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Characterization of bispecific antibody production in cell cultures by unique mixed mode size exclusion chromatography

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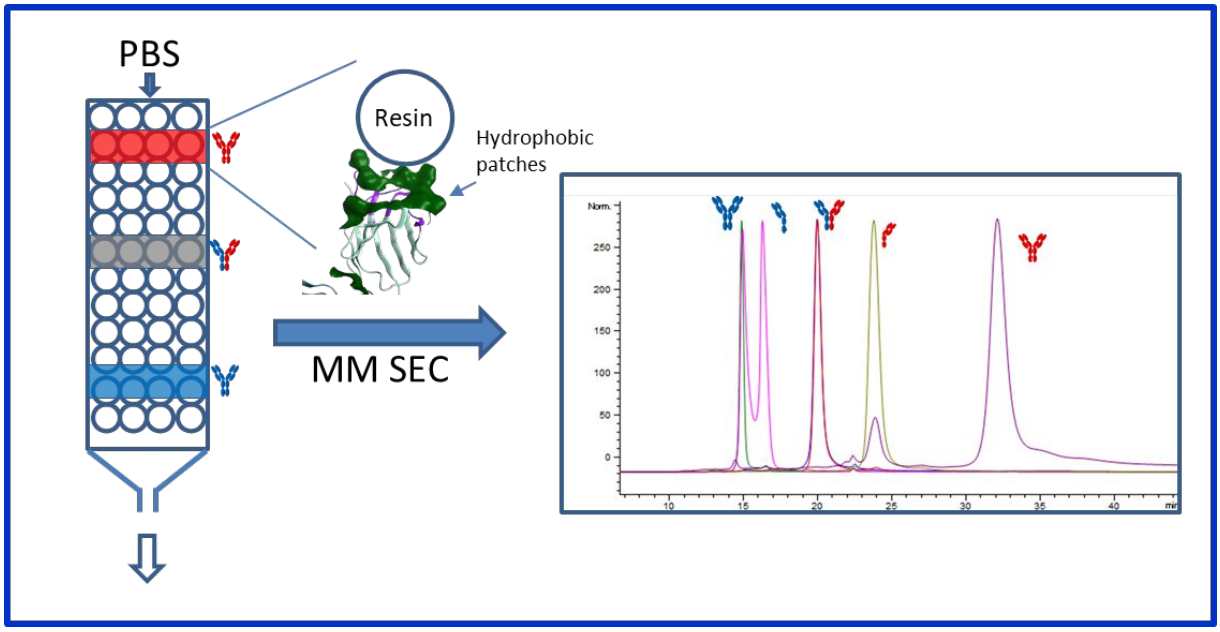
ABSTRACTS Bispecific antibodies have received a wide attention as promising immunotherapeutic agents for their high specificity and the ability to target immune cells to tumors. However, analysis of bispecific antibodies is challenging because multiple forms of antibodies are potentially generated during production in cell culture. Most analyses of bispecific antibodies rely on liquid chromatography with mass spectrometry (LC-MS), which could miss detection or becomes less quantitative if those forms are not physically separated. Here we report a novel and sensitive mixed mode size exclusion chromatography (MM SEC) coupled with multi-angle light scattering (MALS) to analyze different forms of bispecific IgG molecules under native conditions. The method displayed great ability to separate various antibody forms with peak resolutions unmatched by other methods we tested, isolating desired bispecific molecules, parental homodimers, half molecules, and antibodies with mis-paired light and heavy chains. Each peak was analyzed by online MALS and then identified and confirmed by intact or reduced LC-MS of isolated forms. MM SEC in this study performs by a novel mechanism through the interactions of resin with protein surface hydrophobic clusters distributed across CDRs of light chains. This novel MM SEC allows quantitative detection of even low abundance forms and provides a new tool for screening expression profiles of cell culture clones, monitoring purification, and evaluating drug substance purity.



Bispecific antibodies (BsAbs) have become increasingly of interest for both diagnostic and therapeutic applications because of their unique features.[1-3] Traditional BsAbs normally consist of 2 different half molecules (HL) with each binding to a unique antigen. With bivalent binding specificity, BsAbs offer some special biological features that are not available with monospecific antibodies.[4,5] They can also exert unique or synergistic bioactivity by simultaneously interacting with two different targets, or multiple epitopes on the same target.[5,6] They can enhance tumor killing by directing cytotoxic immune cells to the proximity tumor cells through linking cell surface receptors on these cells.[2,6,7] As a result, BsAbs are emerging as an improved alternative strategy for antibody combination therapies and are being developed as the next generation of immunotherapies following successful therapies with immune checkpoint antibodies such as Keytruda/Opdivo.[1-3,6-9]

Because of the rapid advancement of technology in recent years, BsAbs in more than 60 formats have been designed.[1,10,11] They include small proteins harboring two antigen binding domains,[1,12] bispecific IgG, and large molecules containing different antigen-binding sites.[1,2] These bispecific molecules differ in structure, size, valences, and flexibility, as well as in their pharmacokinetic properties. Many bispecific candidates are under clinical trials for different indications.[10,11,13,14] The bispecific IgG format, however, is often adopted since it retains the structure and property of regular antibodies with high stability in serum and optional effector functions.

