Biotech Method

Choosing the right protein A affinity chromatography media can remove aggregates efficiently.

Tomokazu Yada¹
Koichi Nonaka¹
Masayuki Yabuta¹
Noriko Yoshimoto²
Shuichi Yamamoto²

¹Biologics Research Laboratories, R&D division, Daiichi Sankyo Co., Ltd, Oura-gun, Japan
²Bio-Process Engineering Laboratory, Biomedical Engineering Center (YUBEC), Yamaguchi University, Ube, Japan

Correspondence: Prof. Shuichi Yamamoto, Bio-Process Engineering Laboratory, Biomedical Engineering Center (YUBEC), Yamaguchi University, Ube, 755-8611, Japan
E-mail: shuichi@yamaguchi-u.ac.jp

Keywords: arginine, monoclonal antibody, pH gradient elution, protein aggregates, Protein A chromatography

Abbreviations: A280, UV-absorbance at 280 nm; CV, column volume; DBC, dynamic binding capacity; HCP, host cell protein; mAb, monoclonal antibody; PAC, Protein A chromatography; SDS PAGE, Sodium dodecylsulfate-polyacrylamide gel electrophoresis; SEC, Size exclusion chromatography
Abstract

Protein A chromatography (PAC) is commonly used as an efficient capture step in monoclonal antibody (mAb) separation processes. Usually dynamic binding capacity is used for choosing the right PAC. However, if aggregates can be efficiently removed during elution, it can make the following polishing steps easier. In this study a method for choosing the right PAC media in terms of mAb aggregate removal is proposed. Linear pH gradient elution experiments of two different mAbs on various PAC columns were carried out, where the elution behavior of aggregates as well as the monomer was measured. Aggregates of one mAb were more strongly retained compared with the mAb monomer. Another mAb showed different elution behavior, where the aggregates were eluted as both the weakly and strongly retained peaks. In order to remove the two types of aggregates by stepwise elution two protocols were tested. The first protocol A consisted of the sample loading, the wash with the equilibration buffer and the low pH elution. The wash stage of the second protocol B included the wash with 1.0 M arginine. No detectable peaks were observed during the wash stage of protocol A whereas significant peaks were monitored during the arginine wash of protocol B. One of the PAC columns showed a smaller peak during the arginine wash. In addition, both the aggregate removal and the monomer yield were higher with protocol B compared with the other PAC columns. This method was found to be useful for choosing the right PAC column.
1 Introduction

Protein A chromatography (PAC) is generally used as the capture step of monoclonal antibodies (mAbs) as it has high affinity to mAbs and allows direct application of cell culture supernatants to the PAC column [1-3]. A typical platform downstream process for mAbs consists of three chromatography steps, PAC followed by two polishing chromatography steps [1-3]. However, since protein A resins (media) are expensive, it is important to choose the most suitable protein A resin for the target mAb purification process.

A typical PAC operation consists of five periods [sample loading (adsorption), wash, elution (desorption), regeneration and re-equilibration]. One of the important properties of PAC is the dynamic binding capacity (DBC). Higher DBC values at short-residence time values can reduce both the column volume and the process time, which results in higher productivity processes [4-8]. In addition to the DBC values, the recovery and the purity of the eluted mAb are important issues. As mAbs bound to the PAC column is desorbed (eluted) by an acidic solution (pH < ca.4.0), it is desirable to use milder conditions in order to avoid denaturation of mAbs. As for the contaminants, in addition to host cell proteins (HCPs) and DNAs [5,9], product-related contaminants such as aggregates must be removed as aggregates are known to cause immunogenic reactions [10,11]. Aggregates are usually removed by polishing chromatography such as ion-exchange chromatography. However, if aggregates can be removed by PAC, it makes the following polishing chromatography steps easier, which results in efficient and cost-effective downstream processes.

