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Research Article

Versatile, Sensitive, and Robust Native LC−MS Platform for Intact Mass Analysis of Protein Drugs

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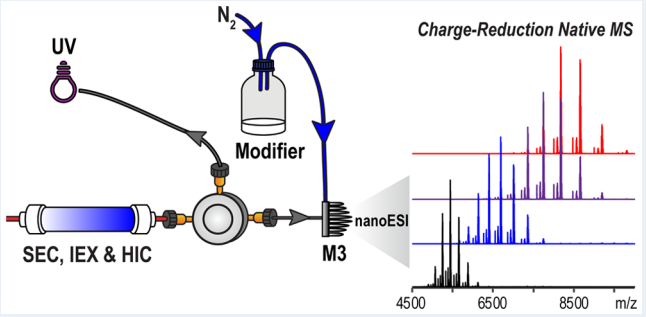
ABSTRACT: Over the past several years, hyphenation of native (nondenaturing) liquid chromatography (nLC) methods, such as size exclusion chromatography (SEC), ion exchange chromatography (IEX), and hydrophobic interaction chromatography (HIC) with mass spectrometry (MS) have become increasingly popular to study the size, charge, and structural heterogeneity of protein drug products. Despite the availability of a wide variety of nLC−MS methods, an integrated platform that can accommodate different applications is still lacking. In this study, we described the development of a versatile, sensitive, and robust nLC−MS platform that can support various nLC−MS applications. In particular, the developed platform can tolerate a wide range of LC flow rates and high salt concentrations, which are critical for accommodating different nLC methods. In addition, a dopant-modified desolvation gas can be readily applied on this platform to achieve online charge-reduction native MS, which improves the characterization of both heterogeneous and labile biomolecules. Finally, we demonstrated that this nLC−MS platform is highly sensitive and robust and can be routinely applied in protein drug characterization.



INTRODUCTION

Over the last couple of decades, electrospray ionization mass spectrometry (ESI MS)-based intact protein analysis has become an indispensable tool to support the development of protein therapeutics.[1] Traditionally, intact mass analysis of protein therapeutics is often achieved on a high-resolution accurate-mass (HRAM) MS system after reversed-phase liquid chromatography (RPLC) separation under denaturing conditions. Using low pH and organic mobile phases, RPLC is highly compatible with MS detection and provides rapid identification of analytes on the basis of accurate mass measurement.[2] However, due to the denaturing nature, this technique is incapable of evaluating the protein drug heterogeneity at its natively folded state, in which the protein drug exhibits its desired therapeutic function. A number of orthogonal liquid chromatography (LC) techniques that separate proteins under near native conditions (nLC), such as size exclusion chromatography (SEC), ion exchange chromatography (IEX), and hydrophobic interaction chromatography (HIC), have long been used in combination with optical detection to study the size, charge, and structural heterogeneity of protein drug products.[3−5] Online coupling of these nLC separation techniques with direct MS detection has several advantages. First, compared to optical detection, MS analysis is highly specific and often can provide unambiguous identification of protein variants based solely on accurate mass measurement. Therefore, the analysis can be achieved in a high-throughput fashion, eliminating the need for offline fractionation and characterization. Second, chromatographic separation often increases the dynamic range of the MS analysis, which facilitates the detection of minor variant species. Due to recent advances in both methodology and instrumentation of native MS techniques, online coupling of various nLC methods to native MS (nLC−MS) has become possible and has seen increasing applications in protein drug characterization.[6,7]

To address the intrinsic incompatibility arising from the use of traditional nonvolatile salt buffers in nLC separation, alternative mobile phases consisting of volatile ammonium-based salts have been widely explored for different separation modes. In particular, ammonium acetate is the most popular additive used in nLC−MS applications, as it is unlikely to induce protein structural changes compared to ammonium bicarbonate or ammonium formate.[8] For example, ammonium-acetate-based mobile phases at concentrations ranging from 20 to 200 mM have been successfully applied in different native SEC−MS (nSEC−MS) applications to study the size heterogeneity of protein drugs.[6,7,9,10] However, it is important

