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Rational methods for predicting human monoclonal antibodies retention in protein A affinity chromatography and cation exchange chromatography Structure-based chromatography design for monoclonal antibodies

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Abstract

Rational methods for predicting the chromatographic behavior of human monoclonal antibodies (hMabs) in protein A affinity chromatography and cation exchange chromatography from the amino acid sequences information were proposed. We investigated the relation between the structures of 28 hMabs and their chromatographic behavior in protein A affinity chromatography and cation exchange chromatography using linear gradient elution experiments. In protein A affinity chromatography, the elution pH of the hMabs was correlated with not only the structure of the Fc region (subclass), but also that of the variable region. The elution pH of hMabs that have LYLQMNSL sequences in between the CDR2 and CDR3 regions of the heavy chain became lower among the same subclass of hMabs. In cation exchange chromatography, the peak salt concentrations I_R of hMabs that have the same sequences of variable regions (or that have a structural difference in their Fc region, which puts them into a subclass) were similar. The I_R values of hMabs were well correlated with the equilibrium association constant K_e, and also with the surface positive charge distribution of the variable region of the heavy chain (corrected surface net positive charge (*cN*) of the VH region). Based on these findings, we developed rational methods for predicting the retention behavior, which were also tested with eight additional hMabs. By considering the information on the number of binding sites associated with protein adsorption as determined experimentally, and the surface positive charge distribution from the three-dimensional structure of Mab A, we hypothesized that hMabs is separated by cation exchange chromatography as the surface positive charge distribution of the VH region is recognized.

Keywords: Ion exchange chromatography; Protein separation; Chromatography models; Human antibodies

1. Introduction

Chromatography is the main unit operation in the purification process of recombinant biopharmaceutical proteins [1–3]. However, as there are many parameters affecting the chromatographic behavior (retention time/volume, elution pH, peak salt concentration, resolution, elution curve), designing chromatography conditions for efficient protein separation remains still difficult and time-consuming. Therefore, rational methods for predicting the chromatographic behavior of protein chromatography are expected to reduce the time needed for protein purification process development. Several methods for predicting the chromatographic behavior of low molecular weight substances using information on the structure and the net charge or the net hydrophobicity have been reported [4–7]. However, these methods are still not established for protein chromatography, as each protein has its own unique three-dimensional structure as well as its (primary) structure (amino acid sequence), and surface

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charge and hydrophobic distribution. For these reasons, process chromatography of proteins is still designed using trial and error approaches [1–3].

Monoclonal antibodies (Mabs) have recently been major targets in the biopharmaceutical industries, and at least 400 Mabs are claimed to be in clinical and preclinical trials [8]. Because the speed with which protein-pharmaceutical drugs reach the market and clinics is critical, the rational process design methods for chromatography separation processes are needed. However, Mabs show different chromatographic behavior in chromatography in terms of the elution pH on protein A affinity chromatography, and the elution salt concentration on cation exchange chromatography. Therefore, as mentioned above, there is no rational or predictive method for purifying Mabs by chromatography. On the other hand, Mabs have similar three-dimensional structures. They are basically Y-shaped proteins, consisting of four polypeptides consisting of two identical light and two identical heavy chains. This indicates that they may have similar surface charges and hydrophobic distributions, and that, therefore, predicting their chromatographic behavior may be possible with information on their amino acid sequences.

We have been investigating the biorecognition of proteins in ion-exchange (or electrostatic interaction) chromatography with the linear gradient elution model [9–11]. It may be possible to use this method to obtain important information on the relation between the structure and chromatographic behavior of Mabs. In the present study, we investigated the chromatographic behavior of 28 human monoclonal antibodies (hMabs) in protein A affinity chromatography and cation exchange chromatography by using linear gradient elution experiments. The data on the model that we developed were analyzed. We then developed methods for predicting the chromatographic behavior (elution pH, peak salt concentration I_R, retention volume V_R and elution salt concentration I_E) from the amino acid sequences information.

2. Experimental

2.1. Chromatography media and columns

MabSelect was used as the protein A affinity chromatography medium, and packed into a column (column size = I.D. 5.0 mm × 50 mm, bed volume V_t = 0.98 mL). Hitrap SP Sepharose FF column (column size = I.D. 7.0 mm × 25 mm, bed volume V_t = 0.96 mL) was used as cation exchange chromatography column and medium. These media are products of Amersham Biosciences (Uppsala, Sweden).

2.2. Materials

All hMabs used in this study were produced at Kirin. Table 1 shows the physiochemical properties of the hMabs which were employed for the experiments. Other reagents used in this study were of analytical grade.

Table 1
Human monoclonal antibodies employed in chromatography experiments

No.	Antibodies	Subclass	Isoelectric points, p*I*
1	Mab A	1	8.2
2	Mab B	2	7.9
3	Mab C	4	7.7
4	Mab D	1	8.3
5	Mab E	1	8.0
6	Mab F	1	8.1
7	Mab G	1	8.0
8	Mab H	2	7.7
9	Mab I	1	8.1
10	Mab J	1	8.1
11	Mab K	1	7.9
12	Mab L	2	7.5
13	Mab M	4	7.7
14	Mab N	4	7.3
15	Mab O	2	7.6
16	Mab P	4	7.3
17	Mab Q	4/2/4[a]	7.5
18	Mab R	4	7.3
19	Mab S	4	7.3
20	Mab T	4	7.8
21	Mab U	1	7.9
22	Mab V	4	7.1
23	Mab W	4	7.1
24	Mab X	4	7.9
25	Mab Y	1	7.9
26	Mab Z	1	8.0
27	Mab AA	1	8.0
28	Mab AB	1	7.9

Note: p*I* values were calculated by amino acid sequences.
[a] Chimeric IgG of IgG_2 and IgG_4.

2.3. Chromatography apparatus

Most of the experiments were performed on fully automated liquid chromatography systems, the ÄKTA explorer 100 or ÄKTA explorer 10S (Amersham Biosciences, Uppsala, Sweden) at room temperature.

2.4. Protein A affinity chromatography experiment

Linear gradient elution experiments were carried out with 28 hMabs on the MabSelect protein A affinity column. The column was equilibrated with binding buffer (0.1 M disodium phosphate–0.1 M sodium acetate–0.1 M glycine–0.15 M NaCl, pH 7.4). The hMabs samples were loaded on the column with 1 mg/mL resin. Elution was performed by linear gradient elution from the binding buffer to elution buffer (0.1 M sodium acetate–0.1 M glycine–0.15 M NaCl, pH 2.5) in a 20 column bed volume. The volumetric flow rate F was 1 mL/min. The elution pH of each hMab was converted to the relative value %B conc. (which was correlated with the elution pH), because accurate and reliable measurements of the pH were not possible due to variations in temperature and other experimental variables.

2.5. Experiment to verify the elution pH predicted for protein A affinity chromatography

The eight hMabs listed in Table 2 were used to verify whether the predicting elution pH was correct in the protein A affinity chromatography. The column was equilibrated with binding buffer (10 mM sodium phosphate, pH 6.0). The hMab sample was loaded on the column with 1 mg/mL resin. Elution was performed with elution buffer (20 mM sodium citrate, pH 3.0 or 3.4) by stepwise elution. The elution pH of the elution buffer was used from the predicted value of each hMab. F was 1 mL/min.

