Complex mixtures of antibodies generated from a single production qualitatively and quantitatively evaluated by native Orbitrap mass spectrometry

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Composite antibody mixtures designed to combat diseases present a new, rapidly emerging technology in the field of biopharmaceuticals. The combination of multiple antibodies can lead to increased effector response and limit the effect of escape variants that can propagate the disease. However, parallel development of analytical technologies is required to provide fast, thorough, accurate, and robust characterization of these mixtures. Here, we evaluate the utility of native mass spectrometry on an Orbitrap platform with high mass resolving power to characterize composite mixtures of up to 15 separate antibodies. With this technique, unambiguous identification of each antibody in the mixtures was achieved. Mass measurements of the intact antibodies varied 7 ppm on average, allowing highly reproducible identification and quantitation of each compound in these complex mixtures. We show that with the high mass-resolving power and robustness of this technology, high-resolution native mass spectrometry can be used efficiently even for batch-to-batch characterization.

Introduction

Monoclonal antibody (mAb)-based therapeutics have rapidly gained importance in the past decade with now more than 30 products approved for clinical use and many more in research and pre-clinical trial phases.1,2 One of the major advantages of mAb-based drugs over more conventional therapeutics is the high target specificity, which allows the possibility of increasing efficacy and patient tolerance while reducing potential immunogenicity issues.3 However, with these advantages come several shortcomings, i.e., mAbs are complicated, large (~150 kDa) biological molecules that potentially harbor a plethora of biologically and chemically induced post-translational modifications (PTMs). The effects of a single mAb therapeutic can be highly dependent on PTMs, such as glycosylation,4 which can be altered based on the manufacturing conditions.5,7 To address these issues, a wide range of complementary, orthogonal analytical methods have been developed to monitor and control the mAb manufacturing process.

In addition to the potential structural constraints, it has become apparent that a single biotherapeutic with high specificity can potentially lose efficacy for mutation-prone diseases, such as some cancers and viruses.6,9 To combat some of these shortcomings, mixtures of therapeutic mAbs have been made and evaluated in the past few years.4,10,11 It has been shown that the use of a mixture of mAbs can have a synergistic effect through the binding of multiple targets.5,12-14 Mixtures of mAbs essentially aim to mimic native immune response of polyclonal serum and to combine improved efficacy with the retention of the high target disease specificity inherent to mAb-based therapeutics, yielding a potential for widespread therapeutic use in the future.11

For use as therapeutics, mixtures of mAbs must be generated by reproducible and robust procedures. Previously, polyclonal antibody serum was isolated from plasma of immunized human donors, but the processes inherently lead to variability in the mixtures and a potential for co-purifying contaminations that could be pathogenic.10,11,15 To overcome these risks and improve product yield, the use of recombinant mAbs for mixture generation has been introduced. Currently, there are two main methods for generating mixtures of recombinant mAbs: (1) development and structural/functional characterization of individual therapeutic mAbs, then the single mAb components are either administered simultaneously or combined as a single drug product that is administered; or (2) development of a mixture of mAbs via a single production platform, i.e., either single batch or single production vessel, followed by structural/functional characterization and administration of the whole mixture.10,16 Both methods have advantages and disadvantages from a production standpoint. The
development and structural/functional characterization of single therapeutic mAbs is a well-defined process, but it can be costly in both time and money when producing composite mixtures. The use of a single production to yield a mixture reduces these costs, but provides a new analytical challenge in the parallel characterization of these multiple, closely related, large biomolecules.8,16

To gain approval for clinical use, as well as for use in research and development, it is essential to have comprehensive tools allowing the analytical characterization of the individual components and the relative amounts of each component in the mixture. Currently, there are no regulatory guidelines for the production of the composite mixtures, though regulatory agencies are recognizing the trend to using multiple antibodies simultaneously, and they are starting to develop guidance for the administration of such mixtures.11,16 While the detailed structural characterization of each antibody in the mixture may be difficult to achieve, some methods for the general characterization of the relative abundance of the components have been used in batch-to-batch analysis. For the most part, cation exchange chromatography (CEX) and ELISA have been used for this purpose.8,15,17 We previously explored a method based on native mass spectrometry (MS) using an electrospray ionization/time-of-flight (ESI-TOF) platform and showed it was capable of both identifying and quantifying individual antibody components in a simple, composite mixture demonstrating the same level of confidence and reproducibility as CEX.18 However, the lack of mass-resolving power achieved on the ESI-TOF platform limited the application of this technique to marginally complex mixtures. We recently demonstrated the incorporation of native MS onto an Orbitrap-based platform, yielding well-resolved mass spectra of intact single mAbs and other larger protein complexes.19,20 With these improvements, high-resolution native MS can now be used to characterize much more complex or composite samples.