Bispecific IgG are produced using DNA recombinant technologies,[1] fusing of hybridoma cells,[15,16] and *in vitro* assembly using controlled Fab arm exchange.[17-19] In most cases, they are produced in a single cell by simultaneous expression of two different sets of light and heavy chains. The introduction of "knobs into holes" mutations on CH3 domain of heavy chains improves the assembly of the desired bispecific antibody by promoting heterodimeric association of heavy chains.[5,20,21] The major challenge of this strategy is the generation of undesired antibody forms from the expressed light and heavy chains via promiscuous recombination/pairing.[1] Production of these undesired forms not only reduces BsAb yields, but also creates great difficulties for the purification process and analytics.

Sensitive and quantitative assays to assess different forms of assembled antibodies and intermediate products are highly critical for screening the cell clones for optimal BsAb production. Thus far, there are few reported methods available to analyze assembled molecules. Most analysis relies on the liquid chromatography in conjunction with mass spectrometry (LC-MS).[22] However, due to the varying ionization efficiencies of different molecules, LC-MS can be constrained by the detection sensitivity or even by failure to detect some antibody forms unless they are physically separated in reverse phase HPLC (RP-HPLC). Separation of BsAbs by ion exchange chromatography has been described[23] but remains challenging, particularly when various antibody forms have similar pIs leading to co-elution. Native SEC-MS[24,25] and ion mobility MS[26] have been described as alternatives to the conventional RPLC-MS. Unfortunately, these methods do not meet our need in analysis of BsAb product profiles. Regular SEC is not a viable option considering similar sizes of testing molecules. In this study, the native MM SEC developed previously to study IgG4 half molecule exchange and antibody oxidation[27-29] was developed and optimized to analyze the complex mixture of potential antibody forms produced in cell cultures. To our surprise, those antibody forms were separated in resolutions unmatched by RPLC methods we have tested. We found that the MM SEC separation mechanism is mainly mediated by a unique interaction of resin with the total surface hydrophobic patches located at CDRs of light chains of antibody forms. BsAb isoforms were characterized by MALS, then isolated, and confirmed by LC-MS. Our results show that MM SEC enables accurate quantitation of our bispecific antibody and other minor forms including homodimers and misassembled antibody, providing several advantages over regular LC-MS in terms of separation and detection sensitivity.

EXPERIMENTAL SECTION

Antibody expression and Protein A chromatography. Bispecific antibody C was made in a single cell expressing two sets of light chain and heavy chain genes for antibody A and antibody B, respectively, following transfection with plasmid vectors at appropriate ratios. The expression of each antibody gene was driven by a Human Cytomegalovirus (CMV) promoter. The heavy chain sequence from antibody A contains "knobs" mutations, and that from antibody B has "holes" mutations, making desired pairing more likely. The homodimers of antibody A or antibody B were also expressed to be used as analytical controls.

The IgG species were purified from the cleared cell culture supernatant by Protein A chromatography using the MabSelect Sure beads (GE Healthcare Life Sciences). Total antibody recovery from the Protein A chromatography was determined by absorbance at 280 nm using UV extinction coefficients of respective antibodies.

Mixed mode size exclusion chromatography coupled with multi-angle light scattering (MM SEC-MALS). The MM SEC chromatography under native conditions was performed on Sepax BioMix™ SEC-300, 3 μm, 7.8x300mm (P/N: 214300-7830) at 25 °C. The column is a modified version of Sepax Zenix SEC-300 MK that was used to assess antibody oxidation and IgG4 half molecule exchange previously.[27-29]

Antibody samples (50 μg in 5-50 μl) were injected on an Agilent1260 HPLC instrument. Elution was made at a flow rate of 0.5 ml/min and monitored by UV trace at both 214 and 280 nm. The mobile phase was phosphate buffered saline (PBS) (8.1 mM sodium phosphate, 1.5 mM potassium phosphate, 2.7 mM potassium chloride, and 137 mM sodium chloride, pH 7.4). The samples were run for 60 min.

The molecular size analysis of different species was performed by MALS with online Light Scattering HELEOS detector (LS) and differential Refractive Index detector (RI) from Wyatt Technologies as described previously.[27,30,31] The protein concentrations of eluting peaks were determined by the UV trace instead of the RI trace because the buffer and salt species from samples eluted at the same positions as some bispecific forms and interfered with the concentration analysis by RI. The molecular mass of detected component peaks was calculated by ASTRA V using their respective UV extinction coefficients for all IgG species.

Intact RPLC-MS. Two μg of samples were injected for intact LC-MS analysis. The LC-MS experiment was performed on Waters H-class UPLC and Xevo G2 XS QTOF mass spectrometer. The column POROS R2/10 (2.1 × 30 mm, Applied Biosystems, PN 1-1112-12) was used for reverse phase

separation. The sample temperature was set at 5 ºC and column temperature was set at 70 ºC. The mobile phase A (MPA) was 0.05% Trifluoroacetic acid (TFA) in water:acetonitrile (90:10), the mobile phase B (MPB) was 0.05% TFA in water:acetonitrile (10:90). The LC gradient was run as follows: 20 to 28% MPB for 2 min, then 28-30% for 8 min, 30-31.6% for 2 min, and 31.6-35% for 4 min, 35-90% for 1 min, and 90% for 2 min, and then equilibrated with 20% MPB for 6 min.

The MS front end conditions were set as the follows: capillary voltage at 2.8 kV, sampling cone at 200 V, source offset at 150, source temperature at 130 ºC, desolvation temperature at 400 ºC, cone gas at 50 L/h, desolvation gas at 800 L/h, m/z range at 600-4000. The mass spec experiment was performed at positive mode and sensitivity mode. The data was analyzed by using MassLynx software.