2.6. Cation exchange chromatography experiment

Linear gradient elution experiments were carried out with the 27 hMabs on an SP Sepharose FF cation exchange column. The column was equilibrated with binding buffer (20 mM sodium acetate, pH 5.0, 6.0 or 7.0). The hMabs sample was loaded on the column with 1 mg/mL resin. Elution was performed by NaCl linear gradient elution from 0 to 0.5 M in binding buffer. The gradient slopes g (M/mL) were chosen so that the gradient volumes were 10, 20, 30 or 40 columns per bed volumes. F was 0.5, 1.0, 1.5 or 2.0 mL/min. The linear mobile phase velocity u was calculated with the cross-sectional area A_c and the column bed void fraction ε as $u = F/(A_c\varepsilon)$. ε was determined from the peak retention volume of Dextran T 2000 pulses (the retention volume is column void volume, V_0). The data of the linear gradient elution experiments of each hMab was analyzed by the linear gradient elution model (see Section 3).

2.7. Experiment to verify the chromatographic behavior predicted for cation exchange chromatography

The eight hMabs listed in Table 2 were used to verify whether the predicting peak salt concentration of the linear gradient elution or elution salt concentration of the stepwise elution are correct in cation exchange chromatography. The SP Sepharose FF column was equilibrated with the binding buffer (20 mM sodium acetate, pH 5.0). hMabs samples were loaded on the column with 1 mg/mL resin. The linear gradient elution was performed with an increasing elution buffer (20 mM sodium acetate, pH 5.0, 0.5 M NaCl) concentration from the binding buffer, and the gradient volume was set at a 17.3 column volume. Stepwise elution was performed with 20 mM sodium acetate, pH 5.0 containing a predicted concentration of NaCl. F was 1.0 mL/min.

Table 2
Human monoclonal antibodies employed in verifying experiments and results of predicted and experimental elution pH in protein A affinity chromatography

No.	Antibodies	Subclass	pI	LYLQMNSL sequences	Elution pH	
					Predicted	Experimental
1	Mab AC	1	9.0	No	pH 3.4	S
2	Mab AD	4	8.7	No	pH 3.4	S
3	Mab AE	1	8.5	Yes	pH 3.0	S
4	Mab AF	1	8.8	Yes	pH 3.0	S
5	Mab AG	1	7.8	No	pH 3.4	S
6	Mab AH	1	8.5	No	pH 3.4	S
7	Mab AI	4	8.7	No	pH 3.4	S
8	Mab AJ	1	9.0	No	pH 3.4	S

Note: pI values were calculated by amino acid sequences. S: successfully eluted.

3. Model and calculation

3.1. Linear gradient elution model

We proposed and experimentally verified the methods by which the relation between the distribution coefficient K and the salt concentration I were determined from the protein peak salt concentration I_R in linear gradient elution. The method is explained briefly below. The normalized gradient slope GH in the linear gradient elution [3,9–15] is defined by the following equation.

$$GH = gV_0\left[\frac{V_t - V_0}{V_0}\right] = g(V_t - V_0) \quad (1)$$

V_t is the total bed volume, V_0 the column void volume and calculated as $V_0 = \varepsilon V_t$, $G = gV_0$ and $H = (V_t - V_0)/V_0$ is the phase ration. g (M/mL) is the gradient slope of the salt, which is defined by the following equation.

$$g = \frac{I_F - I_0}{V_g} \quad (2)$$

I_F is the final salt concentration, I_0 the initial salt concentration, and V_g the gradient volume. The linear gradient elution experiments were performed at different gradient slopes (GH values) at a fixed pH. I_R is determined as a function of GH. The GH–I_R curves thus constructed do not depend on the flow velocity, the column dimension, the sample loading at non-overloading conditions, or I_0 provided that the sample is initially strongly bound to the column [3,13]. The experimental GH–I_R data can usually be expressed by the following equation [3,9–11,13].

$$GH = \frac{I_R^{(B+1)}}{A(B+1)} \quad (3)$$

From the law of mass action (ion exchange equilibrium) [1–3,12,16–19], the following relationship can be derived.

$$A = K_e\Lambda^B \quad (4)$$

Here, B is the number of sites (charges) involved in protein adsorption, which is basically the same as the Z number in the literature [16], K_e is the equilibrium association constant, and Λ is the total ion exchange capacity. From Eqs. (3) and (4), I_R can be expressed as follows:

$$I_R = [GH\,K_e\Lambda^B(B+1)]^{1/(B+1)} \quad (5)$$

From the ion-exchange equilibrium model [3,12,16–19] and Eq. (4), the following equation was derived [3,12].

$$K - K' = K_e \Lambda^B I^{-B} \quad (6)$$

where K is the protein distribution coefficient, K' the distribution coefficient of salt, and I the ionic strength (salt concentration).

From Eq. (6), K–I curves were constructed, and from the K–I relationship, a stepwise elution condition (salt concentration I_E) was determined as the protein distribution coefficient at I_E is approximately K' (= 0.72) [3,12].

If we are only interested in predicting the peak retention in the linear gradient elution, Eq. (3) can be used with A and B as experimental values [3,12,13]. However, if we construct the GH–I_R curves as a function of mobile phase pH, and determine the pH–I_R, pH–B and pH–K_e relationships, quite important information can be obtained on the retention (or biorecognition) and resolution of proteins as a function of the mobile phase pH [9–11].

4. Results and discussion

4.1. Gradient elution data analysis for MabSelect protein A affinity chromatography

Linear gradient elution experiments were carried out with 28 hMabs to investigate the elution pH of various hMabs in MabSelect protein A affinity chromatography. For evaluation of the elution behavior, relative %B conc. values compared to %B conc. of Mab A were adopted, and the results are shown in Fig. 1. The value of the relative %B conc. ($V_{r\%B}$) indicates the degree of the affinity to protein A compared to Mab A. Namely, when a hMab has 1 $V_{r\%B}$, it means that the hMab has the same protein A affinity as Mab A; when the $V_{r\%B}$ of a hMab is below 1, the hMab has a lower protein A affinity, and furthermore, when the $V_{r\%B}$ of a hMab is above 1, the hMab has a higher protein A affinity.

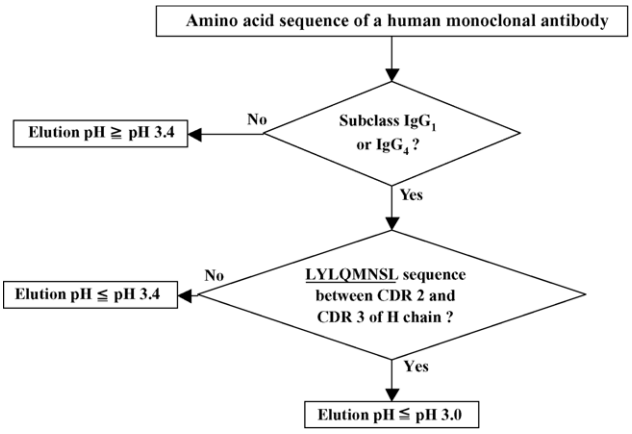


Fig. 2. Flow-chart for predicting protein A affinity chromatography elution pH of human monoclonal antibodies on the basis of amino acid sequences. H chain: heavy chain.