Although protein A specifically recognizes the Fc domain of mAb, it is also known to have interaction with the Fab domain [12, 13]. Alkaline stable protein A ligands have been developed, which permits the wash of the PAC column with sodium hydroxide solutions [13-15]. Such ligands are claimed to lose the Fab affinity [13-15]. Non-specific adsorption
of contaminants is another concern [5]. This is due to both the protein A ligand and the base matrix of PAC media (packing materials or beads). In order to suppress such non-specific adsorption a solution containing amino acids such as arginine is often used for wash as well as elution [16-18]. For these reasons, aggregate removal performance is expected to depend on the ligand and the base matrix properties.

In this study we have proposed a method for choosing the most suitable PAC in terms of aggregate removal and the monomer content. Linear pH gradient elution experiments of mAbs on various PAC columns were carried out, where the aggregate elution behavior was measured by using size exclusion chromatography (SEC) in order to identify the elution pH of aggregates. Stepwise elution experiments with two different protocols (wash with and without arginine) were performed in order to remove aggregates efficiently at high monomer yields.

2 Materials and methods

2.1 Monoclonal antibodies

Three humanized monoclonal antibodies (mAbs) were used in this study. They are referred to as mAb-A, mAb-B and mAb-C. For mAb-A, NS0 cell, which had been pre-adapted to serum-free and suspension culture, was cultured by using a serum-free medium for 12 days at 37 °C. From the clarified cell culture fluid, mAb-A (0.64 g/L) was purified by PAC and two ion-exchange chromatography. mAb-A was used for DBC measurement. For mAb-B and mAb-C, CHO cells, which had been pre-adapted to serum-free and suspension culture were cultured by using a serum-free medium for 14 days at 37 °C. The obtained filtered culture supernatants were used for linear pH gradient elution experiments and stepwise elution experiments. The concentration of mAb-B and mAb-C were 0.89 g/L and 1.36 g/L, respectively.
2.2 Chemicals

All the reagents were of analytical grade. Monoclonal antibodies

2.3 Protein A chromatography media and columns

The five PAC media used in this study are shown in Table 1. Pre-packed columns of 1 mL were used for pH gradient elution experiments and DBC measurements. For stepwise elution experiments, 5-mL pre-packed columns were used. All pre-packed columns were provided by the supplier.

2.4 Protein A chromatography experiment

Experiments were carried out on AKTA explorer 100 and AKTA FPLC (GE Healthcare) at room temperature.

2.5 Dynamic binding capacity (DBC) measurement

The prepacked 1 mL PAC column was equilibrated with an equilibration buffer (0.02 M sodium phosphate / 0.14 M sodium chloride, pH 7.5). Purified mAb-A was loaded onto the column until the UV-absorbance at 280 nm (A280) of the column effluent reached ca. 10 % of A280 of the sample loading solution. Then the column was washed with the equilibration buffer and eluted with 0.05 M sodium acetate / 0.06 M sodium chloride at room temperature. Due to the limited quantities of the sample, the experiment was not repeated.

2.6 Linear pH gradient experiment

The prepacked 1 mL PAC column was equilibrated with 0.02 M sodium phosphate / 0.02 M citric acid (pH 6.0). Then, the mAb sample (15 mg) was loaded to the column. After loading, the column was washed by the equilibration buffer of 5 column volumes (CVs). Then a linear pH gradient from the equilibration buffer (pH 6.0) to the 0.02 M sodium citrate (pH 3.5) was carried out. The gradient volume was 20 CVs. The flow rate was 0.33 mL / min, which corresponds to a residence time of 3 min. The fraction was collected (fraction volume 1 mL) and the absorbance at A280 was measured. The monomer content
of the fraction (the ratio of aggregates to the total mAb in the fraction) was analyzed by SEC as shown below. Due to the limited quantities of the sample, the experiment was not repeated.