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to note that, as ammonium-based volatile salts often exhibit considerably lower ionic strength compared to traditional nonvolatile salts, more pronounced electrostatic interactions, occurring between the residual silanol groups from a silica-based SEC column and the protein analytes, are often observed, which can potentially lead to compromised protein recovery.[11] In particular, analysis of basic proteins by nSEC−MS may require the use of ammonium salts at much higher concentrations to reduce such electrostatic interactions. On the contrary, by intentionally applying a higher ammonium salt concentration (e.g., 300 mM), hydrophobic interactions between the protein hydrophobic patches and the chemical groups (e.g., short alkyl chains or linkage of functional groups) from a surface-derivatized SEC column can also be induced and utilized for improved separation and detection of monoclonal antibody (mAb) variants on the basis of their hydrophobicity.[12] Additionally, nSEC−MS can also be operated in "desalting mode" to achieve online buffer exchange prior to native MS detection.[13−19] Notably, rapid nSEC−MS methods using short columns (or SEC guard columns), operated at high flow rates, have presented great potential for native MS-based high-throughput screening applications in studying protein−ligand interactions.[20] Compared to SEC, developing a successful IEX method compatible for online native MS detection is less straightforward. Reported native IEX−MS (nIEX−MS) methods have explored the use of ammonium-based volatile salts to achieve three different elution modes, namely, (1) salt-gradient,[21] (2) pH-gradient,[22−27] and (3) salt-mediated pH-gradient.[28−31] For routine applications of nIEX−MS in protein drug characterization, both chromatographic resolution and MS compatibility need to be taken into consideration. For instance, a pH-gradient method developed by Fussl et al.[24,25] has been particularly successful in nIEX−MS analysis of mAbs. This approach has great MS compatibility, as the applied mobile phases are prepared using mixtures of ammonium acetate, bicarbonate, and hydroxide at relatively low concentrations (≤25 mM). Alternatively, salt-mediated pH-gradient methods, which supplement the pH gradient with changes in ionic strength, have also gained a lot of popularity in nIEX−MS analysis of mAbs. Compared to the pH-gradient method, this approach may require harsher source conditions for sufficient desolvation, due to the use of higher salt concentrations in mobile phase B (e.g., 150 mM). Nevertheless, it may offer improved separation of protein charge variants in some cases due to the application of an orthogonal elution mechanism.[28−31] Finally, high concentrations of ammonium acetate have also been successfully explored for HIC separation of mAbs and related products, followed by native MS detection (nHIC−MS).[32−34] Notably, due to the high salt concentrations required for protein retention, direct coupling of HIC to MS has been particularly challenging. In pioneering studies, Chen et al.[32,35] adopted novel HIC columns with more hydrophobic ligands so that only 1 M of ammonium acetate was required for successful retention of mAbs. A capillary column format was also adopted in these studies to improve MS sensitivity. Alternatively, Wei et al.[36] reported an isocratic elution strategy that could separate mAb variant species on a conventional HIC column using only 150 mM ammonium acetate. Recently, we also reported a makeup and splitting flow strategy that enabled nHIC−MS application on a conventional, analytical scale HIC column via online salt concentration reduction to 500 mM prior to MS detection.[34]

These reported nLC−MS methods are valuable additions to the analytical assay portfolios for protein drug characterization. However, the different nLC conditions applied in each method, including mobile phase compositions and flow rates, inevitably require MS source parameters to be optimized on an individual basis. The main goal of this study was to develop an integrated nLC−MS platform that could cope with a wide range of salt concentrations and LC flow rates used in various nLC−MS methods. Briefly, the different LC flow rates were addressed by applying a postcolumn splitting strategy, which reduced the flow rates to a range that could be accommodated by a multinozzle emitter for nanoelectrospray ionization (NSI). Additionally, we showed that the concentrations of ammonium acetate can be readily tolerated for up to 600 mM, thanks to the application of NSI. Moreover, via desolvation gas modification, this platform can readily achieve online charge-reduction native MS, which facilitates analysis of labile and heterogeneous molecules. Finally, we demonstrated that nLC−MS methods achieved on this developed platform are highly sensitive and robust so that it can be routinely applied in protein drug characterization.

EXPERIMENTAL SECTION

Materials. Deionized water was provided by a Milli-Q integral water purification system installed with a MilliPak Express 20 filter (Millipore Sigma, Burlington, MA). NIST Monoclonal Antibody Reference Material 8671 (NISTmAb, humanized IgG1κ monoclonal antibody) was purchased from National Institute of Standards and Technology (Gaithersburg, MD). Ammonium acetate (LC/MS grade) and SigmaMAb Antibody Drug Conjugate (ADC) Mimic were purchased from Sigma-Aldrich (St. Louis, MO). Peptide *N*-glycosidase F (PNGase F) was purchased from New England Biolabs Inc. (Ipswich, MA). Invitrogen UltraPure 1 M Tris-HCl buffer, pH 7.5 and acetonitrile (ACN; Optima LC/MS grade) were obtained from Thermo Fisher Scientific (Waltham, MA). Ammonium hydroxide (NH_3; 30%) was purchased from J.T. Baker (Center Valley, PA). 2-Propanol (IPA; HPLC grade) was purchased from Honeywell (Muskegon, MI). All other monoclonal antibodies including both IgG1 and IgG4 subclasses were produced at Regeneron (Tarrytown, NY).

