BRIEF REPORT

Controlled conductivity at low pH in Protein L chromatography enables separation of bispecific and other antibody formats by their binding valency

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ABSTRACT
The complex molecular formats of recent therapeutic antibodies, including bispecific antibodies, antibody fragments, and other fusion proteins, makes the task of purifying the desired molecules in a limited number of purification steps more and more challenging. Manufacturing these complicated biologics can be substantially improved in the affinity capture stage if the simple bind-and-elute mode is accompanied by targeted removal of the impurities, such as mis-paired antibodies and oligomers or aggregates. Here, we report a method, based on the binding valency to Protein L resin, of separating proteins during the elution step by simply controlling the conductivity at low pH. We show that the method efficiently separated targeted antibodies from mis-paired and aggregated species. Notably, the number of Protein L binding sites can be built into the molecule by design to facilitate the purification. This method may be useful for purifying various antibody formats at laboratory and manufacturing scales.

Introduction
A variety of molecular formats have been applied to therapeutic biologics, including bispecific antibodies (BsAbs) in the conventional IgG format, antibody fragments, fusion proteins, and scaffold proteins.1,2 The high potential of therapeutic drugs in the BsAb format in particular has been shown for applications such as crosslinking cancer cells and T cells in cancer immunotherapy, neutralizing two different proteins/signaling pathways, bringing two different proteins together to initiate molecular reactions.3,4 In fact, increasing numbers of BsAbs in various formats are being developed and tested in numerous clinical and preclinical studies.5

Because a conventional IgG-type BsAb consists of two heavy chains (HCs) and two light chains (LCs), 10 possible combinations of different pairings of these chains may result when the four-chain BsAb is expressed.6–8 Of the 10 combinations, only one is the desired molecule, while the remaining nine are unwanted by-products that must be efficiently and selectively removed to produce the desired BsAb. One approach to reduce the number of variants that can be formed in a bispecific antibody is to apply antibody engineering to facilitate targeted pairing of heterologous chains (for review see Ref. 9). In this way, a simpler oligomeric mixture that is enriched for the correctly paired oligomeric form can be obtained.9 Another approach is to remove these undesired by-products during the purification process through a combination of BsAb design and specific purification methods. For example, to selectively purify heterodimerized HCs of a BsAb, Sampei et al. introduced a difference in the isoelectric points of the two HCs for better separation with ion exchange chromatography, while Tustian et al. ablated the binding to protein A from one of the two HCs in the BsAb so that one of the HC homodimers, which lacks Protein A binding, and also another HC homodimer, which binds to the Protein A column by avidity, could be removed by letting it flow through the Protein A column or by gradually lowering the pH during the elution step, respectively.10–12 While both of these strategies function elegantly, the process of optimization required to ensure the purity of the target molecule is generally laborious. Similarly, the use of two different LCs in a BsAb can cause similar mis-pairing of undesired HC and LC pairs, but so far there seems to be no available option for isolating the antibodies that have the correct HCs and LCs in one chromatography step.

Moreover, antibodies that are missing a crystallizable fragment (Fc), such as antigen-binding fragments (Fab), F(ab’)2, single-chain variable fragment (scFv), or Fab/scFv fusion proteins, tend to have less favorable physico-chemical properties compared to conventional IgGs, and are therefore prone to form oligomers or aggregates.13 Generally speaking, such oligomers/aggregates can be removed by gel filtration chromatography at the laboratory scale, but scalability issues prevent it being commonly used at the manufacturing scale. Although

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a combination of ion exchange, hydrophobic interaction, or mixed-mode chromatography would most likely be able to remove such oligomers and aggregates, a simple and robust method that efficiently purifies these molecules with a high monomer rate is critical to meet the required purity, either as a therapeutic drug or even as a reagent in laboratory experiments.\(^{14-17}\)

Protein L was isolated from the bacterial species *Peptostreptococcus magnus*, which binds to certain subtypes or allotypes of Ig \(\kappa\) LC, such as \(\kappa_1\), \(\kappa_3\), or \(\kappa_4\), but not to \(\kappa_2\) or \(\lambda\) LC.\(^{18,19}\) A structure study demonstrated that Protein L has a wide interaction surface mainly with the framework region of the variable domain in an Ig LC.\(^{20}\) Although resins conjugated with Protein L have been utilized to purify not only IgG, but also antibody fragments that carry Ig \(\kappa_1\), \(\kappa_3\), or \(\kappa_4\), the use was mostly limited to a simple bind-and-elute mode.\(^{17,21}\)