In this report, we evaluate the use of this high resolution native MS to qualitatively and quantitatively characterize composite mixtures of therapeutic antibodies generated in a single production vessel. By using native MS, the antibody signal is condensed into a few ion signals in the mass spectrum, making interpretation more straightforward and possibly reducing overlap between adjacent mAb species. The acquired high resolving power allows differentiation between antibodies that are very close in mass, e.g., base-line resolution of two mAbs differing by 42.27 Da on a total mass of ~150 kDa. Concomitant to the increases in resolving power, mass accuracy and reproducibility are also increased, yielding unequivocal identification and quantitation of each component, even in a mixture containing up to 15 antibodies. We believe these results highlight the versatility of this new native MS Orbitrap-based platform for antibody mixture characterization and hint at the ability for both batch-to-batch characterization and mixture screening.

Results and Discussion

Production of complex antibody mixtures using Oligoclonics® technology

Because mAb mixtures are being explored as biotherapeutics, it is necessary to optimize efficient production methods that are qualitatively and quantitatively consistent in the generation of the composite mixture. Producing each mAb individually in a cell line then mixing them after purification limits the number of compounds used per mixture, largely due to prohibitive increases in cost and time. As described previously in detail,8 Oligoclonics® cell lines can be used to generate mixtures containing up to three IgG species, two monospecific IgG and one bispecific IgG, via a single cell line. For this study, the capabilities of Oligoclonics® were expanded through the transfection of multiple plasmids coding for different heavy chains and a common light chain to produce mixtures that contain up to 15 separate IgG species (Fig. 1). Common light chain antibodies all use an identical light chain; this prevents mis-pairing of heavy and light chains, and, therefore, all antigen binding sites are functional. Mixed expression of common light chain antibodies also results in the formation of heavy chain heterodimers, allowing the formation of bispecific antibodies and greatly increasing sample complexity. This increase in sample complexity comes at only a marginal increase in cost over production of a single mAb and at a drastic reduction in cost and time compared with generating these mixtures by more conventional methods.8,16 For this study, three separate mixtures were generated by co-expression of 3, 4, or 5 common light chain antibodies, resulting in 6, 10, or 15 Ab species per mixture, respectively. The masses of these intact, deglycosylated antibodies range from 144381.84 Da to 146965.22 Da. The smallest mass difference between two mAbs is 20.94 Da, and the largest difference was 1149.41. With increasing sample complexity, it is crucial that the analytical tools to
characterize these mixtures are capable of reproducible qualitative and quantitative analyses.

High resolution native mass spectrometry of complex antibody mixtures

Native mass spectra were acquired on an Orbitrap-based (modified Exactive Plus) instrument, and, in a few cases, on a TOF-based (LCT) instrument for comparison. Both instruments had been modified for analyses of large intact proteins under “native” conditions. In the mass spectra, the ion signals from the mAbs centered, as expected, around m/z 6000 with a narrow charge distribution (22+ to 26+) and well-resolved peaks. Experimental native ESI mass spectra, generated on these two mass analyzers, are shown in Figure 2, highlighting the different levels of resolving power achieved. For all measured mixtures, the achieved resolving power of the TOF-based instrument was significantly lower compared with that achieved by the Orbitrap-based platform to the point that not all of the mAbs could be resolved and identified on the TOF-platform. With the Orbitrap at the longest applied transient times, i.e., 256 ms, even minor mass differences could be resolved. As seen in Figure 2F, the six high-mass antibodies (numbered 10–15), which differ in mass by an average of 50 Da, are still baseline-resolved, yielding unambiguous identification of each individual mAb. With this high mass-resolving power, it is also possible to differentiate, albeit not baseline-resolve, two mAbs that differ by 20.94 Da (Fig. 2C, antibodies 5 and 6). The ability to resolve these two species is near the achievable physical limit as the isotopic envelope for an antibody is ~25 Da at full width at half maximum (FWHM) prohibiting baseline resolution. This illustrates the exceptional and differentiating resolving power of this technique over potential orthogonal methods.

The experimental peak width (FWHM) of a single mAb acquired on the Orbitrap-based instrument at the longest transient time applied is thus similar to that originating solely from
the isotopic distribution, as illustrated in Figure 3. It can also be
seen that the peak width for the signal acquired with the TOF-
based instrument is not only much wider, but also the centroid
of the peak is somewhat shifted to higher mass. These effects
are most likely due to inefficient desolvation and adduct forma-
tion but may also be caused by detector effects.23 We believe
that the improved apparent resolving power achieved using the
Orbitrap-based instrument vs. the TOF-instrument is not due to
the inherent instrumental resolution, which on both instruments
is significantly higher than the observed resolution, but due to
the more efficient desolvation, i.e., less adduct formation, for
the Orbitrap-based platform. With this improved desolvation, it
is possible to probe highly complex mixtures with unequivocal
identification and reliable quantitation.