2.7 Stepwise elution experiment

5 mL self-packed columns shown in Table 2 were employed. The residence time was 4 min except for the small-particle size glass based PAC column (2.5 min). The clarified cell culture supernatants (mAb-B and mAb-C) were used as the sample. The two protocols having different wash methods were employed.

Protocol A: After the column was equilibrated with the equilibration buffer (0.02 M sodium phosphate buffer pH 7.5 containing 0.14 M NaCl), the sample was loaded. After the sample loading, the column was washed with the equilibration buffer of 7.5 CVs. The elution was carried out with a 0.05 M sodium acetate buffer (pH 3.5) containing 0.06 M NaCl. Protocol B: After the sample loading, the column was washed with the equilibration buffer of 4 CVs, 1.0 M arginine (pH 7.5) of 5 CVs and the equilibration buffer of 4 CVs. The loading and elution protocols were the same as protocol A.

For both protocols the desorbed fraction was collected as the product pool when the UV absorbance at A280 was greater than 1.0. The experiments were repeated twice for Prosep, MabSuRe, KanCapA columns, and the average values were calculated for the monomer yield and content.

2.8 Analysis of mAb concentration

The concentration of mAb was determined by analytical protein G affinity column (POROS G20, 4.6 mm I.D × 50 mm, Applied Biosystems). Equilibration and wash buffer was 50 mM sodium phosphate buffer pH 7.0 containing 0.15 M NaCl. Elution buffer was 2 % hydrochloric acid pH 2.0 containing 0.15 M NaCl. The UV absorbance was monitored at A280. The flow rate was 5 mL/min. The experiment was performed at room temperature.
2.9 Analysis of mAb monomer content

The mAb monomer content was analyzed by SEC with a TSKgel Super SW 3000 column (7.8 mm id × 300 mm, Tosoh Bioscience, Japan). The mobile phase was 0.05 M sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl. The flow rate was 0.5 mL/min. The experiment was performed by Agilent1100 and 1200 series (Agilent, USA) at room temperature. The A280 was monitored.

2.10 Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS PAGE) analysis

SDS PAGE was performed using a 4-12 % Bis-Tris NuPAGE pre-cast polyacrylamide gel (Life Technologies, USA) in combination with NuPAGE MOPS running buffer. Samples were diluted to a final mAb concentration of 1 mg/mL with a sodium phosphate buffer (pH 7.0). Low concentration samples such as flow-through fractions were not diluted. The sample (65 μL) was mixed with a four times concentrated NuPAGE lithium dodecylsulfate (LDS) sample buffer (25 μL) and NuPAGE sample reducing agent (10 μL), and incubated at 70 °C for 10 min. In the case of non-reduced SDS PAGE, NuPAGE sample reducing agent was replaced by Milli Q water. As a molecular weight marker SeeBlue Plus 2 Pre-Stained Standard (Life Technologies) was used. The sample (10 μL) was loaded to the gel and electrophoresed at 150 V for 65 min. Protein bands were visualized by GelCode Blue Stain Reagent (PIERCE, USA). Chemicals

3 Results

3.1 Measurement of DBC

The first important property of PAC is the DBC. Most commercial PAC columns have DBC > 35 - 40 mg/mL at the residence time of 4 min [4, 6]. The DBC values at 10 % breakthrough obtained with mAb-A are summarized in Table 1. The DBC value for glass-based PAC
(ProSep UltraPlus) is somewhat higher than other four PAC media, which is consistent with the values reported in the literature [19].

3.2 Linear pH gradient elution PAC

Linear pH gradient elution PAC experiments were carried out with mAb-B and mAb-C as the samples. The results are shown in Fig. 1 (A) and (B). For mAb-B, aggregates were eluted after the main mAb peak. Namely, aggregates more strongly bound to the PAC column so that lower pH was needed for the desorption (elution). Similar elution behavior was reported by several researchers [20-23].