Reduced RPLC-MS. Sample was diluted to 0.5 mg/mL in a solution containing 6 M guanidine hydrochloride, 50 mM Tris buffer (pH 8.0), and 20 mM Dithiothreitol (DTT) and incubated at 56 °C for 30 min with shaking at 300 rpm. Two µg of samples were injected for reduced LC-MS analysis. The LC-MS experiment was performed on Waters H-class UPLC and Xevo G2 XS QTOF mass spectrometer. The column Zorbax 300SB-C8 (2.1 × 150 mm, Aglient, PN863750-906) was used for reverse phase separation. The sample temperature was set at 5 ºC and column temperature was set at 75 ºC. The mobile phase A was 0.1% TFA in water:acetonitrile (90:10). The mobile phase B (MPB) was 0.1% TFA in in water:acetonitrile (10:90). The LC gradient was run as follows: 25 to 32% MPB for 2 min, then 32-40% for 6 min, 40-90% for 1 min, and 90% for 1 min, and then equilibrated with 25% MPB for 5 min.

The MS front end conditions were set as the follows: capillary voltage at 2.8 kV, sampling cone at 150 V, source offset at 100, source temperature at 130 ºC, desolvation temperature at 400 ºC, cone gas at 50 L/h, desolvation gas at 800 L/h, m/z range at 600-4000. The mass spec experiment was performed at positive mode and sensitivity mode. The data was analyzed by using MassLynx software.

Hydrophobic patch analysis by MOE. The MOE antibody modeler was built based upon the use of a precompiled antibody structure database, Ig, immunoglobulin, provided within the MOE software (version 2019.0101). This database was used as a source of framework and hypervariable region structural templates. The hydrophobic batches were then analyzed by using the protein patch analyzer in the software. Both hydrophobic clusters of light chains and CDR motifs are shown to demonstrate their location and distribution.

RESULTS AND DISCUSSION

The potential forms of the antibodies generated in cells. Our BsAb C was produced from cells co-transfected with genes for two sets of the light chains and heavy chains, representing antibody A (mAb A) and antibody B (mAb B). The knobs-into-holes (KiH) mutations were introduced into each of HC chains at various locations of Fc to enhance heterodimeric pairing. However, the other undesired forms of full antibodies including those with mis-paired H-L chain or homodimeric parental antibodies, and partially assembled products, such as half antibodies, HC chain dimers, are expected to be formed in different percentages (Figure 1).

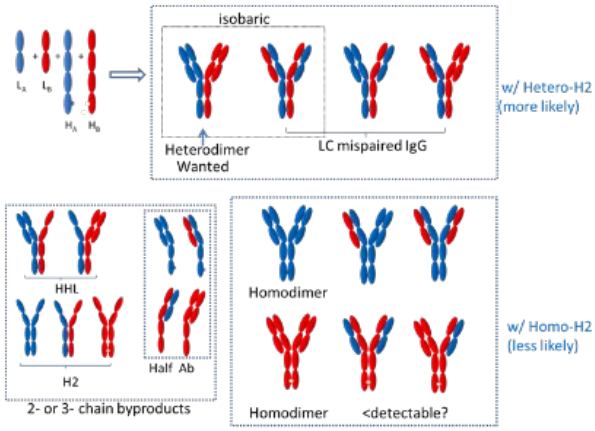


Figure 1 Potential forms of antibodies or byproducts assembled in cells. Both light chains and heavy chains of mAb A and mAb B are co-expressed in independent vectors under CMV promoter with different selection markers. The heavy chains from these two mAbs contain the knobs into holes mutations in the CH3 domain respectively as depicted. The antibodies assembled from hetero- or homo-heavy chain dimers were boxed. Some likely partial byproducts like HL half molecules or 3-chain products are also listed. The desired heterodimer or isobaric and non-cognate HL paired antibodies are indicated.

Separation of BsAb using MM SEC and mass analysis by MALS. To analyze the assembly efficiency of the desired bispecific antibodies in the cell culture, the secreted IgG in harvested cell culture fluid was purified by Protein A chromatography. The Protein A pools were analyzed on a Sepax Biomix SEC column using PBS as mobile phase. The parental antibodies A and B were produced from the cell cultures transfected with only one set of light and heavy chain genes for the respective antibodies (Figure 2A and 2B). On MM SEC, purified parental mAb A homodimer eluted drastically earlier than mAb B homodimer (Figure 2A and 2B). Both parental mAbs A and B displayed two major peaks (peaks 1 and 2).

The molecular size of each peak was determined by coupled multi-angle light scattering detector (MALS), which is a native method to estimate the molecular mass of eluting material (Figure 2C and Table 1). Peak 1 of purified mAb A homodimer had a mass of approximately 146 kDa, and peak 2 had a mass of approximately 75 kDa, likely representing a half molecule of mAb A (Figure 3A). Peaks 1 and 2 of mAb B were identified as the half molecule (75 kDa) and homodimer (147 kDa) respectively. The relative amounts of peaks 1 and 2 in both purified mAb A and mAb B varied significantly among batches as shown in Figures 2 and clearly reflected their different stabilities. Purified mAb A homodimer was more stable than mAb B homodimer. The latter readily dissociated into half molecules (Figure 2B). Interestingly, mAb B half molecules were more stable than mAb A half molecules (Figure 2C). This indicates that two half molecule association in mAb B antibody was not stable. Approximately 50% mAb A half molecules associated to form homodimers.

