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Evaluation of immunoglobulin adsorption on the hydrophobic charge-induction resins with different ligand densities and pore sizes

Hui-Li Lu[a], Dong-Qiang Lin[a,*], Dong Gao[b], Shan-Jing Yao[a]

[a] State Key Laboratory of Chemical Engineering, Department of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310027, China
[b] Key Laboratory of Synthetic and Natural Functional Molecular Chemistry of Ministry of Education, Institute of Modern Separation Science, Northwest University, Shaanxi Key Laboratory of Modern Separation Science, Xi'an 710068, China

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ABSTRACT

Hydrophobic charge-induction chromatography (HCIC) is a novel technology for antibody purification. The ligand densities and pore properties of HCIC resins have significant effects on the separation behavior of protein, however, the understandings are quite limited. In the present work, new HCIC ligand, 2-mercapto-1-methylimidazole (MMI) was coupled to three agarose matrices with different pore sizes. A series of MMI resins with different ligand density and pore size was prepared by the control of ligand coupling. The adsorption isotherms and kinetics on the series of MMI resins were investigated with bovine serum immunoglobulin as the model IgG, and the effects of salt addition were studied. The Langmuir equation and pore diffusion model were used to fit the experimental data, and the influences of ligand density, pore size and salt addition on the saturated adsorption capacity, the dissociation constant and the effective diffusivity were discussed. It was found that the adsorption capacities and the effective pore diffusion coefficient increased with the increase of ligand density and pore size. The effects of salt addition on the adsorption behaviors were dependent on the ligand density. For low ligand density the IgG adsorption was salt-promoted, while the resins with high ligand density showed a salt-independent property. The results indicated that for a given protein the ligand density and pore size of HCIC resins should be optimized for improving the protein adsorption.

1. Introduction

Antibodies and related proteins have been the biotechnology drivers over decades due to the growing demand in clinical trials and therapeutics [1–3]. Currently the commercial successes and dramatic improvements in cell culture productivity have put massive pressure on the downstream processes [4,5]. As we know, chromatographic techniques are widely employed in the separation of antibodies for both laboratory and industrial scale operations [6,7]. Among them, Protein A affinity chromatography has been adopted as the classical platform approach for the capture of antibody with high specificity and efficiency. However, there are still some disadvantages on the Protein A capture, such as high cost, low reusability, unavoidable ligand leaching, harsh elution condition and some difficulties on the regeneration. Thus, some novel chromatographic techniques were developed to separate antibody in more economical and efficient way [3,8].

Burton and Harding [9,10] introduced the hydrophobic charge-induction chromatography (HCIC) for the selective capture of antibodies under physiological conditions in 1998. The basic characteristic of HCIC is the pH-dependent behavior of dual-mode ionisable ligands that combine the hydrophobic and electrostatic interactions together. Normally the HCIC ligands, such as 4-mercapto-ethyl-pyridine (MEP) with the pKa of 4.8, show non-charge at neutral condition but become positively charged at low pH. The target protein can be adsorbed on the uncharged ligand at neutral pH by the hydrophobic forces, and desorbed by the electrostatic repulsion between target protein and the charged ligand at acidic condition. The adsorption capacity was often independent on the ionic strength over a broad range, which was called"salt-independent" or "salt-tolerant" properties [11–14]. Up to now, HCIC has been successfully applied to the purification of antibodies [12,13,15] and other proteins [14] as a cost-effective alternative to Protein A chromatography. High adsorption capacities and good selectivity were often achieved. However, the properties of HCIC resins still need to be optimized in order to improve the separation efficiency for therapeutical uses.

As an efficient resin, most important properties are the ligand density and pore size, which have been found to have significant influences on the separation efficiency in many kinds of chromatographic techniques, including ionic exchange chromatography [16–21], hydrophobic interaction chromatography [22,23],

* Corresponding author. Tel.: +86 571 87951982; fax: +86 571 87951982.
E-mail address: lindq@zju.edu.cn (D.-Q. Lin).

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HCIC [24] and affinity chromatography [25,26]. In general, ligand density has a positive effect on the adsorption of protein [16,19,20]. Franke et al. [18] and Lu et al. [21] found that the adsorption capacity could reach the maximum at certain ligand density range, which might be explained that the accessible surface area for protein binding decreased with the increasing of ligand density. On the other hand, the pore size directly influences the mass transport in the resin and consequently affects the separation resolution and dynamic capacity of protein in the column [27]. The accessibility and transport of protein are facilitated for large pores, while small pores could increase the specific surface area and corresponding adsorption capacity for protein binding. It is obvious that the adsorption behaviors of chromatographic resins are dominated by two factors together, the amount of available ligand and the accessible surface area for target protein. In our previous works [21,28], a parameter *N*, defined as the ratio of ligand density to the square of pore size, was introduced to describe the integrative effects of pore size and ligand density on the saturate adsorption capacity for anion exchange chromatography. Except the properties of stationary phase, the conditions of liquid phase (such as pH and salt concentration) have also obvious influences on the adsorption of protein. Therefore, considering the combinational effects of stationary phase (ligand density and pore size) and mobile phase (pH and salt addition) on the protein adsorption is quite necessary. Unfortunately, till now a systematical work on this aspect and the corresponding experimental data are still lacking, especially for the HCIC, which would certainly hinder the development of new resins and the improvement of separation efficiency.