As shown in Fig. 1, subclass IgG_2 hMabs had a lower protein A affinity than those of subclasses IgG_1 and IgG_4. $V_{r\%B}$ of subclass IgG_1 and IgG_4 hMabs, which have identical variable sequences, and both groups of (Mab A and Mab C) and (Mab U and Mab V), were almost equal, or subclass IgG_4 hMabs showed slightly higher values. The eight hMabs enclosed in the circle had higher values than the other hMabs. A different elution pH of hMabs among the subclasses has already been reported [20]. The higher elution pH of subclass IgG_2 hMabs than subclasses IgG_1 and IgG_4 seems to be due to the different amino acid sequences of the protein A binding domain on the Fc region of the hMabs (although the sequence of subclasses IgG_1 and IgG_4 are equal; sequence data not shown). In addition, the difference in the elution pH among the same subclass of hMabs suggests that the affinity to protein A is dependent not only on the structure of the Fc region, but also that of the Fab region. There was no relation between the elution pH and the number of hydrophobic amino acids, charged amino acids, or isoelectric points (pI) of the Fab region (data not shown). When we compared the variable region of the eight hMabs to those of the other hMabs,

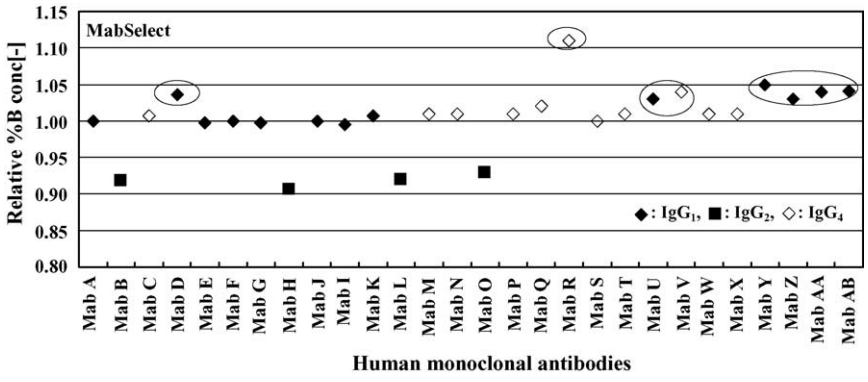


Fig. 1. Protein A affinity for human monoclonal antibodies from different subclasses and variable regions. %B conc. is peak elution pH of antibody on protein A affinity chromatography with pH gradient elution. Relative %B conc. = %B conc. of each antibody/%B conc. of Mab A. Column: MabSelect, 5.0 mm diameter × 50 mm length; binding buffer: 0.1 M disodium phosphate–0.1 M sodium acetate–0.1 M glycine–0.15 M NaCl, pH 7.4; elution buffer: 0.1 M sodium acetate–0.1 M glycine–0.15 M NaCl, pH 2.5. Flow rate: 1 mL/min; gradient: 20 column volume.

we found that specific consensus sequences (LYLQMNSL) of the eight hMabs existed on the frame region between CDR2 and CDR3 of the heavy chain. The hMab that have the LYLQMNSL sequences are classified into the VH3 family, and some Fab fragments in the VH3 family have protein A affinity [21], which may be the reason why the lower elution pH of hMabs which have the sequences, probably the LYLQMNSL region, may be a protein A binding domain.

In the preparative process of protein A affinity chromatography, stepwise elution with an acidic pH buffer was generally applied instead of pH gradient elution. Therefore, elution experiments were performed with pH stepwise elution using

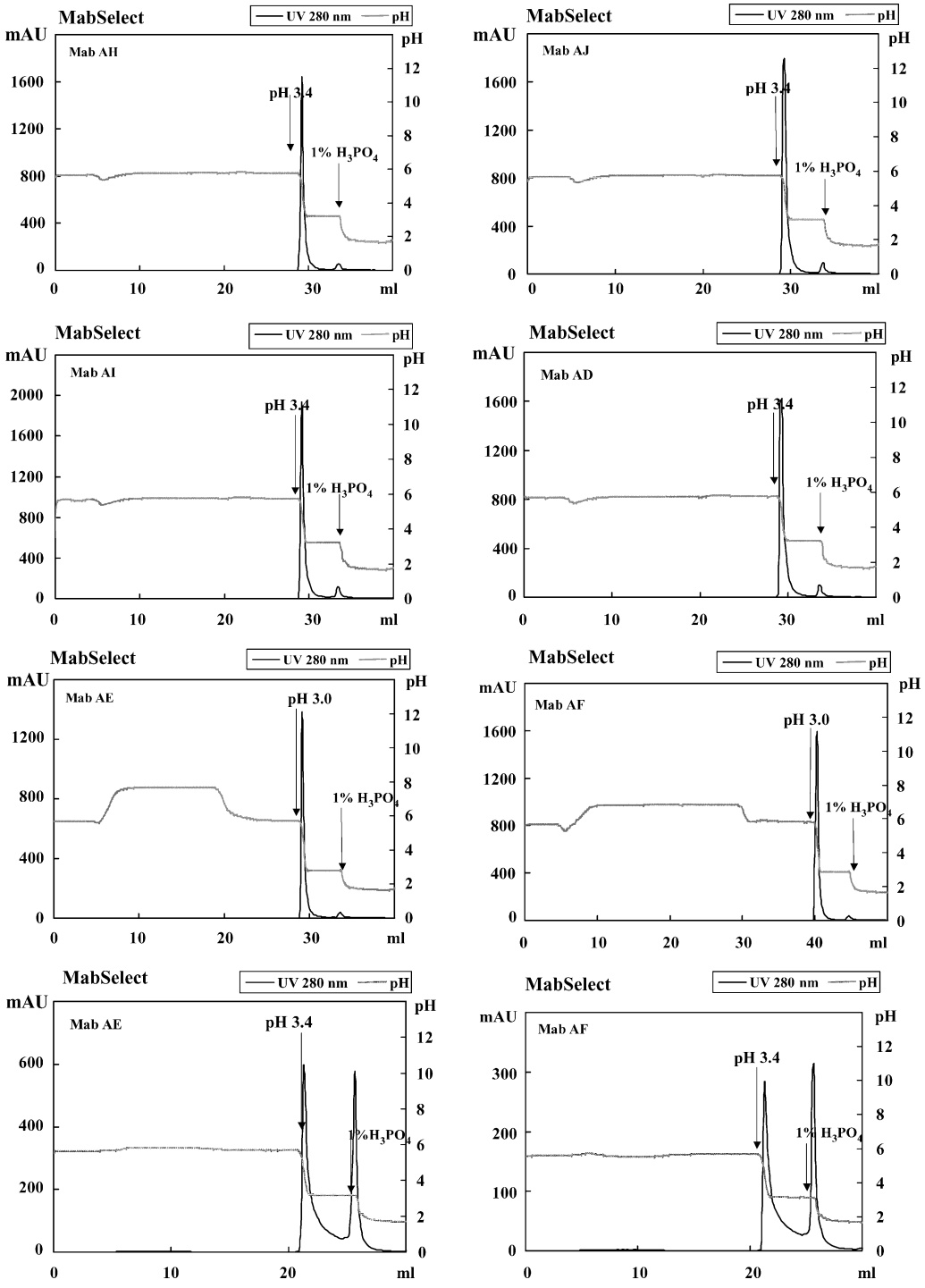


Fig. 3. Elution curves of human monoclonal antibodies with protein A affinity chromatography. System: ÄKTAexploer 10S; column: MabSelect, 5.0 mm diameter × 50 mm length; binding buffer: 10 mM sodium phosphate, pH 6.0; elution buffer: 20 mM sodium citrate; washing buffer: 1% H_3PO_4; flow rate: 1 mL/min (300 cm/h).

a sodium citrate buffer of pH 3.4 or 3.0. The structure of hMabs and the elution pH were as follows.

Subclass IgG_2 hMabs eluted over pH 3.4, subclass IgG_1 and IgG_4 hMabs eluted under pH 3.4; subclasses IgG_1 and IgG_4 hMabs, which have LYLQMNSL sequences on the frame region, eluted under pH 3.0 (data not shown).

From these results, a method for predicting the elution pH of hMabs on MabSelect could be deduced from the amino acid sequences of an arbitrary hMab. Fig. 2 shows a flow chart for predicting the protein A affinity chromatography elution pH of hMabs on the basis of the amino acid sequences. The procedure is carried out as follows:

1. If a target hMab does not belong to either subclass IgG_1 or IgG_4, then the elution pH is $\geq$3.4.
2. If a target hMab belongs to either subclass IgG_1 or IgG_4, but lacks the LYLQMNSL sequence in the frame region between CDR2 and CDR3 of the heavy chain, then the elution pH is $\leq$3.4.
3. If a target hMab belongs to either subclass IgG_1 or IgG_4 and has the LYLQMNSL sequence in the frame region between CDR2 and CDR3 of the heavy chain, then the elution pH is $\leq$3.0.