In a cell culture sample of BsAb C obtained by co-transfection with a mixture of plasmids for mAb A and mAb B, 5 major peaks (peaks 1 to 5) were clearly observed by UV trace (Figure 3A). Peaks 1 and 2 had the same retention times as peaks 1 and 2 for mAb A and were confirmed by MALS as likely a homodimer and half molecule from mAb A, respectively (Figures 3B and Table 1). Peak 3 had 147 kDa in

mass, corresponding to a new whole antibody that was absent in either mAb A and mAb B. Peak 4 and 5 corresponded to peaks 1 and 2 in mAb B, respectively. Peak 4 had a mass of 78 kDa. Peak 5 at 52 min likely represented a homodimer of mAb B, although the molecular weight was not measured due to the low abundance.

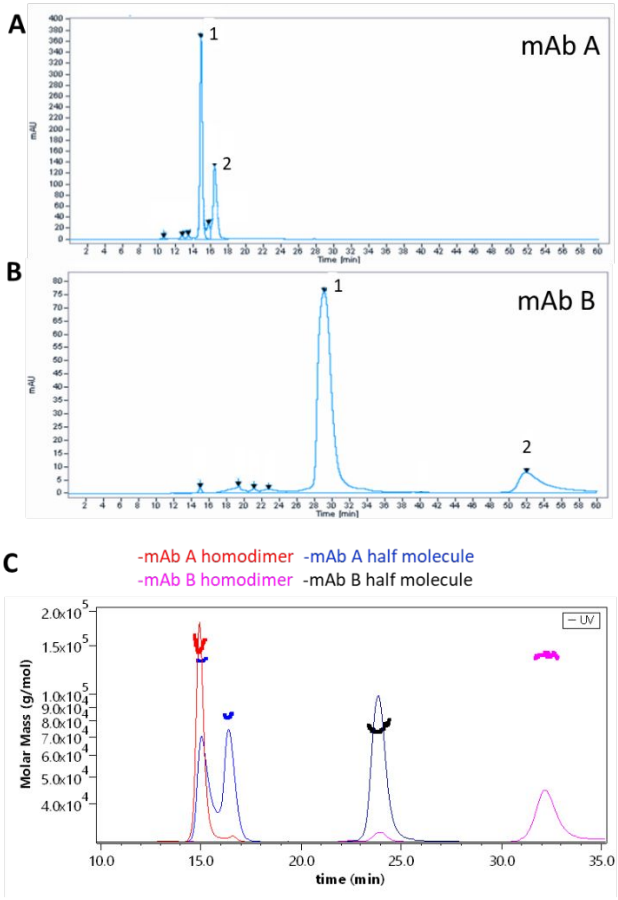


Figure 2 Separation of antibody forms by MM SEC. Antibodies were isolated from cell culture transfected with plasmids for parental mAb A homodimer batch 1 (A), mAb B homodimer, batch 1 (B). The molecular mass of each peak was analyzed by MALS using batch 2 of mAbs A and B (C). Purified half molecules of mAbs A and B were also analyzed. The major peaks are labeled and the mass distribution across each peak are indicated by a short-colored line corresponding to each antibody peak in the same color.

Although majority of antibodies shows a preference of HC (knob)-HC (hole) association as expected, homodimer antibodies from the HC (knob)-HC (knob), or HC (hole)-HC (hole) associations are observed at various efficiencies. In our assessment of cell samples, homodimer mAb B derived from the HC (hole)-HC (hole) association were detected at much less amounts than homodimer mAb A from HC (knob)-HC (knob) association. This is counterintuitive, as it would be expected that the knob to knob interaction would be less favored because of steric hindrance. Likely, other mutations other than "knobs into holes" mutations in the CH3 domain of these antibodies might impact the association of HC chains.

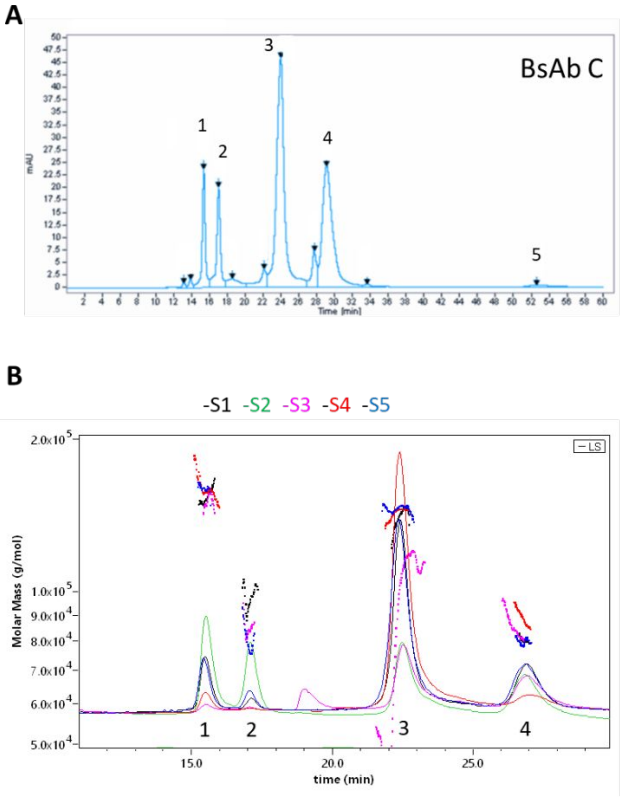


Figure 3 Separation of bispecific antibody BsAb C on MM SEC by A280 (A) and molecular size measurement by MALS for different cell culture samples (S1-5) of BsAb C (B). In panel B, the peaks corresponding to peaks in panel A are labeled. The mass distribution across each peak is shown by the colored dot lines, representing different samples.