However, for mAb-C, the elution behavior of aggregates was complicated compared with that of mAb-B (Fig. 1 (B)). There were two peaks as shown in the data for MabSelect Sure. The elution volume of the first peak was slightly smaller than that of the main product peak. This indicates that there are two different types of aggregates for mAb-C, one of which has the weaker affinity to the PAC.

3.3 Stepwise elution PAC for aggregate removal

In order to remove both types of aggregates we examined the protocol for the wash stage after the sample loading. Several washing protocols have been proposed for removing non-specifically adsorbed contaminants during the wash stage (9, 18). Since arginine is known to be an effective additive, which can suppress protein-protein interaction and/or hydrophobic interaction, it is often added to the washing buffer [16-18]. We have examined the effect of arginine on aggregate removal during the washing state by using protocol B already described in Materials and Methods and the legend of Fig. 2. For all the PAC columns, significant amounts of aggregates were eluted during the washing stage for the genetically engineered (alkaline stable) protein A ligand PAC columns. Consequently, it was possible to improve the monomer content for the engineered ligand PAC columns as shown in Table 2. For the native protein A ligand columns (Prosep Ultra Plus and MabSelect), the addition of arginine was not effective.
Although arginine was found to be effective, the washing protocol without arginine is preferred when the cost of arginine is considered. The monomer content value obtained with protocol A (without arginine) for KanCapA was almost the same as the value with arginine (protocol B). It is likely that the first aggregates were washed from KanCapA-PAC during the sample loading and the washing (without arginine) stages. In order to confirm this, the fractions eluted from the KanCapA-PAC column from the start of the sample loading till the end of the washing stages were collected and used as the sample for MabSelect SuRe-PAC (Fig. 3 (A)). As shown in Fig. 3 (B), the sharp peak was eluted from MabSelect SuRe-PAC, which contained mostly aggregates.

Fractions eluted from Prosep Ultra Plus-, MabSelect SuRe-, and KanCapA-PAC columns were analyzed by SDS-PAGE (Non-reduced, and Reduced). The non-reduced SDS-PAGE results (lane 2 of Fig. 4A) show that the flow-through fraction of KanCapA contained significant amounts of aggregates (high molecular weight species). However, heavy chain was not detected in lane 2 of Fig.4B (Reduced SDS-PAGE). Instead, there was a species smaller than heavy chain. We analyzed this species by N-terminal sequence analysis and confirmed that this is a truncated heavy chain, whose Fc domain was truncated. Therefore, it was concluded that mAb based-aggregates were not bound to KanCap A and eluted in the flow-through fraction. Although similar results were obtained for MabSelect Sure, the amount of aggregates in the flow-through fraction was much smaller compared with that by KanCap A as shown in lane 6 of Fig.4A and B. SEC analysis (supporting information) also clearly showed that both types of aggregates (tri- and tetra-mers) were removed completely by KanCap A without arginine wash whereas they were only removed with arginine by MabSelect Sure. It was not possible to remove them by ProSep Ultra Plus even with arginine.
4. Discussion

It is considered to be difficult to remove mAb aggregates by PAC operation as aggregates are expected to bind more strongly to PAC [20-23] and may be co-eluted with mAb monomer.

In this study we observed a different type of aggregates, which has weaker affinity to PAC. It is likely that this type of aggregates is different in terms of the number of Fc regions accessible to PAC as shown in Fig. 5 (C) (c) as type 2 aggregates. The Fc regions might be buried inside or partially lost. Or the protein structure was drastically changed [24]. Consequently, the affinity to PAC becomes weak. Linear gradient pH elution experiments have shown the possibility of removing aggregates by stepwise elution chromatography without arginine wash when the right PAC column is chosen. Several researchers have already pointed out the importance of the wash protocol for improving the purity of the recovered mAb pool [9,16-19]. In most cases their concern is host cell protein clearance as removing aggregates by PAC is considered difficult [3,11]. For strongly retained aggregates (B) (b) in Fig. 5, it may be possible to design the elution protocol, which allows the selective elution of monomer as shown by Teeters et al.[20].