4.2. Verification of predicted elution pH

To verify the method shown in Fig. 2, the elution pH of the eight hMabs listed in Table 2 were predicted, and the chromatography experiments were carried out. The predicted elution pH of the eight hMabs are also shown in Table 2.

From the results of these predictions, we performed chromatography experiments with an elution pH of 3.4 or 3.0. As examples of the elution curves of the eight hMabs on MabSelect, those of six hMabs are shown in Fig. 3. In addition, the results of the chromatography experiments are shown in Table 2. All chromatography were successfully performed with the pH elution buffer predicted. Moreover, Mab AE and Mab AF, whose predicted elution pH was 3.0, could not be eluted using pH 3.4 buffer. These results indicate that the elution pH of hMabs in MabSelect protein A affinity chromatography can be predicted from the amino acid sequence information, as shown in Fig. 2. The proposed method is applicable under other protein A affinity resin and different chromatographic conditions from those of the present experimental conditions (e.g., flow rate, column size, binding and elution buffer composition) with the exception of the elution buffer pH. The present method can also be applied on a wide range of scales, i.e., from a laboratory to a manufacturing scale. It should be noted that the elution pH of protein A affinity chromatography is determined only by the structure of protein A and the Mabs themselves [20]. Although the method described here may not be applicable to mouse and chimeric Mabs, it may be applicable to humanized Mabs due to their similar structural properties to those of hMabs.

4.3. Gradient elution data analysis for SP Sepharose FF cation exchange chromatography

Linear gradient elution experiments of SP Sepharose FF were performed using 27 hMabs, and the relation between the chromatographic behavior and structure of the hMabs was investigated with the linear gradient elution model.

Fig. 4 shows typical GH–I_R curves. The GH–I_R curves did not depend on the flow velocity, as mentioned in Section 3.1. This indicates the applicability of the model to the present experimental system. Using Eqs. (3)–(5) in Section 3.1, B, K_e and I_R (at $GH = 0.02$) were determined, and the relation among pI, B, K_e and I_R of the hMabs employed in this study were investigated.

As shown in Fig. 5, less correlation was obtained between I_R (at $GH = 0.02$) of the various hMabs on SP Sepharose FF in the mobile phase pH 5.0 and pI values calculated by amino acid sequences of the hMabs. This indicates that the chromatographic behavior of hMabs cannot be determined and predicted simply by net charge properties.

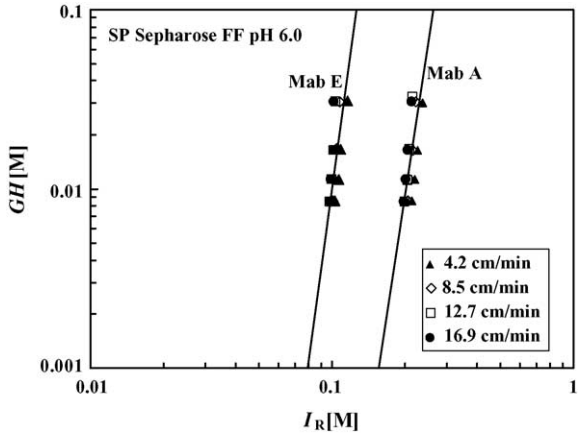


Fig. 4. GH–I_R curves of cation exchange chromatography column.

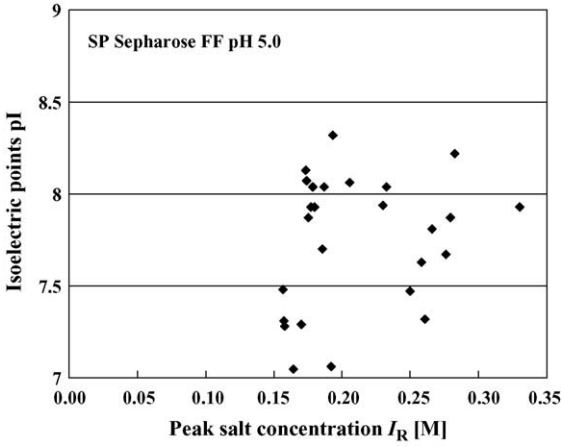


Fig. 5. Peak salt concentration I_R and isoelectric points pI relationships of human monoclonal antibodies. Column: SP Sepharose FF, 7.0 mm diameter × 25 mm length; mobile phase: 20 mM sodium acetate pH 5.0, salt: NaCl; I_R: at $GH = 0.02$.

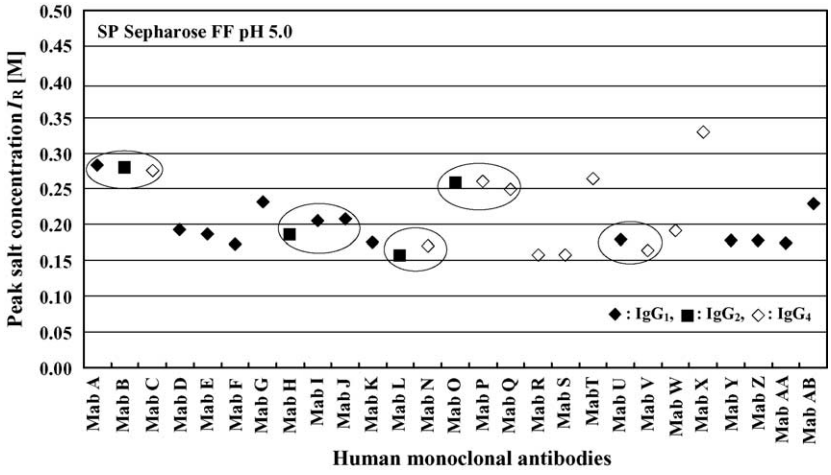


Fig. 6. Peak salt concentration I_R of human monoclonal antibodies from different subclasses and variable regions in cation exchange chromatography. Column: SP Sepharose FF, 7.0 mm diameter × 25 mm length; binding buffer: 20 mM sodium phosphate, pH 5.0; elution buffer: 20 mM sodium phosphate, pH 5.0, 0.5 M NaCl; gradient: GH = 0.02; flow rate: 1 mL/min. *Note*: Each hMab enclosed in a circle has the same sequences of the variable region.

Fig. 6 shows the I_R (at $GH = 0.02$) of various hMabs on SP Sepharose FF in the mobile phase pH 5.0. The results of I_R (at $GH = 0.02$) of various hMabs in the mobile phase pH 6.0 and 7.0 indicated the same tendency (data not shown). The investigated hMabs indicated that the I_R is in the range of 0.15–0.35 M. I_R of the hMabs which have the same variable region were equivalent. Fig. 7 shows the elution curves of six kinds of hMabs. Mab A, Mab B and Mab C, which have the same variable region, revealed equivalent elution curves. These results suggest that the elution behavior of hMabs on SP Sepharose FF do not depend on the subclass, but on the variable region. In other words, SP Sepharose FF would not recognize the Fc region, but would recognize the variable region of hMabs.

Fig. 8 shows the relation between the K_e and I_R (at $GH = 0.02$) of various hMabs on SP Sepharose FF. When the *Y*-axis is on a logarithmic scale, a positive correlation can be obtained between the I_R and the K_e. Especially, in the range of over 0.15 M I_R or in mobile phase pH 5.0, a high correlation coefficient could be obtained ($R^2 \geqq 0.95$). These results indicate that chromatographic behavior can be calculated using the linear gradient elution model and the I_R–K_e relation. Also, considering the results that the I_R of hMabs that had the same variable region were equivalent, if the relation between I_R and the amino acid sequences of the variable region becomes clear, we will be able to predict the chromatographic behavior of hMabs on SP Sepharose FF from the amino acid sequences.