Identification of peaks from MM SEC by Intact LC-MS. As described above, each major peak in the MM SEC chromatogram were preliminarily identified by the mass of each peak from MALS analysis and its elution time compared to purified homodimer and half molecules, the more accurate mass and identities of these peaks still needed to be confirmed by LC-MS (Figure 4). First, each major peak was isolated by MM SEC from a cell culture sample and concentrated by UF-centrifugation (Figure 4A). The separation efficiency was verified by reinjection of each fractionated peak on the MM SEC column (Figure 4B). The fractions were then analyzed by intact (non-reduced) RPLC-MS (Figure 4C) and reduced RPLC-MS (Figure 4D).

Table 1 Molecular mass (kDa) of eluting peaks by MALS

Antibody	Peak 1	Peak 2	Peak 3	Peak 4
mAb A	146	75	-	-
mAb B	78	147	-	-
BsAb C	146	75	147	78

The minus sign "-" indicates the peak not detected

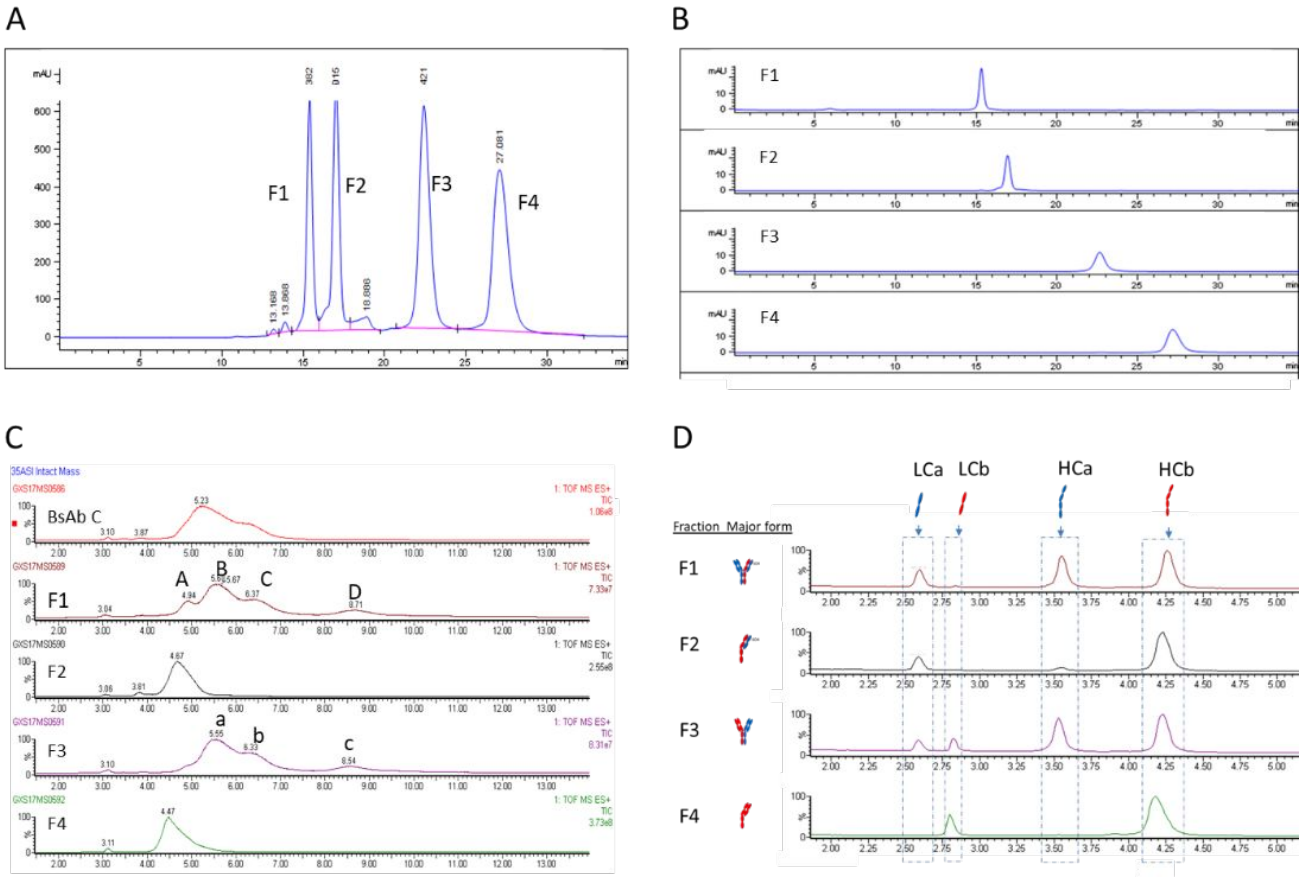


Figure 4 Fractionation of MM SEC and further analysis by LC-MS. A cell culture BsAb C sample purified by Protein A chromatography was fractionated by MM SEC (A). The major peaks were fractionated as labeled, concentrated, and re-analyzed by the MM SEC (B) or analyzed by intact LC-MS (C) and reduced LC-MS (D). The major identified antibody forms in each fraction are shown by the colored antibody diagrams, and the dissociated LC and HC for each antibody in the reduced LC-MS are indicated by the respective diagrams.