If Protein L can be used to separate proteins by their binding valency, it may make it possible to remove BsAbs with mis-paired HC-LC and oligomers or aggregates from antibody fragments. Given that the interaction between Protein L and an Ig \(\kappa\) LC consists mainly of hydrophobic interactions and hydrogen bonds, it may be possible to achieve the separation by modifying these interactions.\(^{20}\) Achieving a separation process of this nature would potentially have a large impact on how we manufacture BsAb and other biologics with the Protein L binding motif.

**Results**

Separation of BsAb and monospecific antibodies by Protein L chromatography based on the valency of the interaction between Protein L and Ig \(\kappa\) LC.

We hypothesized that when the electrostatic and/or hydrophobic interactions of proteins are controlled, the proteins can be separated according to the valency of the binding between Protein L resin and the \(\kappa\) LC. However, we also considered that chaotropic agents may have a negative impact on the physico-chemical properties of proteins depending on its strength and concentration, so we prioritized controlling the conductivity during acid elution using mildly chaotropic salt based on Hofmeister series, such as sodium chloride (NaCl), when testing this hypothesis.\(^{22-24}\) Furthermore, for the same reason, we kept the conductivity level below the physiological level. We also noted reports showing that, when IgG was purified with Protein A, elution efficiency was highly compromised when a high concentration of salt was added to the acid because the hydrophobic interaction was overly enhanced, which was another reason to keep the salt concentration at less than the physiological level.\(^{25}\)

First, we tested our concept by evaluating the separation of IgG-type BsAbs. As Figure 1 shows, Ab#1 has a chimeric \(\text{Vk}1-\text{Cl}\) LC that binds Protein L on one arm and a \(\lambda\) LC on the other arm; Ab#2 is an antibody with the same \(\text{Vk}1-\lambda\) LC on both arms. Ab#1 and #2 interact in a monovalent or bivalent manner, respectively, with a Protein L-conjugated resin. Since the constant region of \(\kappa\) LC only contributes in a minor way to Protein L binding, it should be noted that the binding of chimeric \(\text{Vk}1-\lambda\) LC to Protein L resin is similar to that of a full \(\kappa_1\) LC (data not shown).\(^{20}\) Equivalent amounts of Ab#1 and Ab#2 were mixed and loaded onto a Protein L column followed by elution with Na-acetate buffer at pH 2.4, 2.7, 3.0, or 3.3, and at the same time the NaCl concentration was decreased linearly from 100 mM to 0 mM (Figure 2a-d). As a result, we observed a trend that proteins were eluted earlier when the pH was lower, and eluted later when the pH was higher. Interestingly, at pH 2.4 a peak appeared very soon after the pH reached the target value (Figure 2a), but at pH 3.3 the proteins only started to elute when the salt concentration was less than 50 mM (Figure 2d), suggesting that even 50 mM NaCl can influence the interaction between Protein L under acidic pH conditions. In addition, while pH 2.4 and pH 3.3 showed peaks with a shoulder, pH 2.7 and pH 3.0 showed two distinct peaks. Of the two peaks that appeared at pH 2.7 and pH 3.0, in both cases the first peak, which occurred at a conductivity level of 8.63 mS/cm in pH 2.7 and at 6.64 mS/cm in pH 3.0, consisted mainly of Ab#1, which binds monovalently to Protein L resin, while the second peak, which occurred at 3.76 mS/cm in pH 2.7 and at 2.53 mS/cm in pH 3.0, consisted mainly of Ab#2, which binds bivalently to the Protein L column. This result suggested that controlling the conductivity level at acidic pH has the potential to separate antibodies that bind monovalently to the Protein L column from those that bind bivalently. Notably, the same phenomenon was observed under similar conditions using one-arm antibodies, which have one Protein L binding site, and two-arm antibodies, which have two Protein L binding sites (Supplementary Figures 1 and 2).