In the present work, three crossed-linked agarose gels with different agarose concentrations (6%, 4% and 3.5%) would be used as the matrices to represent the varying pore sizes. The matrices were activated by allyl bromide (AB), and then coupled with 2-mercapto-1-methyl-imidazole (MMI) to prepare new HCIC resins. With the control of the activation reaction, series of MMI resins were obtained with different ligand densities and pore sizes. The adsorption isotherms and adsorption kinetics of bovine immunoglobulin G (IgG) would be investigated with series of MMI resins prepared. The effects of salt addition were also studied. The Langmuir equation and pore diffusion model (PDM) were used to fit the experimental data. The influences of ligand density, pore size and salt concentration on the saturated adsorption capacity, the dissociation constant and the total effective diffusivity were discussed. The potential adsorption mechanism was discussed, too.

2. Experimental

2.1. Materials

Three kinds of crossed-linked agarose gel with different agarose concentrations, Bestarose 3.5HF, Bestarose 4FF and Bestarose 6FF, were purchased from Bestchrom Bio-Technology Co., Ltd. (Shanghai, China), and used as the chromatographic matrices. 2-mercapto-1-methylimidazole (MMI) was purchased from Aladdin (Shanghai, China). Bovine γ-globulin (bovine IgG, M_w = 153.1 kDa), which is bovine normal immunoglobulin G (IgG > 95%), has been chosen as a model protein to investigate the adsorption behavior of antibodies on HCIC resins. The polyclonal antibody mixture was purchased from Sigma (Milwaukee, WI, USA). Other reagents were of analytical reagent grade and purchased locally.

2.2. Electrophoresis and SEC-HPLC analysis of bovine IgG

Bovine IgG used in the present work is a mixture of polyclonal IgGs. The purity of bovine IgG was analyzed by 8% non-reducing SDS-PAGE gel and SEC-HPLC. For SDS-PAGE, the protein solution was diluted to 0.5–2 mg/mL, and protein migration was performed at 220 V for 35 min. The gel was stained with Coomassie Blue R-250, and destained. The stained gel was imaged with the Gel Doc 2000 imaging system (Bio-Rad, Hercules, CA, USA). SEC-HPLC was performed with Agilent 1100 system (Agilent Technologies, Santa Clara, CA, USA) using the TSK G3000SW XL column (7.8 mm × 30.0 cm, TOSOH, Japan). The operation flow rate was 0.5 mL/min. 0.1 M sodium phosphate buffer (pH 6.7, containing 0.1 M Na_2SO_4) was used as the mobile phase after 0.22 μm membrane filtration and degassing.

The distribution of isoelectric points (pIs) of bovine IgG used was analyzed by isoelectric focusing electrophoresis (IEF). The IEF with precast polyacrylamide gel (pH 3–10, Bio-Rad, Hercules, USA) was conducted on a Multiphor II apparatus (GE Healthcare, Uppsala, Sweden) connected to a thermostated water-cooling system and a 3000 V power supply (GE Healthcare, Uppsala, Sweden) [25]. The precast gel was pre-run at a constant power of 700 V for 20 min. Then the sample was loaded onto the gel and the protein migration was performed at 500 V for 20 min and 2000 V for 90 min. After that, the gels were stained with Coomassie Brilliant Blue R-250, and destained. The stained gel was imaged with the Gel Doc 2000 imaging system (Bio-Rad, Hercules, USA). The pIs of the sample components were estimated by comparison with standard proteins of known pI and the pH gradient curves.

2.3. Preparation of HCIC resins

The HCIC resins were prepared according to the method of Burton and Harding [9,10] with some modifications [15,29]. The synthesis scheme is shown in Fig. 1. At first, agarose gels were activated by allyl bromide (AB) at the basic condition. 10 g gels were mixed with suitable amount of allyl bromide and sodium hydroxide in 20% dimethyl sulfoxide (DMSO) solution, and the mixture was continuously agitated at 180 rpm and 30 °C for 24 h. Secondly, the allyl-activated agarose gels were brominated with 1.2 molar excess of N-bromosuccinimide (NBS) over the allyl groups in 50% acetone at 180 rpm and 30 °C for 1 h. Then the brominated gels were washed with deionized water several times. Finally, 3 molar excess of MMI molecules over the allyl groups were coupled to the brominated gels in 1 M carbonate buffer (pH 10) at 180 rpm and 30 °C for 24 h, and then the HCIC resins was obtained and named MMI-B. In the present work, the amount of allyl bromide in the activated reaction was optimized to obtain the activated agarose gels with different densities and further to prepare series of the MMI resins with different ligand densities.

The olefinic bond of the activated matrices and the ligand density of MMI resins were determined by the titration method [15,29]. In particular, to determine the olefinic bond of the activated matrices, 1 g drained activated matrix was mixed with 120 μL of mercaptoacetic acid (MMA) and 1 mL of 25 mg/mL ammonium persulfate, and the mixture was incubated at 180 rpm and 60 °C. After 8 h, the matrix was washed with deionized water, 0.1 M NaOH and 0.1 M HCl, respectively, and then drained and transferred to a vial. 5 mL 0.5 M NaCl solution was added, and the mixture was titrated with 0.1 M Tris to pH 6.4. For the measurement of MMI ligand density, 1 g MMI resin was washed repeatedly with deionized water, 0.1 M HCl and 0.1 M NaOH, respectively, and then drained and transferred to a vial. 5 mL 0.5 M NaCl solution was added, and the mixture was titrated with 0.1 M HCl to pH 3.8.

2.4. Adsorption equilibrium experiments

The adsorption isotherms of bovine IgG with series of MMI resins were measured with the procedure as the reference [30]. MMI resins were pre-equilibrated with an appropriate buffer, then 0.04 g drained resins was added to 0.8 mL aliquots of 20 mM phosphate