High mass accuracy for unequivocal identification of
antibodies

As can be seen in Figure 3, the improved desolvation efficiency
of the Orbitrap-based instrument yields not only higher resolving
power, but also more accurate masses. Because the experiment-
al FWHM is similar to the predicted isotopic FWHM, we can
assume that the mAb signal is due to “bare” ions with no effects
due to adducts, thereby imparting very high mass accuracy. We
note that incomplete desolvation so far has been the most limit-
ing factor in acquiring accurate masses in native MS of larger
proteins and protein complexes.23 As seen for data acquired on
the TOF-based instrument, not only is the peak wider, but the
centroid is also shifted to higher mass, thereby decreasing the
mass accuracy.

High mass accuracy is crucial for unambiguous identification,
especially in composite mixtures, in which each of the compo-
nents is highly related. In preliminary data analyses of these mix-
tures, using the sequence-derived “theoretical masses,” the mass
accuracy was not only quite large but also varied substantially
for a few of the mAbs. It was noticed that the mass accuracy,
and therefore experimental mass deviation, fell into one of three
levels (Fig. 4A). This demarcation led us to believe that there was
a mismatch between the theoretical and experimental masses,
possibly originating from an unexpected PTM. Upon further
inspection of the sequence, it was noticed that some of the heavy
chains possessed an N-terminal glutamine, which is known to
be able to cyclize to a pyroglutamate.24 Once this PTM was
 accounted for, the mass accuracy improved to an average error of
7 ppm (Fig. 4B).

Besides the identification of an unanticipated PTM, the
high resolving power and mass accuracy of the Orbitrap-based
instrument make this platform better suited for the analysis of
complex mixtures. Therefore, it may be possible to exclude one
of the few sample preparation steps used in our native analy-
sis, i.e., deglycosylation. To explore this possibility, a mixture
containing six antibodies was analyzed both before and after
deglycosylation with PNGase-F (Fig. 5). While the deglycosy-
lated spectrum yields very nicely resolved peaks (Fig. 5A), it is
still possible to differentiate between the mAb species with the
glycans still attached (Fig. 5B). By calculating the mass differ-
ence between the deglycosylated mAb and the main glycosylated
species, we determined that the major glycosylated form is that
with a G0F structure on each heavy chain (as indicated by the
asterisk in Fig. 5B). The additional glycoforms correspond to the

![Figure 3. Comparison of peak width of a single antibody charge state (24+) on the two different platforms used and at increasing transient
time on the Orbitrap. Measurement at the longest transient time results
in a signal profile similar to that originating from the isotopic distribution
of an intact antibody. We attribute the improvements in mass resolving
power to improved desolvation prior to mass analysis. The FWHM values
of each peak are listed in the upper right.](image1)

![Figure 4. High mass accuracy leads to identification of non-sequence
predicted mass deviations due to PTMs. (A) Original mass error for each
component in a mixture of 15 antibodies. (B) Mass error for each compo-
nent in a mixture of 15 antibodies after N-terminal pyroglutamate for-
mation was taken into account.](image2)
addition of hexoses (as determined by an additional mass of 162 Da) and most likely result from the addition of terminal galactoses to the sugar chain. It was noticed that each mAb in the mixture showed the same three glycoforms with the same relative ratios, and we believe that this lack in glycoform heterogeneity across multiple antibodies is due to the mixture being produced in a single production, in which each mAb produced would undergo the same enzymatic glycosylation during processing.

Notably, when we quantified the relative abundance of each of the components in the mixture, equal results were obtained when comparing the samples before and after deglycosylation, indicating that deglycosylation has no effect on the relative ion intensities measured (Fig. 5A inset). Though complete glycan profiling cannot be obtained with this experimental setup, an assessment of the glycan heterogeneity and quantitation of the various glycoforms can be conveniently performed in parallel.

Figure 5. High-resolution native ESI mass spectra of a mixture of 6 antibodies after and before deglycosylation (A and B, respectively). Mass comparison between the deglycosylated measurement and the major peak of the glycosylated measurement showed that the main peak is due to addition of a G0F glycan to each heavy chain (indicated by the *, panel B inset). Each antibody exhibited three glycoforms separated by 162 Da (with the exception of the third Ab; the signal of the final glycoform overlaps with the signal of the major glycoform of the fourth Ab). The percentage of signal attributed to each glycoform deviated by < 2%, indicating that production of Abs in a single cell clone leads to formation of a common glycan chain. Quantitative comparison of the relative amount of each antibody before and after deglycosylation revealed that the amounts of each antibody are not distorted by the presence of the heterogeneous glycan chain (Panel A inset).
with the quantitation of the different antibody components in a single analytical characterization.