For solving the above relation, the surface charge distribution of the variable region in hMabs was considered. Furthermore, the variable region of hMabs according to the germline family consensus frameworks [22] was classified, and the relative side chain solvent accessibility (SCA) of

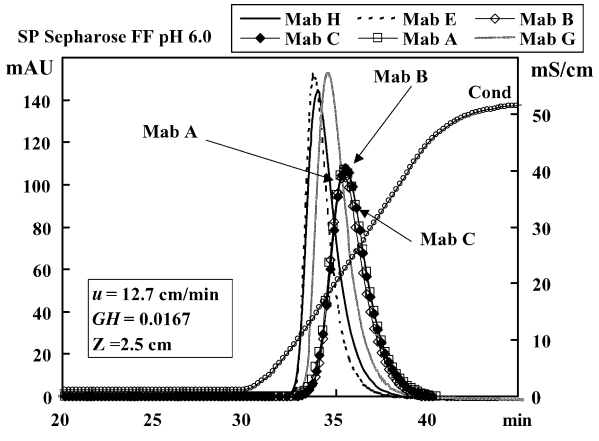


Fig. 7. Comparison of elution curves (chromatogram) of various human monoclonal antibodies with cation exchange chromatography column in linear gradient elution. System: ÄKTA explorer 100; column: SP Sepharose FF, 7.0 mm diameter × 25 mm length; binding buffer: 20 mM sodium acetate, pH 6.0; elution buffer: 20 mM sodium acetate, pH 6.0, 0.5 M NaCl; flow rate: 1.5 mL/min; gradient: 20 column volume.

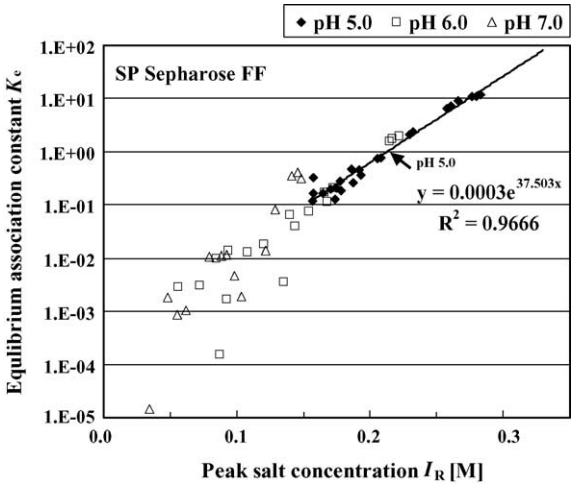


Fig. 8. Equilibrium association constant K_e and peak salt concentration I_R relationships. Column: SP Sepharose FF, 7.0 mm diameter × 2.5 cm length; mobile phase: 20 mM sodium acetate, salt: NaCl; I_R: at $GH = 0.02$.

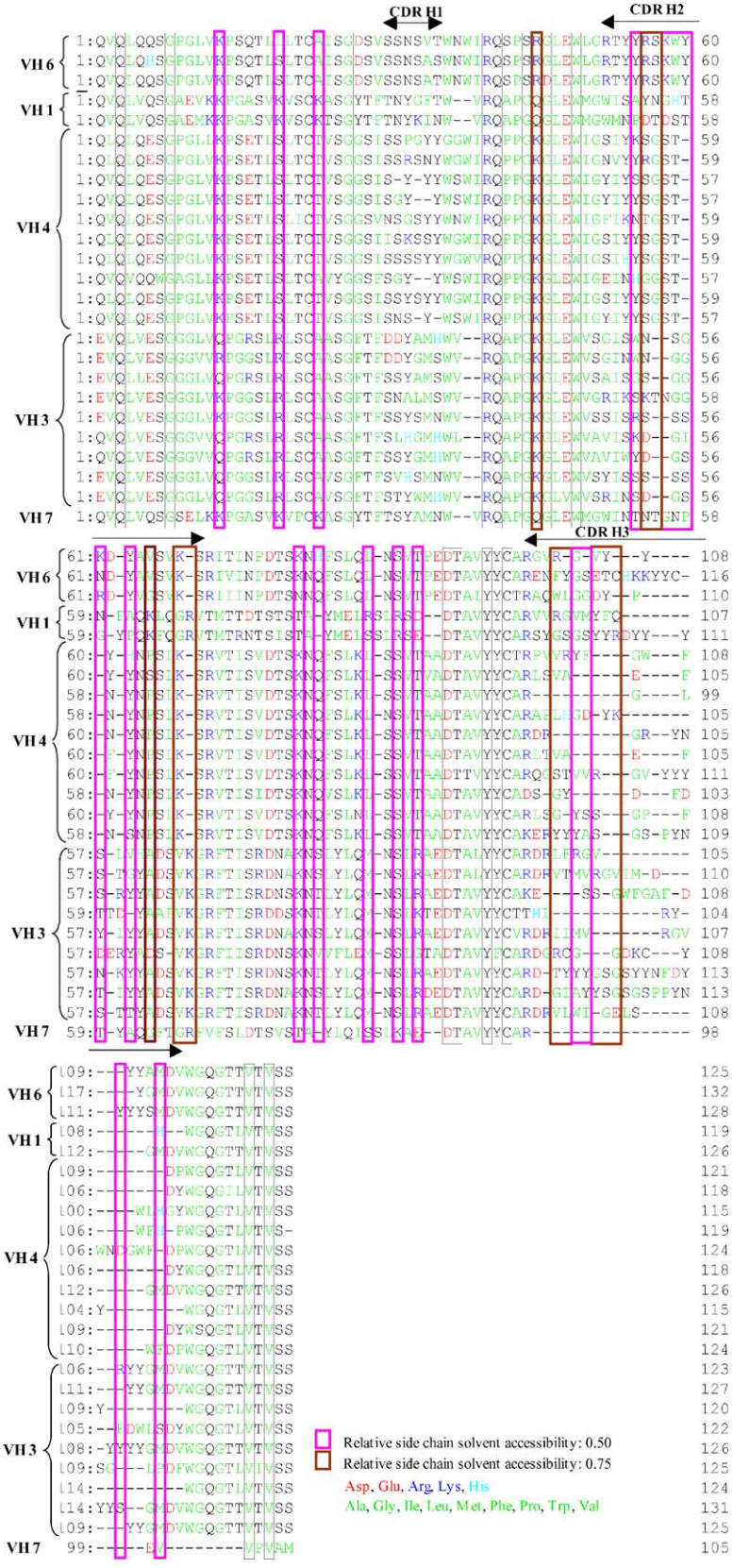


Fig. 9. Picture A: Alignment VH sequences of human monoclonal antibodies. *cN* of VH region: corrected surface net positive charge of variable region of heavy chain = 0.75*a* + 0.50*b*. *a*: number of positive side chains (relative side chain solvent accessibility = 0.75) of VH region; *b*: number of positive side chains (relative side chain solvent accessibility = 0.50) of VH region. *Note*: Relative side chain solvent accessibility at □ residues was estimated 0.50. Relative side chain solvent accessibility at □ residues was estimated 0.75.

