as clustered ligands. This approach allows close control of cluster size, but is uneconomically expensive for some research and most practical applications. In this work, we demonstrate that the inexpensive synthetic analog of the natural polyamine spermine ($H_2N—CH_2—CH_2—CH_2—NH—CH_2—CH_2—CH_2—CH_2—NH—CH_2—CH_2—CH_2—NH_2$) also can serve as the basis of effective clustered adsorbents. The calcium-depleted form of the protein α-lactalbumin, and RNA from baker's yeast, were adsorbed on a spermine Sepharose adsorbent. This adsorbent exhibited enhanced α-lactalbumin binding capacity ($Q_{max}$ > 1.6 and 1.3-fold higher than those for Qiagen DEAE and GE DEAE Sepharose adsorbents of much greater charge density) and higher initial binding affinity ($Q_{max}/K_D$ 2.4 and 2.1-fold higher, respectively). The new spermine-based matrix exhibited a higher value of the *Z* parameter, suggesting an increased number of apparent interaction sites between the protein and the resin, and functioned well in column mode.



## 1. Introduction

The steady increase in titers and sales of recombinant pharmaceutical proteins have increased interest in high-efficiency, high-capacity downstream processes for the recovery and purification of biological products [1–3], particularly since estimates of the fraction of bioprocess costs devoted to purification range as high as 80% [4–6]. The continuing need for selective, scalable and economical unit operations has driven the design of novel bioengineering strategies [7,8].

Chromatographic purification remains the most costly element of biopharmaceutical downstream processing [9,10], and development of optimal chromatographic processes is a central issue in bioprocess engineering. Because it offers strong and easily reversible protein adsorption, ion-exchange chromatography is widely used for the selective purification of biomolecules [11,12]. Traditional ion-exchange adsorbents present a random charge distribution over the particle surface that creates a heterogeneous field of adsorption sites [13]. In order to improve this random charge distribution for protein capture, adsorbents have been modified in the past to present improved characteristics by either increasing ligand density through the use of tentacular adsorbents [14,15] or by attaching polyions such as polyethyleneimine or polylysine in order to enhance product selectivity and recovery [16].

Many proteins present a characteristic high charge-density patch that can play a dominant role in the ion-exchange adsorption of these molecules [11,17–19]. We have found that an adsorbent displaying uniform clusters of positive charge shows a higher affinity and capacity for negatively charged biomolecules than an adsorbent with the same total charge density displayed as dispersed charges [11,20]. Clustered pentaargininamide adsorbents of relatively low ligand density show higher affinity for negatively charged proteins than conventional adsorbents of much higher ligand density [11]. The use of pentaargininamide as a ligand in research and for process scale purification, however, must contend with the cost and base-lability of the ligand, which hampers efficient resin cleaning. In this work we describe spermine as an inexpensive, stable ligand for clustered adsorbents. Spermine is a polycationic polyamine involved in DNA packaging whose four amines (see Fig. 1) can form a cluster of four positive charges [21], and whose low cost and alkali stability make it potentially suitable as a ligand for clustered-charge anion exchange chromatography.

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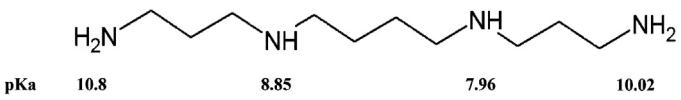


**Fig. 1.** Spermine molecule with corresponding p$K_a$ values for titratable atoms. Note that p$K_a$ values for symmetry-related atoms differ because of the sequential addition of protons to the molecule.

In the present work we demonstrate the superior affinity and capacity of a spermine Sepharose clustered-charge anion exchanger for the anionic protein α-lactalbumin, which presents a modifiable charge cluster. The clustered-charge adsorbent presents enhanced initial binding affinity ($Q_{max}/K_D$) and maximum binding capacity ($Q_{max}$) when compared to dispersed-charge commercial adsorbents, even when the latter have higher ligand density.

## 2. Materials and methods

### 2.1. Materials

Sepharose CL-6B, Q Sepharose Fast Flow and DEAE Sepharose were from GE Healthcare (Piscataway, NJ). DEAE Plasmid Plus resin was from Qiagen (Valencia, CA). All other reagents were from Sigma Aldrich (St. Louis, MO) including salmon sperm DNA (Cat. number: D1626), Baker's yeast RNA (Cat. number: R6750) and $Ca^{2+}$ depleted α-lactalbumin from bovine milk (Cat. number: L6010). α-Lactalbumin was prepared from unpasteurized bovine milk and was purified by ion exchange chromatography on DEAE-agarose. Its purity is ≥85% as was determined by PAGE. The molar mass for the protein used was 14,100 g/mol and the extinction coefficient $\varepsilon^{1\%} = 20.1$ [22].