High technical reproducibility leads to consistent antibody characterization

In addition to the high resolution native MS on the Orbitrap-based platform being highly accurate with respect to component identification, the acquired spectra are also highly reproducible (Table 1). The mass accuracy was previously discussed, but another feature of this technique also leads to the unequivocal identification of each mAb component: the mass error. Across triplicate measurements, the mass error was an average of 7.5 ppm, which given an average mass accuracy of 7 ppm implies that the error in the mass accuracy stems mostly from minor random error in the technique. Thus, high resolution native MS is both highly accurate and precise in mass measurement.

High resolution native MS is also quantitatively reproducible. For each of these experiments, the mass spectra were reduced to a zero-charge spectrum using the Protein Deconvolution 2.0 package (ThermoFisher Scientific). The summed intensities for each species were then used to calculate the relative amount of that species in the mixture. The error in relative amount was less than 1.2% for baseline-resolved species across the triplicate measurements resulting in high confidence that the quantitative results were accurate (Table 1).

**Conclusions**

MS is gaining momentum as an analytical method for the structural characterization of intact protein biotherapeutics. The simplicity of sample preparation (optional deglycosylation and rebuffering) and speed of analysis (maximum acquisition time of eight minutes in this study) make it an enticing method for product screening. So far, due to instrument considerations, detailed intact mass analysis was typically performed under denaturing conditions. With the application of native MS on a high-resolution Orbitrap-based platform, it is now feasible to perform highly precise and accurate qualitative and quantitative assessment of biotherapeutics in their “native-like” form. The improved mass resolving power allows highly complex mixtures to be accurately characterized both in terms of their individual components and the relative amounts of each antibody in the sample. Our results strongly argue for the application of native MS with high-resolution mass analysis as a complementary tool in product characterization and screening of biotherapeutics, particularly when they are produced as mixtures.

**Materials and Methods**

**Antibody production and purification**

The antibodies analyzed were chosen based on functional criteria against their targets, rather than favorably selecting them based on differences in physical properties, e.g., as charge, mass. Antibody mixtures were produced by transient expression; up to five expression constructs encoding common light chain antibodies were mixed and used to transfect HEK293T cells. Mixtures were generated containing either 6, 10, or 15 separate mAb species (from 3, 4, and 5 expression constructs, respectively) with intact masses ranging from 144381.84 Da to 146965.22 Da and mass differences spanning from 20.94 Da to 1149.41 Da. Purification of the mAbs was done by using protein A followed by neutralization. Samples were rebuffered to phosphate buffered saline before further sample preparation required by native MS.

**Native mass spectrometry**

For most of the data presented, the mAb mixtures were deglycosylated prior to mass analysis. N-linked glycans were removed via incubation with peptide N-glycosidase F (PNGase-F; Roche Diagnostics) at 37 °C overnight. Each mixture was rebuffered to 150 mM ammonium acetate buffer, pH 7.5 using a 10 kDa molecular weight cut off (MWCO) spin-filter columns (Millipore).

**Table 1.** Theoretical and experimental values of the 15 antibody mixture. This table includes experimental masses for each technical replicate as well as the average mass and mass error. Also included is the signal intensity of each species (as determined after deconvolution) for each technical replicate along with the average and error of the signal intensity.
was loaded into a gold-coated borosilicate glass capillary (made in-house using a Sutter P-97 puller [Sutter Instruments Co.] and an Edwards Scanoco six sputter-coater [Edwards Laboratories]). An ESI capillary voltage around 1.2 kV was applied. On the modified Exactive Plus, the voltage offsets of the flatapoles and the transport octapole were manually tuned to increase the transmission of larger protein ions. For increased desolvation, in-source dissociation energy was applied, and ions were stored in the nitrogen-filled HCD cell prior to return to the C-trap. The resolution settings were adjusted as required, measuring transient times of either 128 ms or 256 ms prior to Fourier transformation. Data were acquired for 200 scans with an averaging of 5 transients and 10 microscans. In total, the longest acquisition time was 8 min. Prior to analyses, the instrumental calibration was checked using cesium iodide clusters and confirmed to be ~1 ppm. Mass spectra were deconvoluted to zero-charge and analyzed using Protein Deconvolution 2.0 (ThermoFisher Scientific).

For comparison, some of the samples were also analyzed on an ESI-TOF-based instrument (LCT, Waters) using conditions identical to those previously reported.18 The backing pressure on the source on the LCT was increased to 6.0 mbar to aid in desolvation.21 LCT data was processed using MassLynx v4.1 (Waters).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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