The use of gradient elution for purification is more challenging when manufacturing biologics because of the greater economic burden posed by a longer process time, larger volume of buffer, and the need for larger buffer tanks. These

![Figure 1](image-url). Schematic representation of the structures of antibodies used in this study. Ab#1 is a bispecific antibody, Ab#2 is a monospecific bivalent antibody, and Ab#3 is a BITE antibody that comprises two scFv fusion proteins. The target antigen of each paratope is described.
considerations make stepwise elution a preferred option for bioprocessing.

Therefore, we next checked if lowering the conductivity level during the acid elution step in a stepwise manner can separate Ab#1 and Ab#2 (Figure 3). Since pH 2.7 and 3.0 showed good separation in the earlier experiment, these two pH levels were tested. After loading the same set and amount of antibodies as those shown in Figure 2, the elution was conducted in two steps. First, elution was done with Na-acetate buffer at pH 2.7 and ~8.63 mS/cm (Figure 3a) or at pH 3.0 and ~6.64 mS/cm (Figure 3c), which were the conductivity levels observed at the top of the first peak during the gradient elution in Figure 2. Second, the elution buffer as in the first step, but without salt, was used. According to the cation exchange chromatography (CIEX) method for quantification of Ab#1 and Ab#2 (see Materials and Methods, and Supplementary Figure 3), peaks that appeared in each step at pH 2.7 and pH 3.0 showed that the eluate from the first step consisted of Ab#1 at a high purity rate of 92.0% (Figure 3b) and 94.8% (Figure 3d), respectively, while the eluate from the second peak consisted mainly of Ab#2 (Figure 3b,d). These results clearly demonstrated that antibodies can be separated based on the valency of their interaction with Protein L–coupled resin by lowering the conductivity during the acid elution step even in a stepwise manner. These results were closely replicated when the one-arm and two-arm antibodies mentioned earlier were tested under similar conditions (Supplementary Figures 1 and 2). We termed this method of eluting proteins under acidic pH with controlled conductivity as “conductivity elution”.

Separation of monomeric and oligomeric bispecific T-cell engagers by conductivity elution in Protein L chromatography

Bispecific T-cell engagers (BiTEs) consist of two scFvs of different antibodies connected by linker polypeptides. Because BiTE antibodies do not have an Fc domain, they cannot be purified by Protein A affinity chromatography; however, if the scFv includes a Vκ1, Vκ3, or Vκ4 domain, a BiTE can be purified by Protein L chromatography.

It has been reported that BiTE antibodies tend to form oligomers when they are expressed. Indeed, we observed the same phenomenon when analyzing our transiently expressed BiTE molecule Ab#3 (Figure 1) by size exclusion chromatography-high performance liquid chromatography (SEC-HPLC) (Figure 4a); approximately 21.9% of the protein was formed as an oligomer. Since one scFv of Ab#3 has a Vκ1 domain and the other has a Vκ domain, the monomeric form has one Protein L binding motif, whereas the oligomer will have multiple motifs, assuming that the surfaces are not embedded in the structure. Therefore, we tested whether oligomers and aggregates can be removed with Protein L chromatography using the conductivity elution method.

In order to test if a Protein L column with controlled conductivity can separate monomeric and oligomeric BiTE antibodies, Ab#3 used in Figure 4a was applied to a Protein L column and eluted with Na-acetate buffer at pH 2.7, while linearly decreasing the NaCl concentration from 100 mM (~11.2 mS/cm) to 0 mM (Figure 4b). As a result, two distinct protein peaks were observed (Figure 4b). To check whether the BiTE molecules were oligomeric, fractions from each peak
were analyzed by SEC-HPLC (Figure 4c). The results showed that fractions from the first peak contained BiTE antibody with a high rate of monomers (93.75% to 100%), while fractions from the second peak consisted mainly of oligomers (Figure 4c). Thus, this result clearly demonstrated that the combination of Protein L–coupled resin and conductivity elution at low pH can separate monomeric and oligomeric forms of BiTE antibodies, based on their different valency toward Protein L binding.

**Discussion**

Intense competition in the biopharmaceutical industry has made it essential for therapeutic biologics to have complex modes of action (MoA) that differentiate it from other therapeutic products. These new MoA may be realized by new molecular formats for antibodies, including BsAbs, antibody fragments, and antibody fusion proteins, or for non-antibody proteins (e.g., DARPin®, Affibody®). In consequence, clinical and pre-clinical studies using unconventional molecules are increasing.