Sample Preparation. The mAb mixture was prepared by mixing four in-house mAbs at final concentrations of 5 mg/mL per mAb. One aliquot of the mixture was treated with PNGase F (1 IUB milliunit per 10 μg of protein) in 100 mM Tris-HCl (pH 7.5) at 45 °C for 1 h prior to nSEC−MS analysis. Another aliquot was directly subjected to nIEX−MS analysis without any treatment. All other mAb samples, including the SigmaMAb ADC Mimic, were diluted to 2−5 mg/mL with water prior to injection for nLC−MS analysis.

nLC−MS Method. All nLC separations were performed on an UltiMate 3000 UHPLC System (Thermo Fisher Scientific, Bremen, Germany). For nSEC−MS analysis, an Acquity BEH200 SEC column (4.6 mm × 300 mm, 1.7 μm, 200 Å; Waters, Milford, MA) was used at room temperature with an isocratic elution of 150 mM ammonium acetate at 0.2 mL/min. For nIEX−MS analysis, a BioPro IEX SF column (4.6 mm × 100 mm, 5 μm; YMC Co., Ltd., Kyoto, Japan) was used at 45 °C with a linear gradient of 20 mM ammonium acetate (pH 5.6, adjusted with acetic acid) to 150 mM ammonium acetate (pH 6.8) for 16 min at 0.4 mL/min. For desalting SEC−MS analysis, an Acquity BEH200 SEC guard column (4.6 × 30 mm, 1.7 μm, 200 Å; Waters, Milford, MA) was used

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at room temperature with an isocratic elution of 150 mM ammonium acetate with the following flow rates: 0.1, 0.2, 0.4, 0.6, or 0.8 mL/min. To enable online native MS detection, a stainless-steel tee was applied postcolumn connecting two Viper tubing with different dimensions (20 μm × 15 cm and 100 μm × 65 cm; Thermo Fisher Scientific, Waltham, MA) to reduce different analytical flows (≤0.8 mL/min) to a microflow range (sub μL/min) for native MS detection and to divert the remaining high flow for UV detection (280 nm). A Thermo Q Exactive UHMR or Exactive Plus EMR mass spectrometer equipped with a Nanospray Flex Ion Source (Thermo Fisher Scientific, Bremen, Germany) was used for native MS analysis. To achieve nanoelectrospray (NSI) with microflow, a Newomics Microfabricated Monolithic Multi-nozzle (M3) emitter (Berkeley, CA) was applied. To achieve charge-reduction native MS, a dopant-modified desolvation gas (2 L/min, nitrogen), achieved by passing the gas flow through the headspace of a DURAN pressure plus bottle (1 L; SCHOTT North America, Inc., Elmsford, NY) containing 200 mL of various modifiers (e.g., IPA, ACN, or 5% ammonia in ACN), was applied to assist the NSI (Figure 1). All

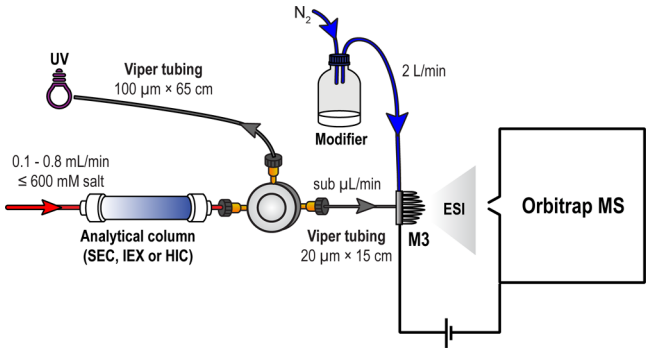

Figure 1. Representation of the nLC–MS platform.

experiments were performed with IPA-assisted native MS unless specified otherwise. The MS resolution was set at 12 500 (UHMR) or 17 500 (EMR), the capillary spray voltage was set at 3.0 kV, the capillary temperature was set at 350 °C, the S-lens RF level was set at 200, the in-source fragmentation energy was set at 100 (or 50 for ADC mimic analysis), and the HCD trapping gas pressure was set at 3. Mass spectra were acquired with an m/z range window between 2000 and 15 000.