As mentioned in Introduction genetically altered alkaline-stable protein A ligands are claimed to lose the Fab affinity [13-15]. B motif was genetically modified for MabSelect Sure whereas C motif was mutated for KanCap A and Toypearl 650. However, the resulting affinity to Fab or different types of aggregates is different from ligand to ligand. Such affinity interaction is not directly connected to DBC as DBC values are similar. It is not possible to predict the affinity of different types of aggregates to PAC in advance. Linear pH gradient elution experiments can provide important information, which can be used for designing the suitable protocol for stepwise elution chromatography of the target mAb.
Another important issue is the elution pH. If it is too low such as pH <3, it may damage the recovered mAb. It is also expected to produce aggregates during and/or after low pH elution [25-27]. The elution pH values from alkaline stable protein A ligands are generally higher than those for native protein A ligands partly because of the loss of the interaction with the Fab region [28, 29]. Choosing low pH also results in co-elution of mAb monomer and aggregates.

5. Concluding remarks
This study has shown the possibility to choose the most suitable protein A chromatography media for the mAb of interest in terms of aggregate removal as well as the DBC and the elution pH. Currently, various types of non-standard mAbs have been developed such as asymmetric antibody and antibody fragment products. Those molecules tend to form aggregates easily compared with the standard mAbs. Therefore, our proposed method summarized below may be useful. Linear pH gradient elution experiments are first carried out in order to examine aggregates elution behavior with several PAC columns. If you find a PAC column, where aggregates are eluted before the mAb peak, choose this column and tune the wash protocol for stepwise elution.

Conflict of interest
The authors declare no financial or commercial conflict of interest.
6 References

**Table 1. Properties of protein A chromatography (PAC) media employed in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Supplier</th>
<th>$d_p$ [μm]</th>
<th>Alkaline base resistance</th>
<th>Matrix</th>
<th>DBC [mg/mL-bed]</th>
<th>RT [min]</th>
<th>$V_t$ [mL]</th>
<th>load* [mg/mL-bed]</th>
</tr>
</thead>
<tbody>
<tr>
<td>ProSep UltraPlus</td>
<td>MerckMillipore</td>
<td>60</td>
<td>No</td>
<td>Glass</td>
<td>55</td>
<td>2.5</td>
<td>5.0</td>
<td>30</td>
</tr>
<tr>
<td>MabSelect</td>
<td>GE-HealthCare</td>
<td>85</td>
<td>No</td>
<td>Agarose</td>
<td>44</td>
<td>4</td>
<td>4.7</td>
<td>30</td>
</tr>
<tr>
<td>MabSelect SuRe</td>
<td>GE-HealthCare</td>
<td>85</td>
<td>Yes</td>
<td>Agarose</td>
<td>46</td>
<td>4</td>
<td>4.7</td>
<td>30</td>
</tr>
<tr>
<td>Toyoperal AF650</td>
<td>Tosoh</td>
<td>40</td>
<td>Yes</td>
<td>Poly-methacrylate</td>
<td>33</td>
<td>4</td>
<td>5.0</td>
<td>25</td>
</tr>
<tr>
<td>KanCapA</td>
<td>KANEKA</td>
<td>75</td>
<td>Yes</td>
<td>Cellulose</td>
<td>45</td>
<td>4</td>
<td>5.0</td>
<td>30</td>
</tr>
</tbody>
</table>

*sample load for stepwise elution chromatography

DBC = dynamic binding capacity at 10% breakthrough, $d_p$ = nominal particle diameter,