Table 2 The mass of MM SEC fractions by Intact LC-MS

Fraction	Identified form	Diagram	Measured mass (Da) of major species	Theoretica mass (Da)
F1	Mismer		148876	148870 (G0F + sulfation)
F2	Half mismer		74306 74468	74305 (G0F + sulfation) 74467 (G1F + sulfation)
F3	Heterodimer (BsAb)		148941	148936 (G0F)
F4	1/2 mAb B		74372 74536 74702	74372 (G0F) 74536 (G1F) 74698 (G2F)

Fraction F1 from MM SEC was resolved into several closely-eluted peaks in non-reduced RPLC, labeled A, B, C, and D. F2 contained mostly one peak. F3 was resolved into three peaks (labeled as a, b, and c) on reverse phase profile. F4 was a single peak.

The mass species present in each non-reduced RPLC peak of the mixed mode SEC fractions were determined by MS (Figure 5) and major species listed in Table 2. The antibody form in each RPLC peak was then identified based on the measured mass. These forms are represented by the antibody diagrams with blue and red colors corresponding to light and heavy chains of parental antibodies, mAb A and mAb B, respectively (Figure 5).

Each non-reduced RPLC peaks of F1 fraction was characterized as follows. 1) Peak A was identified as mAb B half molecules. This is surprising in that mAb B half molecules would be expected to not coelute with F1 as they normally elute much later as described above. Denatured mAb B half molecules formed aggregates that eluted as two small peaks before peak 1 on MM SEC (Figure 4A and data not shown). The mAb B half molecule-containing aggregates could contaminate the F1 fraction during fractionation. 2) Peak B of F1 is the most abundant form, identified as a mis-formed mAb A with one heavy chain replaced by a mAb B heavy chain (HaLa/HbLa) (for clarity, L and H are used for LC and HC; a and b refer to parent antibodies. respectively). This "mismer" (HaLa/HbLa) had a non-cognate or mis-paired HL arm and a sulfation modification [32] on one of La chains. This result contradicted our initial assessment of the peak as mAb A homodimer based on elution time and molecular weights by MALS. 3) Peak C of F1 was identified as the isoform of the mismer HaLa/HbLa with a sulfation modification on a different La. 4) RP peak D was an unknown form but was present at a low level.

The F2 fraction, with one RP peak, was identified as the mis-paired half molecule (HbLa) with a sulfation modification. Again, this result was inconsistent with our initial assignment of F2 as mAb A half molecule. The Hb-La mispairing apparently occurred at a much higher frequency than cognate Ha-La. As a result, half molecule (HaLa) of mAb A were not seen.

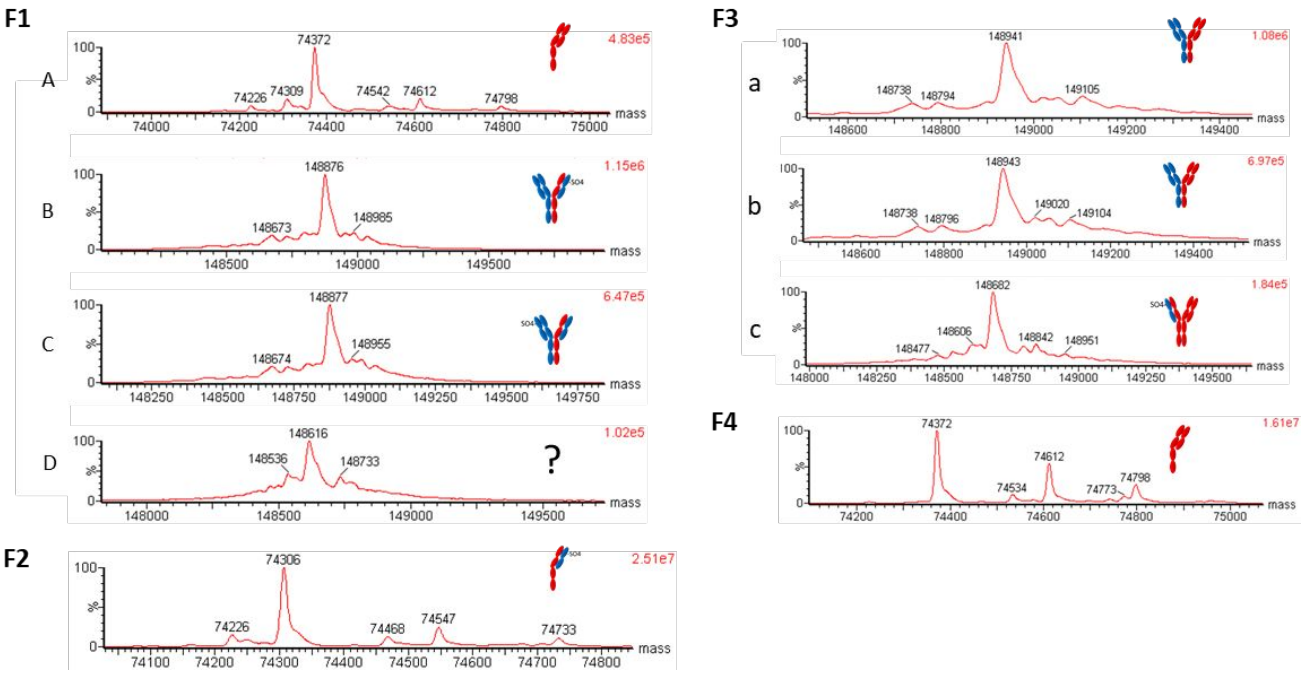


Figure 5 Deconvoluted mass spectra by intact LC-MS of isolated MM SEC fractions. Each peak identified by RP-HPLC (shown in Figure 4C) is indicated if multiple peaks were observed. The major mass species corresponding to different BsAb forms were assigned in Table 2.