RESULTS AND DISCUSSION

Integrated nLC–MS Platform. Analytical scale columns (I.D. of 2.1 mm or 4.6 mm) are preferred for nLC–MS-based characterization of protein drugs for several reasons. First, compared to the capillary column format, a much broader selection of analytical scale columns is readily available, therefore facilitating method development, particularly for challenging molecules with unusual physicochemical properties (e.g., charge, hydrophobicity, etc.). Moreover, analytical scale columns prevail in robustness and throughput and are widely used in quality control (QC) assays. From an assay comparability perspective, although nLC–MS methods are unlikely to exhibit identical chromatographic performance as QC assays, due to the application of MS-compatible mobile phases, performing such analysis on an identical analytical column instead of a capillary column is still more likely to generate reasonably comparable separation profile compared to QC assays. To cope with a wide range of flow rates (0.1–0.8 mL/min) used in analytical scale separation, a postcolumn splitting strategy was applied to reduce the flow rates to a range (<10 μL/min) that can be readily accommodated by a multinozzle emitter (M3) for NSI (Figure 1). The splitting ratio was primarily controlled by the two fixed Viper tubing of different dimensions connected to a stainless-steel tee (Figure 1) and remained unchanged for all tested nLC methods in this study. It is important to note that the multinozzle design of the M3 emitter is critical to achieve NSI at submicro flow rates, as it further reduces the flow rates by splitting the submicro flow into eight separate nozzles before NSI. Additionally, application of NSI not only reduces the harshness of the source conditions to achieve softer ionization but also significantly increases the MS sensitivity, as well as the tolerance toward high salt concentrations, a feature that is critical for accommodating various nLC methods (e.g., SEC, IEX, and HIC). Moreover, unlike a majority of other NSI setups, this M3 emitter-based approach allows desolvation gas to be applied to the spray emitter via a built-in desolvation gas line (Figure 1), which is useful to achieve improved spray stability and desolvation efficiency. This also provided an opportunity to further expand the versatility of the platform through desolvation gas modification. By simply doping the desolvation gas with volatile organic modifiers with charge-reducing capability (Figure 1), using a strategy previously reported,[37] online charge reduction can be readily achieved for native MS analysis. Finally, it is worth noting that, as this platform is built with readily accessible parts, it can be easily maintained or transferred for consistent performance. As described in the following paragraphs, extensive evaluations were performed to demonstrate the versatility, sensitivity, and robustness of this nLC–MS platform.

Platform Versatility for nLC Integration. When various nLC methods (e.g., SEC, IEX, and HIC) are adopted for nLC–MS analysis, the volatile salt concentrations used in mobile phases and/or LC flow rates often need to be optimized to achieve the best chromatographic performance. Hence, it is important that the developed nLC–MS platform can handle a wide range of LC flow rates and salt concentrations. To evaluate the tolerance of this nLC–MS platform toward different flow rates and salt concentrations, two tests were conducted by performing native MS analysis on a mAb1 sample after a rapid online desalting step using a Waters BEH200 SEC guard column (4.6 mm × 30 mm). First, using 150 mM ammonium acetate as a mobile phase and an injection amount of 10 μg, five different flow rates ranging from 0.1 to 0.8 mL/min were tested. Without changing the splitting settings or the MS source parameters, the total ion chromatograms (TICs) generated from the five flow rates all exhibited high signal intensity, spray stability, as well as high resemblance to their corresponding UV profiles (Figure 2a). In particular, the relative intensities of the UV and TIC peaks generally correlated with each other at different flow rates, suggesting that the NSI-MS sensitivity at higher flow rates was not compromised due to less sufficient desolvation. In addition, the averaged raw MS spectra obtained under these five conditions all exhibited highly symmetric m/z peaks and high signal-to-noise ratio (Figure S1), indicating sufficient desolvation even under the fastest flow rate tested (0.8 mL/min). These observations suggest that this developed nLC–MS platform can successfully tolerate an LC flow rate between 0.1 and 0.8 mL/min, which is sufficient for a majority of nLC–

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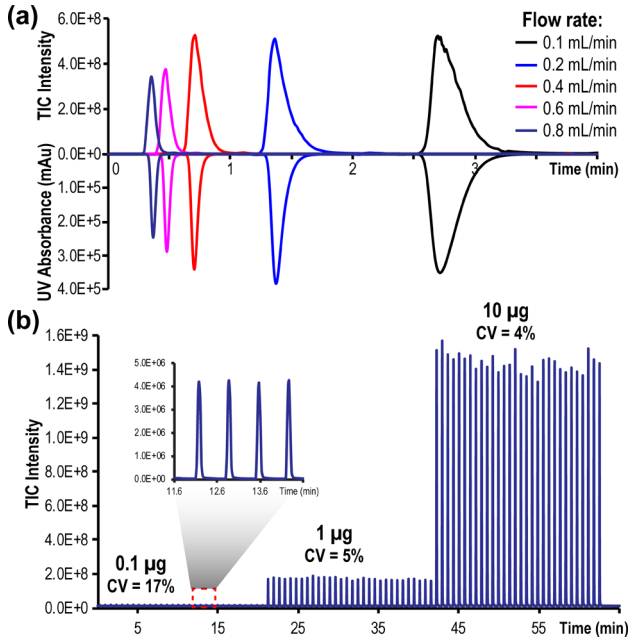

Figure 2. (a) UV and TIC traces generated from desalting SEC−MS analysis of mAb1 under different flow rates. (b) TIC trace from 90 desalting SEC−MS runs of mAb2 at 0.8 mL/min. Each injection amount (0.1, 1, and 10 μg) was repeatedly analyzed 30 times. The inset displays a zoomed-in region containing 4 desalting SEC−MS runs.