RT = residence time (based on the packed bed volume) = $V_t/F$, where

$V_t$ = packed bed volume, $F$ = volumetric flow-rate

**Table 2. Monomer content and yield of mAb-C by stepwise elution chromatography**

<table>
<thead>
<tr>
<th>PAC rein</th>
<th>Alkaline resistance</th>
<th>Monomer content$^a$ [%]</th>
<th>Monomer yield$^b$ [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Protocol A Without arginine wash</td>
<td>Protocol B With arginine wash</td>
</tr>
<tr>
<td>ProSep UltraPlus</td>
<td>No</td>
<td>72</td>
<td>75</td>
</tr>
<tr>
<td>MabSelect</td>
<td>No</td>
<td>79</td>
<td>87</td>
</tr>
<tr>
<td>MabSelect SuRe</td>
<td>Yes</td>
<td>74</td>
<td>95</td>
</tr>
<tr>
<td>Toyoperal AF650</td>
<td>Yes</td>
<td>79</td>
<td>95</td>
</tr>
<tr>
<td>KanCapA</td>
<td>Yes</td>
<td>94</td>
<td>95</td>
</tr>
</tbody>
</table>

$^a$ Monomer content was calculated as the ratio of the recovered monomer to the total mAb recovered (monomer + aggregates) by SEC.

$^b$ Monomer yield was calculated as the ratio of the recovered monomer to the amount of mAb in the load sample.
Figure legends

**Figure 1.** Linear pH gradient elution PAC of mAb-B (A) and of mAb-C (B). Fraction volume was 1 mL. Aggregates content, AC is the ratio of aggregates to the total mAb in the fraction analyzed by SEC.
Figure 2. Stepwise elution PAC of mAb-C with two different wash protocols

Protocol A: The sample (clarified cell culture supernatant of mAb-C) was loaded to the column equilibrated with buffer A (0.02 M sodium phosphate buffer pH 7.5 containing 0.14M NaCl). After the sample loading, the column was washed with buffer A of 7.5 CVs. The elution was carried out with a 0.05 M sodium acetate buffer (pH 3.5) containing 0.06 M NaCl. Protocol B: After the sample loading, the column was washed with buffer A (4 CVs), 1M arginine (pH 7.5) of 5 CVs and buffer A (4 CVs). The loading and elution protocols were the same as protocol A. For both protocols the desorbed fraction was collected as the product pool when the UV absorbance at A280 was greater than 1.0.

Figure 3. Stepwise elution chromatography of flow-through fractions of KanCapA PAC by MabSelect Sure PAC. Protocol A was used for both experiments. The sample for MabSelect Sure PAC was the pooled fraction from the start to the elution volume of 75 mL for KanCapA-PAC stepwise elution. The sample was the clarified cell culture supernatant of mAb-C.
Figure 4. SDS PAGE analysis for the eluted fractions from KanCap A, MabSelect SuRe, and Prosep Ultra Plus stepwise elution PAC

Lane 1: Culture supernatant, Lane 2: Flow-through, Lane 3: Eluate (without arginine wash), Lane 4: Arginine wash peak, Lane 5: Eluate (with arginine wash), Lane 6: Flow-through, Lane 7: Eluate (without arginine wash), Lane 8: Arginine wash peak, Lane 9: Eluate (with arginine wash), Lane 10: Flow-through, Lane 11: Eluate (without arginine wash), Lane 12:
CIP peak (After elution, Non arginine wash), Lane 13: Arginine wash peak, Lane 14: Eluate (with arginine wash). The sample was the clarified cell culture supernatant of mAb-C.

**Figure 5.** Protein A- mAb interaction

(A) native-Protein A has weak interaction with Fab region as well as strong binding with Fc region. (a) Alkaline stable recombinant Protein A does not have weak interaction with Fab. (B) (b) Type 1 aggregates are bound to Protein A more strongly than mAb monomer does as they have more binding Fc sites. (C) Type 2 aggregates are bound to native-protein A with Fab and Fc regions. (c) As Fc regions of Type 2 aggregates are buried or partially lost, the interaction with recombinant-protein A becomes weaker than the interaction between mAb monomer and Protein A.