Peaks a and b in F3 were determined as the bispecific heterodimer of BsAb C, the desired form of the antibody, containing no detectable sulfation. A low amount (<5%) of a mAb B mismer (HbLb/HbLa) with a mis-paired and sulfated La was observed in peak c.

F4, a late eluting fractionated peak, was identified as the half molecule (HbLb) of mAb B. This was consistent with MALS analysis. Peak 5 in Figure 2C that had the longest retention time, was not isolated because of the low quantity, but identified as mAb B homodimer since it eluted at the same position as the purified mAb B homodimer.

Characterization of purified forms by reduced RPLC-MS. In addition to the non-reduced LC-MS, the chain compositions of each antibody form isolated from MM SEC were evaluated by reduced RPLC-MS, where the antibodies were first denatured and reduced (Figure 4D). F1 was dissociated into La, Ha, and Hb, but contained almost no Lb. This agrees with the characterization of this fraction by non-reduced RPLC-MS as a mismer HaLa/HbLa. F2 was dissociated into mostly La and Hb, indicating it was mis-paired HbLa half molecule. F3 had all expected chains, La, Lb, Ha, and Hb. Therefore, it was a desired heterodimer, HaLa/HbLb as identified by MALS. F4 contained Lb and Hb as expected, in agreement with the half molecule, HbLb. The mass of detected light and heavy chains was confirmed and listed in Table 3.

Variation of the bispecific antibody assembly in cell culture samples. The antibody products purified from different cultures varied among the transfection and growth conditions. In terms of BsAb assembly, three major types of cell culture samples were observed (Figure 6). In type 1 samples (Figure 6A), significant amounts of various half molecules were present; assembly of BsAb was inefficient and BsAb content could be as low as 30%. The undesired assembled antibodies including whole or half mismers (with mis-paired H-L), were observed in a significant amount.

Table 3 Characterization of light and heavy chains of MM SEC fractions by reduced LC-MS

Fraction	Mass of Reduced LC-MS Peak (Da)			
	LCa	LCb	HCa	HCb
Theoretical Mass	23779	23925	50802	50463
F1	23779	N/A*	50802	50463
F2	23779	N/A	N/A	50463
F3	23779	23924	50803	50463
F4	N/A	23924	N/A	50463

*: not available

In type 2 samples (Figure 6B, and Figure S1 in supporting information), the major form is the desired BsAb, with some amounts of half molecules of mAb A, and a significant amount of mAb A homodimer and coeluting mismer: However, virtually no half molecules of mAb B were produced.

The type 3 samples were similar to type 2 samples in terms of identified forms (Figure S2 in supporting information), except that the level of mAb A homodimer was much lower and the mismer was not seen (Figure 6C).

The HL mis-paired antibody variants and parental homodimers changed drastically with different expression vectors and transfection conditions. We observed that the non-cognate pairing between heavy and light chains, and homo-pairing of heaving chains could occur at a high frequency during antibody assembly. For example, the mismer HaLa/HbLa could appear in high amounts in both type 1 and type 2 samples but not in type 3 samples. Currently it remains elusive what determines the formation of mismers, but unequal expression of H and L chains could drive the mis-pairing of H and L. Although there is a possibility that every potential form

could be produced, many of forms illustrated in Figure 1 were not observed.

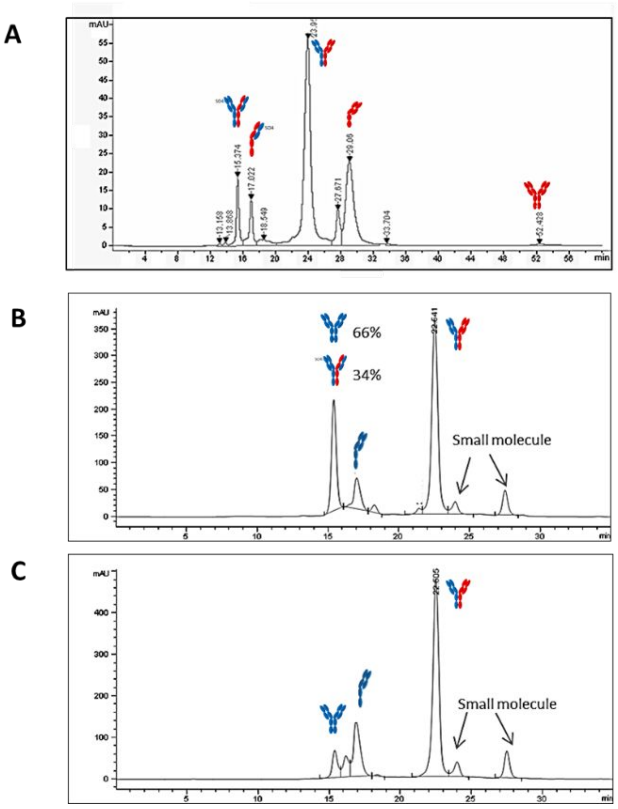


Figure 6 Identified antibody forms in three types of cell culture samples that have different MM SEC profiles of assembled antibody species. Type 1 sample (A) was characterized as shown in Figures 2-4; type 2 sample (B) and type 3 sample (C) were also characterized similarly as type 1 sample (Figures S1 & S2).

Mechanism of MM SEC for separation of different antibody forms. The unique elution behaviors of different bispecific antibody molecules in our MM SEC are determined by an attribute other than the molecule size, likely the specific hydrophobic interactions between the molecules and stationary phase.[24,27] Analysis of oxidized mAbs by MM SEC was also previously explored via the surface hydrophobicity of tested antibodies [27,29] but demonstrating a separation mechanism from those experiments was elusive.