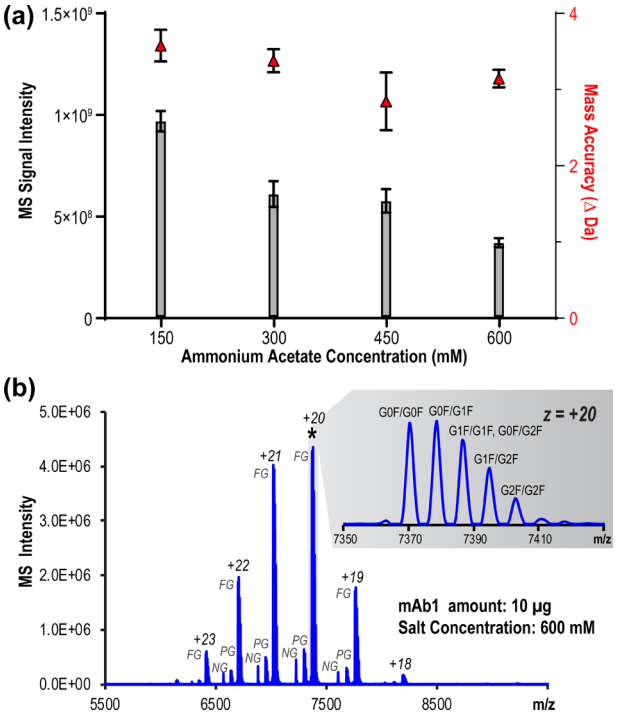

Figure 3. Desalting SEC−MS analysis of mAb1. (a) Comparison of MS signal intensity (left *y*-axis) and mass accuracy (right *y*-axis) achieved using 150, 300, 450, and 600 mM ammonium acetate as mobile phase (triplicate analysis each). The MS signal intensity was based on integrated peak area from the extracted ion chromatogram of the main species of mAb1 (including all charge states). (b) Example of the raw mass spectrum of mAb1 acquired using 600 mM ammonium acetate, showing successful detection of different glycoforms, resulting from the macro- and microheterogeneity of Fc N-glycosylation. FG, fully glycosylated; PG, partially glycosylated; NG, nonglycosylated.

MS applications. Moreover, the success of operating high flow rates on a short SEC column for fast and efficient desalting on this platform presented great promise in native MS-based high-throughput screening applications. As a proof-of-concept, mAb2 samples (2 μL) at three concentration levels (0.05, 0.5, and 5 μg/μL) were repeatedly injected for desalting SEC−MS analysis at 0.8 mL/min. The resulting TIC is presented in Figure 2b. Due to the high flow rate applied, the duty cycle between injections was 42 s, which corresponds to a throughput of ∼2050 samples per day. Although the achieved throughput is not as high when compared to some direct infusion-based MS platforms, this desalting SEC−MS-based approach prevails in minimal sample treatment, which not only saves benchwork time but also reduces risks of introducing sample preparation induced artifacts. Additionally, analysis of the 30 replicates demonstrated both excellent MS sensitivity and repeatability, as evidenced by the low coefficient of variation values (CVs of 17%, 5%, and 4% for 0.1, 1, and 10 μg sample injection amounts, respectively). The higher CV for the 0.1 μg sample injections was largely due to an overall decreasing trend in TIC intensity from the 30 injections. Therefore, it is likely attributed to the gradual decrease in stock protein concentration (0.05 μg/μL), as a result of needle adsorption-induced sample loss from repeated injections.

In the second test, the platform's tolerance toward salt concentrations was evaluated using the same desalting SEC−MS method with the flow rate maintained at 0.4 mL/min. For practical considerations, ammonium acetate concentrations ranging from 150 to 600 mM were studied for the impact on both MS sensitivity and spectrum quality. As shown in Figure 3a, high MS intensity was consistently achieved over triplicate analyses of mAb1 at each salt concentration (150, 300, 450, and 600 mM). Although a noticeable decrease in MS intensity was observed with increasing salt concentrations, presumably due to less efficient ionization at higher salt concentrations, the overall MS signal was still considered sufficient for most nLC−MS applications. Moreover, close examination of the native MS spectrum of mAb1 acquired at 600 mM salt concentration demonstrated good spectrum quality, with symmetric m/z peaks and a high signal-to-noise ratio (Figure 3b). Different glycoforms, resulting from the macro- and microheterogeneity of Fc N-glycosylation, can be well resolved and confidently assigned with accurate mass measurements. Lastly, it is noteworthy that the evaluated ammonium acetate concentration range (150−600 mM) is sufficient in enabling most nLC−MS applications, such as nIEX−MS and nSEC−MS, most of which were reported using mobile phases containing 20−200 mM ammonium-based salts. To enable nHIC−MS analysis using salt concentrations within the tested range, a makeup and splitting flow strategy, as recently introduced by our group,[34] could be applied. Briefly, by introducing a diluent flow (water) postcolumn, the HIC mobile phases (up to 3 M ammonium acetate) could be diluted by 6-fold (up to 500 mM) prior to native MS detection. Applying this strategy, nHIC−MS analysis of multiple mAb samples was performed on this platform and high-quality data were consistently obtained (Figure S2). Therefore, we conclude the developed nLC−MS platform can be versatilely applied to support most nLC−MS applications.