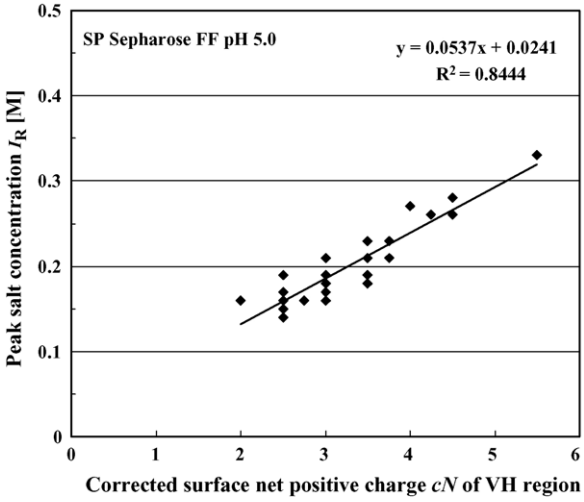


Fig. 10. Relation between peak salt concentration I_R and corrected surface net positive charge *cN* of variable region of heavy chain (VH region). Column: SP Sepharose FF, 7.0 mm diameter × 2.5 cm length; mobile phase: 20 mM sodium acetate, pH 5.0, salt: NaCl; I_R: at $GH = 0.02$.

the amino acid residue of the variable region in each family [22] was considered. From this information, the number of charged amino acid residues in the variable region and the ratio of exposure of these residues from the SCA were calculated. Then, as shown in Fig. 9, by the alignment of the variable region of the heavy chain (VH region) from the hMabs and by simplifying the values of the SCA of amino acid residues at the positions indicated in the Figure, we defined the summation of each simplified SCA values multiplied by the number of positive side chains at the indicated positions (see Fig. 9) as the corrected surface net positive charge (*cN*) of VH region. Here, we found that the *cN* of the VH region has a positive correlation with I_R (at $GH = 0.02$) in pH 5.0 ($R^2 = 0.84$), as shown in Fig. 10. Therefore, I_R and V_R in the arbitrary linear gradient elution condition and the elution salt concentration I_E in stepwise elution can be calculated using the *cN*–I_R relation, I_R–K_e relation, the linear gradient elution model and information on the amino acid sequences of the VH region of hMabs. Fig. 11 shows the protocol for predicting the chromatographic behavior of hMabs on SP Sepharose FF on the basis of the amino acid sequences and the chromatography model. Below is an outline of the proposed procedure.

1. The VH sequence of a target hMab is aligned with Picture A (Fig. 9).
2. The number of positive side chains with an SCA of 0.5 or 0.75, respectively, is counted using Picture A.
3. The *cN* of the VH region is calculated according to the following, where the *cN* of the VH region = $0.75a + 0.50b$.
   a. Number of positive side chains (SCA = 0.75) of the VH region.
   b. Number of positive side chains (SCA = 0.50) of the VH region.
4. The I_R is calculated according to the *cN*–I_R relation ($I_R = 0.0537cN + 0.0241$).
5. The K_e is calculated according to the I_R–K_e relation ($K_e = 0.0003\, e^{37.503 I_R}$).
6. B is calculated according to the GH–I_R relation (Eqs. (3) and (4)). Λ of SP Sepharose FF is 0.215 mmol/mL gel according to the information supplied by the manufacturer.
7. In the case of stepwise elution, the *K*–*I* curve is plotted by Eq. (6).
8. The I_E value is determined based on the *K*–*I* curve, and Eq. (6). I_E is determined as the protein distribution coefficient at I_E, which is approximately the distribution coefficient of salt K' (= 0.72). In this study, I_E was calculated as $K = 0.76$ with Eq. (6).
9. The I_E value is determined as the elution salt concentration in stepwise elution of the target hMab.
10. In the case of linear gradient elution, the linear gradient elution volume is set.
11. The I_R is calculated according to Eqs. (3) and (4) with the previously calculated B and K_e.
12. Calculate V_R using the following equations [3,12,15,23], $V_R = (I_R - I_0)/g + V_0\ (1 + HK')$, $H = (V_t - V_0)V_0$.
13. The calculated I_R and V_R are those of at the set gradient elution volume for the target hMab.

Table 3
Results of predicted and experimental cation exchange chromatography

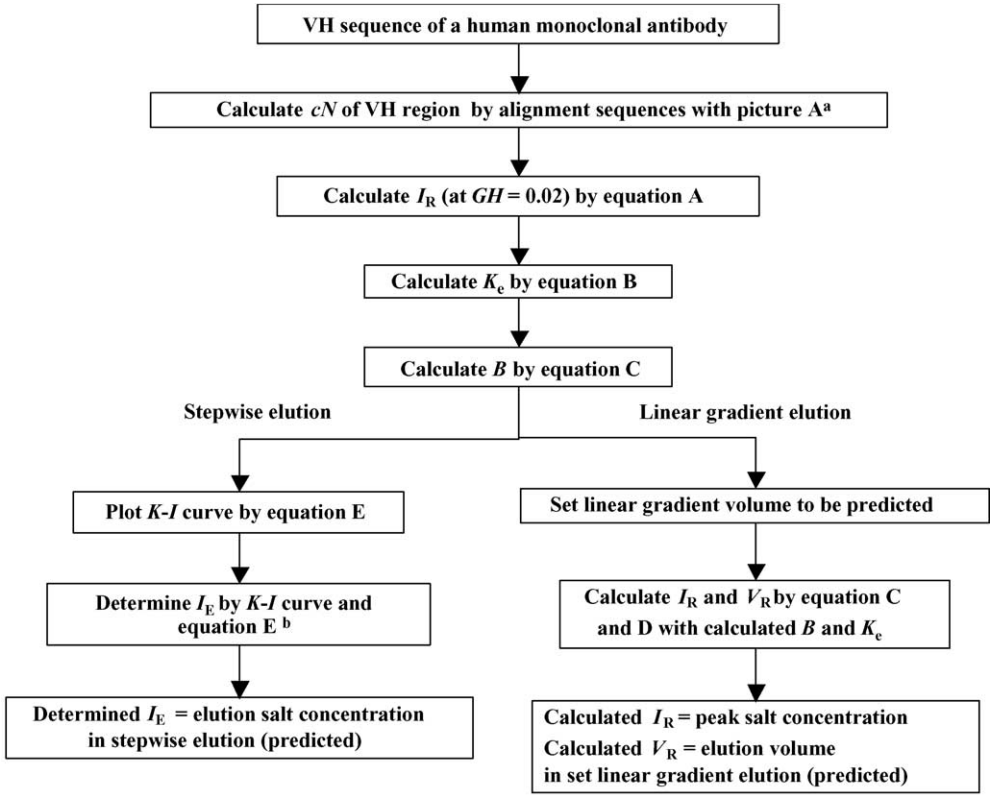
Antibodies	*a*	*b*	*cN* of VH region	Calculated K_e	Calculated *B*	I_R (M) (gradient)		I_E (M) (stepwise)	
						Predicted	Experimental	Predicted	Experimental
Mab AF	3	4	4.25	3.64E+00	6.00	0.25	0.26	0.53	S
Mab AE	3	4	4.25	3.64E+00	6.00	0.25	0.21	0.53	S
Mab AH	2	2	2.50	1.22E−01	7.09	0.16	0.15	0.25	S
Mab AJ	2	3	3.00	3.22E−01	8.87	0.19	0.19	0.27	S
Mab AC	2	3	3.00	3.22E−01	8.87	0.19	0.21	0.27	S
Mab AI	2	2	2.50	1.22E−01	7.09	0.16	0.14	0.25	S
Mab AG	2	2	2.50	1.22E−01	7.09	0.16	0.16	0.25	S
Mab AD	1	4	2.75	1.98E−01	7.10	0.17	0.16	0.27	S

S: successfully eluted; *cN* of VH region: corrected surface net positive charge of variable region of heavy chain = $0.75a + 0.50b$. *a*: number of positive side chains (relative side chain solvent accessibility = 0.75) of VH region; *b*: number of positive side chains (relative side chain solvent accessibility = 0.50) of VH region; I_R: peak salt concentration; I_E: elution salt concentration.