However, the potential value of biologics with new molecular formats tends to be offset by problems in production. In the case of BsAb, these problems are the generation of misassembled by-products when the number of polypeptide chains of a similar character has increased and the formation of oligomers and aggregates due to poor physico-chemical properties. Therefore, strategies to specifically remove such unwanted impurities are critical to the manufacturing of these molecules. One approach is to apply antibody engineering, which can be used to create differences in the isoelectric point (pI) of two Ig HCs or ablate one of the Ig HC’s Protein A binding in order to separate between heterodimer from undesired homodimers. As such, options for the isolation of desired Ig HC heterodimers are available, but a way to purify BsAb with two different Ig LCs using one chromatography step is still unavailable.
In this study, we showed that Protein L is not only able to purify proteins containing Ig κ1, κ2, or κ4, such as antibodies (e.g., IgG, IgM), antibody fragments (e.g., Fab, F(ab’)2, scFv), and other fusion proteins in a simple bind-and-elute mode, but is also able to efficiently separate proteins by discriminating the difference in their valency when binding to Protein L-coupled resin when the conductivity elution method we have developed is used. With the results demonstrated here, it is reasonable to believe that this purification method can be applied to various molecular formats that include one or more Protein L binding motifs. It should also be noted that this conductivity elution gives similar results on multiple types of Protein L columns that are commercially available (data not shown).

The concept of protein separation by valency has also been reported by Tustian et. al using a Protein A resin and gradually lowering the pH during the acid elution step.\textsuperscript{11,12} Interestingly, while the pH difference used to separate monovalent binders to Protein A resin from bivalent binders seemed not to be so large (less than 1.0), addition of 500 mM of calcium or magnesium chloride during the pH gradient elution somewhat improved the valency-dependent separation, which led to successful purification of BsAb at high purity even at the manufacturing scale, with a specially developed Protein A resin. The authors hypothesized that, as Protein A is known to interact with IgG by hydrophobic interaction, the effect was caused because salt modified the hydrophobic interaction.\textsuperscript{11,33} This suggests that our conductivity elution may also be influencing the hydrophobic interaction between Protein L and the Ig κ LC. However, it should be noted that Tustian et al. use a much higher concentration of chaotropic salt (500 mM CaCl\textsubscript{2}) than was used in this study (50 ~ 100 mM NaCl), which suggests that the interaction of Protein A with Ig γ HC may have a stronger influence on ionic interaction than that of Protein L with Ig κ LC. Understanding the molecular mechanism of using conductivity elution with a Protein L resin will be investigated in the future. At the same time, it should be noted that the concept of the conductivity elution may be applicable to other affinity purification chromatography that uses protein-protein interaction.

There are several clear advantages of using conductivity elution with Protein L to separate proteins by binding valency over using pH gradient elution with Protein A. First is the simplicity of setting up the conditions required for stepwise elution. Stepwise elution is obviously economical in terms of process time and cost at the manufacturing scale.\textsuperscript{26} We have shown in this study that, by testing the conductivity gradient elution from 100 mM to 0 mM NaCl at acidic pH ranging between 2.4 to 3.3, it is possible to identify the levels of pH and conductivity that are suitable for stepwise elution. It should also be noted that the buffer for stepwise elution can be generated very easily, as it can be prepared by simply adding NaCl or other salt to the acid solution. This simplicity should bring a clear benefit for the process design and optimization when manufacturing various biologics drugs in the future.

Secondly, it is clear that Protein L can purify molecules, e.g., “Fc-less” antibodies, that cannot be purified by Protein

\begin{figure}[h]
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\caption{Separation by conductivity elution of monomer and oligomer/aggregates of an antibody fragment in BiTE format. (a) A chromatogram by SEC-HPLC shows the rate of aggregates and monomers of Ab#3 at pH 2.7. (b) A chromatogram shows the separation of aggregates and monomers of Ab#3 by conductivity gradient elution. A key in the figure gives the meaning of each line. The conductivity value of each peak is described. Arrows show fractions that were selected for further analysis. (c) SEC-HPLC chromatograms of Ab#3 in fractions from peak 1 and peak 2 selected in (b) and its monomer rate is shown. The results of an analysis of the molecular weight marker (MWM) is also shown at the bottom.}
\end{figure}
A. We have shown that conductivity elution can also remove oligomers or aggregates, based on their valency difference from monomers. As these unconventional molecules tend to form oligomers or aggregates more often than conventional IgGs, it can be advantageous if the oligomers and aggregates can be removed during the affinity capture step.