Platform Versatility for Online Charge-Reduction Native MS Analysis. Charge reduction is a strategy

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frequently used to facilitate the native MS analysis of labile protein complexes or highly heterogeneous protein samples. Via charge reduction, noncovalent interactions present in protein complexes can be better preserved during native MS analysis due to reduced Coulombic repulsion. In addition, by shifting the charge state envelope toward a higher m/z region, the spatial resolution between adjacent charge states can be greatly improved, therefore reducing the spectrum complexity. The most common approach to enable charge-reduction native MS is by adding charge-reducing reagents, such as imidazole or triethylamine (TEA), into the bulk analyte solution prior to ESI.[38,39] By using this developed nLC−MS platform, charge-reduction native MS can be readily achieved online by doping the desolvation gas with a variety of organic modifiers. To evaluate the charge-reducing effects of three different modifiers (IPA, ACN, and 5% (v/v) ammonia in ACN), a mixture of four different mAbs with pI values ranging from 6.8 to 8.5 were analyzed by both nSEC−MS and nIEX−MS analyses with dopant-modified desolvation gas. Subsequently, the average charge state of each mAb under each charge-reduction condition was calculated and compared with that from the control experiments, which were performed using unmodified nitrogen as desolvation gas (Figure 4). This study demonstrated that, regardless of the mAb analytes (e.g., different pIs), the solvent conditions for NSI (e.g., different pH and salt concentration from IEX gradient), or the nLC methods (e.g., SEC vs IEX), a consistent order of charge-reducing capability was observed. ACN/NH_3 (5% ammonia in ACN) resulted in the greatest extent of average charge reduction (~9.5 charges), followed by ACN (~8 charges) and IPA (~5 charges). Similar charge-reduction effects have also been observed in a previous study, where charge reduction was achieved by exposing the ESI droplets to neutral solvent vapor.[40] As expected, charge reduction also led to a corresponding decrease in overall MS intensity (Figure 4), which was likely attributed to less efficient transmission and detection of larger m/z ions by an Orbitrap instrument. Even under the greatest charge-reduction conditions, as achieved by ACN/NH_3-modified desolvation gas, the overall MS intensity from analyses of 5 μg of mAb samples still reached low E8 levels with high-quality MS spectra (Figure S3). It is worth noting that, unlike TEA that is difficult to remove once introduced to MS instruments,[41] charge reduction by this approach is considered completely contamination free. Interestingly, it was found that modifying desolvation gas with IPA or ACN could also reduce adduct formation, which tends to occur over extended analysis time (Figure S4). This adduct-reduction effect, as achieved by solvent-modified desolvation gas, was also consistent with the previous report.[40] Therefore, the mildest charge-reducing modifier, IPA, was applied by default for all nLC−MS applications on this platform.

The ability to achieve online charge-reduction native MS on this platform is valuable for the analysis of labile and/or heterogeneous protein drugs. For example, Cys-linked antibody-drug conjugates (ADCs) often present challenges for intact protein analysis because of their instability in the gas phase due to the lack of interchain disulfide bonds, as well as the high-mass heterogeneity arising from the different number of payloads. Using SigmaMab ADC Mimic as a model system, we evaluated the advantages of applying online charge reduction for native MS analysis of Cys-linked ADCs (Figure 5). This ADC Mimic consists of a mixture of drug-loaded species with 0−4 pairs of payloads (~668 Da) conjugated at