### 2.2. Adsorbent preparation

Aldehyde activation of Sepharose was based on the method of Miron and Wilchek [23]. For activation, DI water-washed Sepharose CL-6B (400 μl in 1.6 mL water) was treated with 0.2 M sodium periodate for 3 h at room temperature, then washed with DI water and resuspended in 50 mM borate buffer pH 9.5 ± 0.2. To 400 μl of activated Sepharose CL-6B settled resin, 1.6 mL of spermine tetrahydrochloride (45 mg/mL) solution in 50 mM borate buffer, pH 9.5 was added and incubated on a gyratory rotator at room temperature for 2 h and centrifuged 2 min at 16,000 × *g*. The gel with the bound spermine was treated for 3 h with 2 mL of 2 mM sodium borohydride in 50 mM borate buffer, pH 9.5 to reduce the initially formed Schiff base into a stable secondary amine linkage. Spermine modified Sepharose was washed with 20 mM phosphate buffer, pH 7.0 and stored at 4 °C.

### 2.3. Ligand density determination

The ligand density of spermine Sepharose was determined by titration with 5 mM NaOH in column mode using an ÄKTA purifier FPLC equipped with a post-column pH monitor. Spermine Sepharose was packed in a column (5 × 20 mm) and equilibrated with 5 column volumes of 0.1 M HCl, and then 5 mM NaOH was run through a bypass in order to equilibrate the extra-column volume. After the equilibration, 5 mM NaOH was passed through the column to determine the moles of base required to titrate the acidified adsorbent. The amount of 5 mM NaOH required for titration of unmodified Sepharose (typically about 1.2 mL) was subtracted from that required to titrate the spermine Sepharose (typically about 6.8 mL) to obtain the millimoles of NaOH used to titrate spermine.

### 2.4. Adsorption isotherm measurements

Aliquots (25 μl) of 60% (v/v) spermine Sepharose suspension in binding buffer (10 mM Tris, 10 mM NaCl, pH 8) were placed in 1.5 mL Eppendorf tubes. To these tubes different amounts of protein stock solution (10–180 μl of 5 mg/mL $Ca^{2+}$-depleted bovine α-lactalbumin (or RNA) in binding buffer) were added, followed by binding buffer up to 1 ml. Tubes were placed on a gyratory rotator at room temperature for 1 h, then centrifuged for 10 min at 16,000 × *g*. Protein concentration in the supernatant was quantified by 280 nm absorbance using a Tecan Infinite M200 Pro microplate reader (Tecan, USA). The adsorbent pellets were washed with 1 mL of binding buffer followed by recentrifugation (the wash contained only a small fraction, 3.1% ± 1.7%, of the added protein). Bound protein was eluted with 1 mL of elution buffer (binding buffer + 1 M NaCl) and adsorbed protein content was determined. In order to determine the completeness of protein recovery, mass balances were performed by dividing the sum of the protein remaining in the supernatant after adsorption equilibration + protein recovered in the wash + protein in the elution fraction, by the original amount of protein added. The ionic strength of the 10 mM Tris buffer, 10 mM NaCl, pH 8 is estimated at 0.015 M.

### 2.5. Data analysis

The mass balance recoveries for all points in all experiments closed in the range 86–111%. Protein adsorption data were fit to the Langmuir (Eq. (1)), Langmuir–Freundlich (Eq. (2)) isotherms and the steric mass action (SMA) model of Cramer et al. (Eq. (3)) [24].

$$y = \frac{Q_{max}X}{K_D + X} \tag{1}$$

$$y = \frac{Q_{max}X^{n_H}}{K^nH + X^nH} \tag{2}$$

$$y = \left(\frac{Q_\alpha}{K}\right)\left(\frac{X}{\Lambda - (\nu+\sigma)Q_\alpha}\right)^\nu \tag{3}$$

In these equations, $y$ is the bound protein concentration, $X$ is the free protein concentration, $Q_{max}$ is the maximum binding capacity (in μmol/ml of settled bed adsorbent), and $K_D$ is the dissociation constant. In Eq. (2), $n_H$ is the Langmuir–Freundlich heterogeneity parameter. In Eq. (3), $Q_a$ is the equilibrium adsorbent-bound protein concentration, K is the equilibrium constant, $\Lambda$ is the total ionic capacity of the adsorbent in μmol/ml of settled bed adsorbent, $\sigma$ is the dimensionless steric factor that reflects the number of sites on the surface that are shielded by the adsorbate and prevented from exchange with salt counter-ions in solution and $\nu$ is the dimensionless characteristic charge that reflects the number of sites that the protein interacts with on the surface. Fitting used Igor Pro (WaveMetrics, Lake Oswego, OR; version 6.05) which uses the Levenberg–Marquardt algorithm to search for parameters which minimize the $\chi^2$ values, as described previously [11,20]. The initial guess vectors for the parameters were varied manually to ensure that a true global optimum was found.

Boardman and Partridge [25] and Regnier [26] have described the stoichiometric displacement model which emphasizes the importance of electrostatic interactions between proteins and adsorbents in terms of ionic equilibria. If an electrolyte such as NaCl is used, the equilibrium of $Na^+$ ions and target proteins (a polyion represented by $P^{n+}$) between the mobile phase (m) and the adsorbent (ad) can be represented by: $Z\text{Na}_{ad} + \text{P}_m \leftrightarrow \text{P}_{ad} + Z\text{Na}_m$. Beginning with this equilibrium an expression was derived which relates the binding affinity of the adsorbent to the molarity of the displacing salt in the mobile phase which was then linearized to the