4.4. Verification of predicted chromatographic behavior

To verify the method in Fig. 11, chromatography experiments were performed using the eight hMabs listed in Table 2. In this study, the gradient volume was set as a 17.3 column volume for simplicity. Fig. 12 shows the *K–I* curves of the eight Mabs plotted with the calculated parameters K_e and B (Table 3). From the *K–I* curves and Eq. (6), the predicted I_E was determined. The predicted I_R and I_E determined with the method and the results of the chromatography experiment of the hMabs are summarized in Table 3. The prediction and verification of V_R in the linear gradient elution with the linear gradient elution model has already been reported [3,12,15,23].

In the linear gradient elution, experimental I_R of each hMab was approximately equivalent to the predicted I_R. In the experiment for the stepwise elution, all hMabs were successfully eluted by the predicted I_E. These verification results indicate that I_R and I_E on SP Sepharose FF can be successfully predicted from the information on the amino acid sequences



Equation A: $I_R = 0.0537\,cN + 0.0241$ (See Fig. 10)

Equation B: $K_e = 0.0003e^{37.503IR}$ (See Fig. 8)

Equation C: $GH = I_R^{(B+1)}/[K_e \Lambda^B (B+1)]$ (See Eq. (3) and (4)) [C]

Equation D: $V_R = (I_R - I_0)/g + V_0(1+HK')$ [References 3, 12, 15, 23]

Equation E: $K - K' = K_e \Lambda {}^B I^B$ (See Eq. (6))

VH: Variable region of heavy chain
I_R: Peak salt concentration
B: Number of binding sites
I: Salt concentration
I_E: Elution salt concentration
V_t: total column volume
I_0: initial salt concentration
V_0: column void volume

cN: Corrected surface net positive charge
K_e: Equilibrium association constant
K: Distribution coefficient
K': Distribution coefficient of salt
$GH = g(V_t - V_0)$
Λ: ion exchange capacity
g: gradient slope (M/mL)
$H = (V_t - V_0)/V_0$

Fig. 11. Flow-sheet for predicting cation exchange chromatographic behavior of human monoclonal antibodies on the basis of amino acid sequences and chromatography models. *Note*: [a]The calculation method of *cN* is as follows: (1) Count positive side chain whose relative side chain solvent accessibility of 0.5 and 0.75, respectively, with the alignment of amino acid sequences of VH region of a objective hMab using Fig. 9 (Picture A). (2) Calculate corrected surface net positive charge *cN* of VH region according to the equation in the Fig. 9. [b]Stepwise elution condition (salt concentration I_E) is determined as the protein distribution coefficient at I_E is approximately distribution coefficient of salt K' (= 0.72). In this study, I_E was calculated as $K = 0.76$ with Eq. (6). [c] Λ of SP Sepharose FF is 0.215 mmol/mL gel according to the information supplied by the manufacturer.

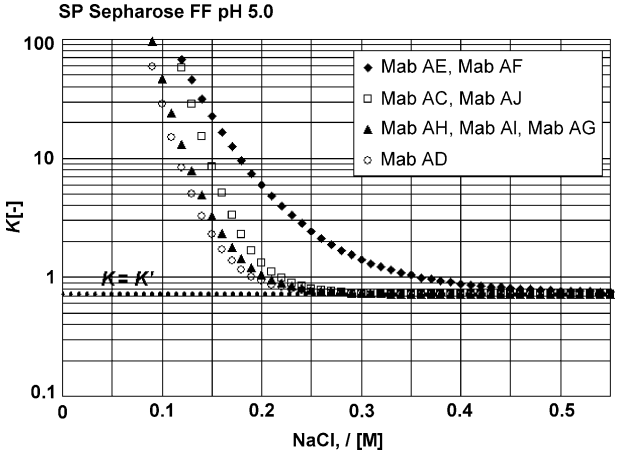


Fig. 12. Distribution coefficient K–salt concentration I curves.

in hMabs. Therefore, it was concluded that the this novel approach may, within a certain level of accuracy, predict I_R and I_E with the use of SP Sepharose FF cation exchange chromatography based on the amino acid sequences of arbitrary hMabs. The proposed method is applicable and scalable to different chromatographic conditions from those of the experimental conditions presented here (e.g., under different flow velocity, column dimension, gradient steepness, the sample loading at non-overloading conditions, or I_0 provided that the sample is initially strongly bound to the column). This variation is possible because the present method was developed based on the linear gradient elution model. Although this method may not apply to mouse and chimeric Mabs, it may apply to that of humanized Mabs, due to their sequence similarity with the hMabs used here. The same approach may also be applied with other cation exchange chromatography resins and different mobile phase pH conditions. The mobile phase pH of the method developed here is pH 5.0 (Generally, mobile phase pH around pH 5 is commonly used, in the preparative cation exchange chromatography of hMabs, especially for biopharmaceutical purification process including protein A affinity chromatography). In fact, as shown in Fig. 8, a positive correlation can be obtained at pH 6.0 and 7.0, although the correlation coefficients were lower than that at pH 5.0 (pH 6.0: $R^2 = 0.82$, pH 7.0: $R^2 = 0.83$, respectively). The I_R range of pH 6.0 and 7.0 shown in Fig. 8 was 0.03–0.22 M. At levels under the 0.1 M I_R range, there may be less of an interaction

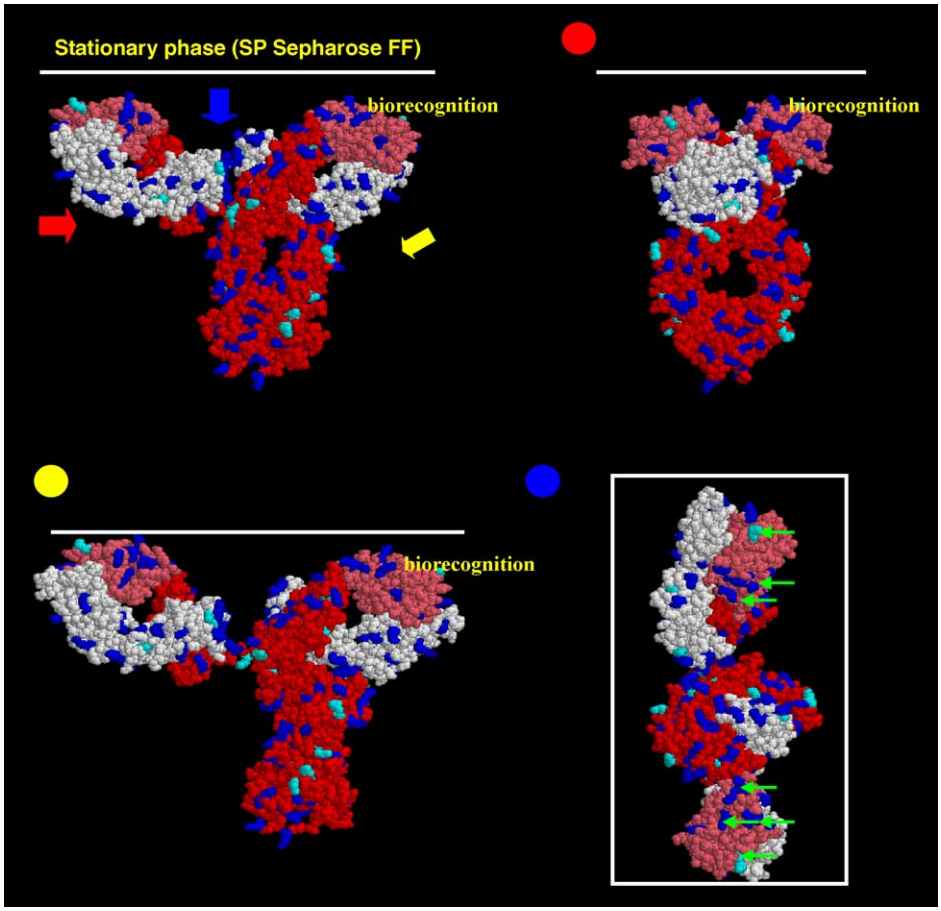


Fig. 13. Three dimensional structure of Mab A. *Note*: Red, blue and yellow circle or arrowhead indicate viewing directions toward the molecule. Red segment of the antibody molecule indicates constant region of heavy chain, Pink segment is variable region of heavy chain, white segment is light chain. Blue segment is Lys and Arg residue on the molecular surface, Cyan segment is His residue on the molecular surface. White straight line and white square line is diagrams of stationary phase which is drawn by considering the size of hMabs molecule and pore size of stationary phase.

between the hMabs and the resin, which may account for the lower correlation coefficients at pH 6.0 and 7.0 than at pH 5.0. Therefore, if the chromatography conditions required for stronger interactions could be optimized at various pH values, and if a correlation between cN–I_R at the various pH values could be obtained, then this approach may be applicable to various mobile phase pH values.