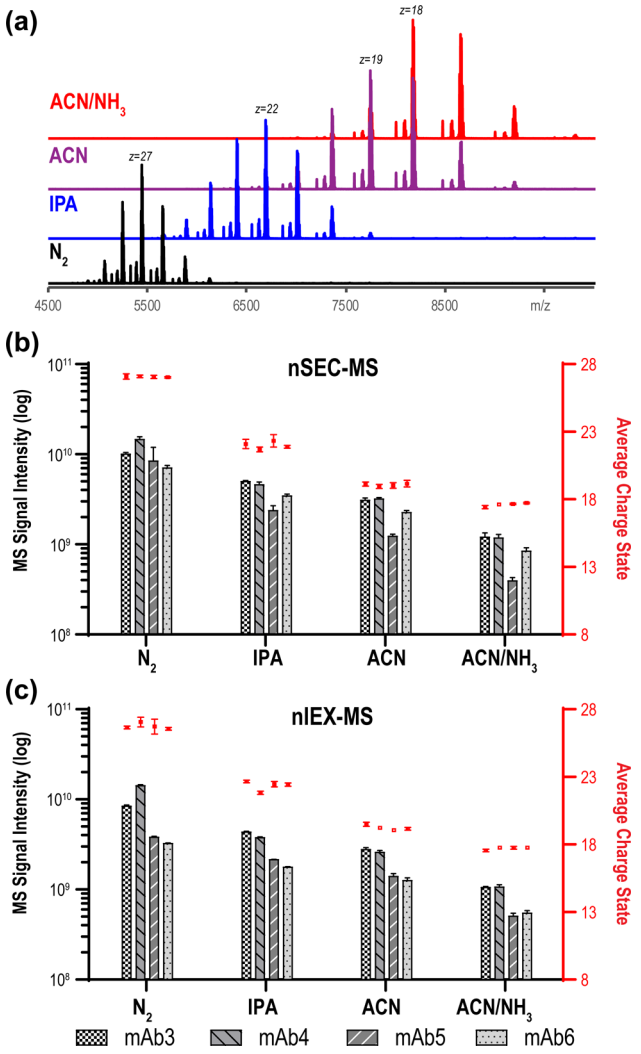

Figure 4. (a) Representative native mass spectra of mAb5 obtained under different charge-reduction conditions. (b and c) Evaluation of different organic modifiers for online charge-reduction ability by performing nSEC−MS and nIEX−MS analysis of a four-mAb mixture sample. The MS signal intensity (left y-axis) and average charge state (right y-axis) of each mAb molecule are measured in triplicate and plotted under each condition. The MS signal intensity was based on an integrated peak area from the extracted ion chromatogram of the main species for each mAb (including all charge states).

interchain disulfide bond Cys residues from an IgG1 mAb. Without removing the Fc N-glycosylation from the molecule, nSEC−MS analyses were performed with and without online charge reduction. It was shown that, when analyzed without charge reduction, dissociation of the noncovalent ADC complex was readily observed even with minimal in-source energy (SID = 50 eV) applied, leading to the formation of a highly charged light chain (LC) species and a charge-striped H2L species (Figure 5a, bottom panel). In contrast, when ACN/NH_3-assisted charge reduction was applied, such dissociation events were dramatically reduced even with a higher in-source energy applied (SID = 100 eV, optimized for efficient desolvation; Figure 5a, top panel). By calculating the relative MS intensity of the released LC against the intact ADC, this dissociation propensity was estimated to decrease from 8.3% to 0.4% upon charge reduction. This increased stability of ADC complex in the gas phase is likely attributed to

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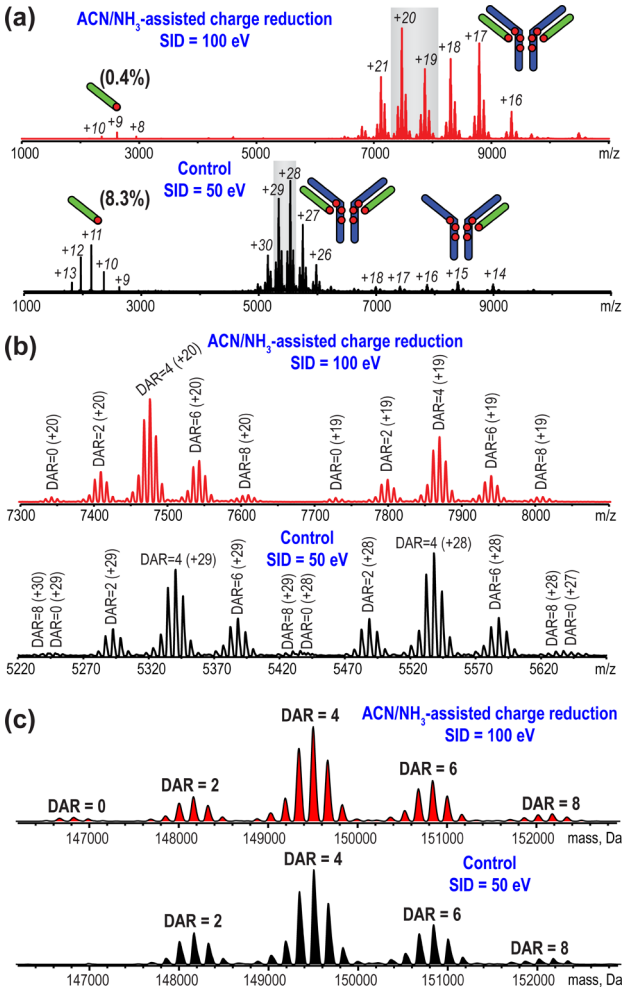