Several unexpected features were found with this MM SEC that enable separation of different antibody forms effectively as described above. The forms with the same molecular size did not elute at the same elution times. For example, mAbs A and B eluted approximately 16 min apart from each other in spite the fact that they have similar molecular weights (Figure 2). Similarly, the half molecules of mAbs A and B eluted at very different times. Another unexpected observation is that the homodimer mAb B eluted much later than its counterpart the half molecule (Figure 2C). This is in stark contrast to the conventional SEC where the large size molecules elute earlier than small ones. However, mAb homodimer (HaLa/HaLa) and mismer (HaLa/HbLa) had the same retention time, and so did BsAb (HaLa/HbLb) and mismer (/HbLa /HbLb). By observing the following elution order: HaLa/HaLa = HaLa/HbLa > HaLa = HbLa >HaLa/HbLb = HbLa/HbLb >HbLb >HbLb/HbLb, it became evident that it was the number of Lb chains present in the molecules that determined the elution order. The greater number of Lb is present in a molecule, the more surface hydrophobicity it has, and the later it elutes. If the Lb number is the same, the size of the molecules dictates the elution time. The mAb A homodimer elutes earlier than its half molecule as both contained no Lb. Similarly, both HaLa/HbLb and HbLb have one Lb chain, but former elutes earlier because of larger size. Apparently, the species of heavy chains have little impact on the elution order.

To understand which domain of light chains of mAb A and mAb B was involved in the interaction with the column resins, we performed the hydrophobic patch analysis of both light chains by using MOE similar to hydrophobicity cluster analysis.[33] The sequences of La and Lb chains show common frameworks and differ only in CDR regions. It is evident that the hydrophobic clusters on CDRs of La and Lb are significantly different. Lb chain has much more of these hydrophobic clusters on the antigen binding region formed by CDRs than La (Figure 7). The surface hydrophobic clusters drive separation of different antibody forms via the interaction with silanol groups on the resin of the MM SEC column. The weak ionic interactions between antibody and charged resin groups could play a limited role but are not the major force for the separation of molecules in our MM SEC analysis since these interactions only slightly changed the elution time.

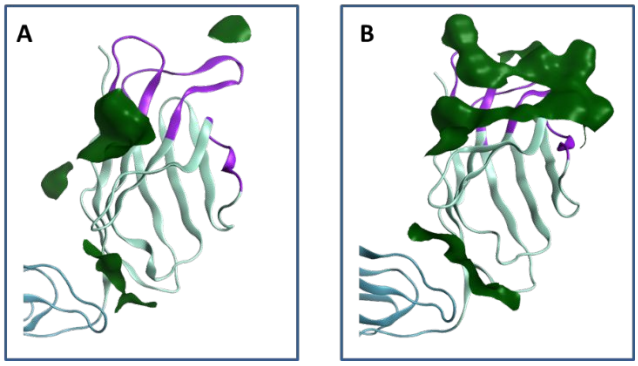


Figure 7 Hydrophobic clusters of light chain, La from mAbs A (A) and light chain, Lb from mAb B (B), analyzed by MOE. Only are Fab domains shown, with CDRs indicated as purple colors. The hydrophobic patches are shown in the green color.

During our early stage of studies, it was found that the MM SEC column demonstrated some variation and peak shift during long terms of usage. Through the more robust coating methods, the column manufacturer was able to significantly improve the stability and repeatability of the columns. Sepax Biomix columns offer the most obvious hydrophobic interactions with the antibodies compared to Waters BEH and YMC Pack diol 200.

The antibody-resin associations are impacted by salts in mobile phases. As we are preparing the manuscript, a recent study also reported use of MM SEC to analyze the bispecific antibodies using ammonium acetate (NH_4Ac) as mobile phase so that MS of each species can be measured.[24] Separation was drastically affected by NH_4Ac concentration.[24] Sufficient NaCl salt concentration in PBS is required for optimal resolution of oxidized mAbs by mixed mode SEC.[29] The current salt concentration in PBS in our study enabled the sufficient separation of our bispecific antibody forms.

CONCLUSIONS

In summary, a native MM SEC has been developed to separate bispecific antibody molecules based on their hydrophobic interactions with column resin. In the current study, the peak assignment in the MM SEC was performed at different levels by native SEC-MALS, as well as by denaturing non-reduced, and reduced LC-MS.

MM SEC-MALS has several advantages compared to other methods because of the unique separation mechanism in which the major driving force for the molecule separation is the special interaction of the surface hydrophobic clusters with the resin under native conditions. First, it allows effective separation and direct measurement of molecular weights of species in the peak by light scattering. Detection of some antibody forms, for example, the mismer (HaLa/HbLa) that is present in a relatively high amount in the samples, is only achieved by MM SEC, the form is either not detected or underestimated by RPLC-MS. Secondly, MM SEC uses UV absorbance to monitor peak elution enabling a more quantitative estimation than the MS method. The ability to isolate different native forms under non-destructive conditions by MM SEC enables other assessments like potency assays. Finally, the MM SEC can be implemented in a regular laboratory without the expensive MS instruments. We have successfully implemented the method in routine cell culture screening, and purification process development, and characterization of final purified drug substance.

ASSOCIATED CONTENT

Supporting Information. The characterization of MM SEC peaks by LC-MS for sample types 2 and 3 is described in Figure S1 and Figure S2, respectively

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Notes

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