Finally, while conductivity elution with Protein L–coupled resin was designed to improve the purification of various formats of antibodies, it may also have additional advantages in the discovery of the antibodies themselves. The main advantage is that the number of Protein L–binding sites can be controlled, which is the primary consideration when designing a molecule in the discovery stage that can be purified by conductivity elution using Protein L resin. For example, one arm of a BsAb should bind to Protein L but the other arm should not. Since we can use an endogenous Ig LC sequence for the Protein L–binding arm and the non-binding arm, it is not necessary to apply additional modifications to the amino acid sequence of the Ig LC, thus any immunogenicity risk of the therapeutic antibody is unchanged. Another advantage is that, when Protein L replaces Protein A chromatography in the future, it may be possible to use the Protein A binding surface of Ig HC located at CH2-CH3 domain for protein engineering to apply novel functions.

In summary, while Protein L resin is already widely used to purify conventional antibodies and antibody fragments lacking an Fc-domain but containing Ig κ1, κ2, or κ4, we have demonstrated in this study that applying conductivity elution enables efficient separation of various types of antibodies based on their valency in binding to a Protein L-conjugated resin. Further studies currently underway aim to elucidate the molecular mechanism of conductivity elution and investigate its applicability to the manufacturing setting.

Materials and methods

Expression and purification of antibodies

Figure 1 summarizes the antibodies used in this study. All proteins were constructed and cloned into Chugai’s in-house mammalian expression vector using traditional methods, and then transiently expressed with an ExpI293 expression system (Thermo Fisher) according to the manufacturer’s recommendation. This step was followed by purification with Protein A and gel filtration for Fc-containing proteins or with Ni-NTA for the BiTE molecule, which is fused with a His-tag on its C-terminus. The final formulation buffer for all antibodies was 1x phosphate-buffered saline (PBS)(−) (FUJIFILM Wako Pure Chemical).

Protein L chromatography

The equipment used for Protein L chromatography in this study was AKTA Avant 25 or AKTA explorer 10S (GE Healthcare) connected with a 0.7 × 2.5 cm Protein L-Agarose HC (ProteoNova; Kagawa, Japan) column [column volume (CV) = 1 mL]. The flow rate was constant at 1 mL/min. Buffers used in this study are shown in Supplementary Table 1, and their conductivity was measured using LAQUAtwin B-771 or LAQUAact D-74 (Horiba Scientific), while their pH was measured using S220-Bio (Mettler Toledo) or LAQUA F-72 (Horiba Scientific). Conductivity and pH were also monitored using Unicorn software (GE Healthcare), which accompanies the purification equipment AKTA Avant 25 and AKTA explorer 10S.

Protein analysis

Purified proteins or eluates from Protein L experiments were analyzed, depending on the purpose, by either SEC-HPLC using a TSKgel G3000SW XL column (Tosoh) with SEC mobile phase (300 mM NaCl, 50 mM sodium phosphate buffer, pH 7.0) at a flow rate of 0.5 mL/min, or by CIEX-HPLC using a ProPac WCX-10 LC Column (Thermo Fisher) with linear gradient from CX-1 pH Gradient Buffer A (pH 5.6, Thermo Fisher) to CX-1 pH Gradient Buffer B (pH 10.2, Thermo Fisher) at a flow rate of 1.0 mL/min, or by SDS-PAGE followed by Coomassie staining. The CIEX analysis results of Ab#1 and Ab#2 are shown in Supplementary Figure 3.

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Abbreviations

BiTE bogenerator T-cell engager
BsAb bispecific antibody
CIEX cation exchange chromatography
Fab antigen-binding fragments
Fc crystallizable fragment
HC heavy chain
HPLC high performance liquid chromatography
Ig immunoglobulin
LC light chain
NaCl sodium chloride
PBS phosphate-buffered saline
scFv single-chain variable fragment
SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC size exclusion chromatography

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