4.5. Mechanism of hMabs biorecognition

In this study, the relation between I_R and cN of the variable region of the right chain (VL region), cN of the VL region and VH region, and the surface charge distribution considering the negative charge was also investigated. However, less correlation was obtained compared to cN of the VH region (data not shown). In addition, the I_R of two hMabs, Mab Y and Mab Z, whose structures differed only in the light chain, were equivalent (Fig. 6). These results suggest that I_R of hMabs on SP Sepharose FF is determined by the surface positive charge distribution of the VH region. In other words, hMabs seem to be separated on SP Sepharose FF with the biorecognition of the surface positive charge distribution of the VH region. Fig. 13 shows the Mab A molecule sterically from four directions. It can be understood that the stationary phase (in this case, SP Sepharose FF) recognizes only a part of the hMab molecule, rather than the entire molecule, because of the molecules' three-dimensional structure. From the hypothesis mentioned above, hMabs and the stationary phase would biorecognize (bind) each other, as shown in Fig. 13. From the results of the linear gradient elution model, the B value of Mab A on SP Sepharose FF were ca. 7 in the mobile phase pH 5.0, ca. 6 in pH 6.0, and ca. 4 in pH 7.0, respectively. The number of our hypothesized binding positive sites (green arrowhead in the figure) from the three-dimensional structures were similar to the B value in pH 5.0. Moreover, the fact that the B value is reduced while the mobile phase pH is increasing can be explained by the non-dissociation of the His residue, which is located on the biorecognition (binding) sites (green arrowhead and cyan residue in the figure) in a higher pH solution. Although biorecognition has broad meaning, the present study has shown that the biorecognition in the present Mab A-SP Sepharose FF system is based on the molecular recognition due to electrostatic interaction between positively charged His residues and negatively charged ionic groups.

5. Conclusion

From the viewpoint of the biorecognition of hMabs in protein A affinity chromatography and cation exchange chromatography, the relation between the structures of 28 hMabs and their chromatographic behaviors in these chromatography modes using a linear gradient elution experiment was investigated.

In protein A affinity chromatography, the elution pH of the hMabs was related by not only the structure of the Fc region (subclass), but also that of the variable region. The elution pH of hMabs with LYLQMNSL sequences in between the CDR2 and CDR3 regions of the heavy chain was lower among the same subclass of hMabs. Based on these results, a method for predicting the elution pH on MabSelect on the basis of the amino acid sequences of hMabs was developed and verified using eight hMabs.

In cation exchange chromatography, I_R of each hMab with the same sequences of variable regions were equivalent to each other. I_R of the hMabs was correlated with K_e of the hMabs. The I_R was also correlated with cN of the VH region. With the results of this investigation, and using the linear gradient elution model, a method for predicting I_R/V_R of the arbitrary linear gradient elution condition and for predicting I_E of stepwise elution on SP Sepharose FF on the basis of the amino acid sequences was developed. The predictive ability of the method was then verified with additional chromatography experiments using eight hMabs.

The hypothesis that hMabs are separated on SP Sepharose FF with the biorecognition of the surface positive charge distribution of the VH region was supported by the result of the B value of Mab A, and the consideration with the three-dimensional structure of Mab A.

The results reported here thus provide the first line of evidence to demonstrate that chromatographic behavior can be predicted quantitatively from the amino acid sequences of proteins. In addition, the present investigation demonstrated, for the first time, the reliability of a novel methodology for structure-based chromatography for hMabs. The approach described here may enable the use of amino acid sequences to predict the chromatographic behavior of hMabs that can be expected when using the chromatography modes described above. Furthermore, the present method may contribute to accelerating the purification process development of hMabs for biopharmaceutical industries, as it eliminates the necessity of carrying out a trial-and-error approach to determine the appropriate chromatography conditions.

References

[1] G. Sofer, L. Hagel, Handbook of Process Chromatography, Academic Press, San Diego, CA, 1997.
[2] E. Karlsson, L. Ryden, J. Brewer, in: J.-C. Janson, L. Ryden (Eds.), Protein Purification, second ed., Wiley-VCH, New York, 1998.
[3] S. Yamamoto, K. Nakanishi, R. Matsuno, Ion-Exchange Chromatography of Proteins, Marcel Dekker, New York, 1988.
[4] M. Salo, H. Siren, P. Volin, S. Wiedmer, H. Vuorela, J. Chromatogr. A 728 (1996) 83.
[5] R. Berges, V. Sanz-Nebot, J. Barbosa, S. Wiedmer, J. Chromatogr. A 869 (2000) 27.
[6] T. Baczek, R. Kaliszan, J. Chromatogr. A 962 (2002) 41.
[7] R. Put, C. Perrin, F. Questier, D. Coomans, D.L. Massart, Y. Vander Heyden, J. Chromatogr. A 988 (2003) 261.
[8] T. Gura, Nature 417 (2002) 584.
[9] S. Yamamoto, T. Ishihara, J. Chromatogr. A 852 (1999) 31.
[10] S. Yamamoto, T. Ishihara, Sep. Sci. Technol. 35 (2000) 1707.

[11] T. Ishihara, S. Yamamoto, Kagaku Kogaku Ronbunshu 27 (2001) 186 (in Japanese).
[12] S. Yamamoto, M. Nomura, Y. Sano, AIChE J. 33 (1987) 1426.
[13] S. Yamamoto, Biotechnol. Bioeng. 48 (1995) 444.
[14] S. Yamamoto, P. Watler, D. Feng, O. Kaltenbrunner, J. Chromatogr. A 852 (1999) 37.
[15] S. Yamamoto, Bioseparation binran, Kyoritsu shuppan, Tokyo, Japan, 1996, p. 379 (in Japanese).
[16] W. Kopaciewicz, M.A. Rounds, J. Fausnaugh, F.E. Reniger, J. Chromatogr. 266 (1983) 3.
[17] N.K. Boardman, S.M. Partrige, Biochem. J. 59 (1955) 543.
[18] C.M. Roth, K.K. Unger, A.M. Lenhoff, J. Chromatogr. A 726 (1996) 45.
[19] S.R. Gallant, S. Vunnum, S.M. Cramer, J. Chromatogr. A 725 (1996) 295.
[20] P. Gagnon, Purification Tools for Monoclonal Antibodies, Validated Biosystems, 1996.
[21] R. Kontermann, S. Dubel (Eds.), Antibody Engineering, Springer, Berlin, 2001.
[22] A. Honegger, A. Pluckthun, J. Mol. Biol. 309 (2001) 657.
[23] S. Yamamoto, K. Nakanish, R. Matsuo, T. Kamikubo, Biotechnol. Bioeng. 25 (1983) 1465.