Figure 5. nSEC–MS analysis of SigmaMab ADC Mimic with ACN/NH_3-assisted charge reduction (top panels) and without charge reduction (bottom panels). (a) Full range raw mass spectra. (b) Zoomed-in raw mass spectra of two adjacent charge states as highlighted in (a). The cluster of peaks within each charge state were attributed to the Fc N-glycosylation microheterogeneity. (c) Deconvoluted mass spectra. The cluster of peaks within each DAR species were attributed to the Fc N-glycosylation microheterogeneity.

the reduced Coulombic repulsion at lower charge states, as well as the decreased internal energy of the ions through evaporative cooling (by charge-reducing reagents).[42] Moreover, because of the increased mass heterogeneity introduced by a different number of payloads, when analyzed without charge reduction, the charge state envelopes of different drug-to-antibody ratio (DAR) species overlapped in certain m/z regions. For example, charge states of +30, + 29, and +28 from DAR8 species appeared in similar m/z regions as charge states of +29, + 28, and +27 from DAR0 species, respectively (Figure 5b, bottom panel). Overlapping m/z regions can cause difficulty for spectral deconvolution, in which case, the DAR0 species were not detected after deconvolution (Figure 5c, bottom panel). In contrast, with ACN/NH_3-assisted charge reduction, the entire charge state envelope was shifted to higher m/z regions where the spatial resolution between adjacent charge states was greatly improved. As a result, no overlapping between charge states from different DAR species (e.g., z = +20 from DAR8 and z = +19 from DAR0) was observed (Figure 5b, top panel), facilitating confident spectral deconvolution (Figure 5c, top panel). Minimizing the undesired chain dissociation and spectral overlapping are both important to accurately measure the average DAR and characterize the payload distribution in Cys-linked ADCs. Chain dissociation in the gas phase will likely lead to underestimation of the average DAR due to preferential dissociation of the high DAR species. Spectral overlapping will lead to ambiguity or even loss of information during deconvolution. In this example, although the average DAR values obtained under the two conditions were comparable (DAR = 4.3 from charge reduction method vs DAR = 4.2 from control method), the payload distributions were different. Without charge reduction, the DAR0 species was not observed after deconvolution, while the relative abundances of the high DAR species were underestimated due to their preferential dissociation. These two causes affected the average DAR calculation in opposite directions and led to a comparable average DAR. However, only under the charge-reduction conditions, both the average DAR and the payload distribution of this Cys-ADC Mimic were accurately characterized.

Platform Sensitivity and Dynamic Range. In-depth characterization of protein drug heterogeneity requires both high sensitivity and a large dynamic range from the analytical methods. High sensitivity allows for characterization to be achieved with minimal sample consumption, while a large dynamic range enables the identification of low-abundance variants. To evaluate the sensitivity and dynamic range of the developed nLC–MS platform, NISTmAb reference standard was used as a test article and was subjected to both nSEC–MS and nIEX–MS analyses. Without any sample treatment, 10 μg of NISTmAb was injected onto a Waters BEH200 SEC column (4.6 mm × 300 mm) followed by IPA-assisted native MS analysis. The TIC from the nSEC–MS analysis revealed two high molecular weight (HMW) peaks and two low molecular weight (LMW) peaks that were separated or partially separated from the main peak (Figure 6). The main peak, detected at a total ion intensity of ~1E9, was attributed to NISTmAb monomer. HMW Peak 1 and HMW Peak 2, present at ~0.1% and ~1.3%, respectively, on the basis of UV-based quantitation, were both assigned as dimeric forms of NISTmAb, which were presumably separated by SEC due to their different conformations. Notably, even at such low levels, high-quality MS data were obtained for these two dimeric species, exhibiting glycoform-resolved mass peaks after deconvolution (Figure 6, insets). In addition, two LMW peaks also exhibited high spectrum quality and were unambiguously identified as two complementary fragments resulting from a series of clipping events occurring at the upper hinge region (Figure 6, insets). Subsequently, nIEX–MS analysis of 10 μg of untreated NISTmAb was carried out by adopting a reported strong cation exchange method[29] into this nLC–MS platform. Multiple charge variant species present at very different levels, including the same truncated mAb fragments, were all confidently identified on the basis of high-quality MS data (Figure S5). Finally, the size and charge variants in NISTmAb, as identified by these two nLC–MS methods, were summarized in Table S1, with their relative abundances quantified by corresponding UV peaks. Notably, despite the low abundances of size/charge variants, good mass accuracy was consistently achieved with native MS detection, facilitating confident identification (Table S1). As variant species could be readily detected at levels down to ~0.02%, a dynamic range of approximately 4 orders of magnitude could

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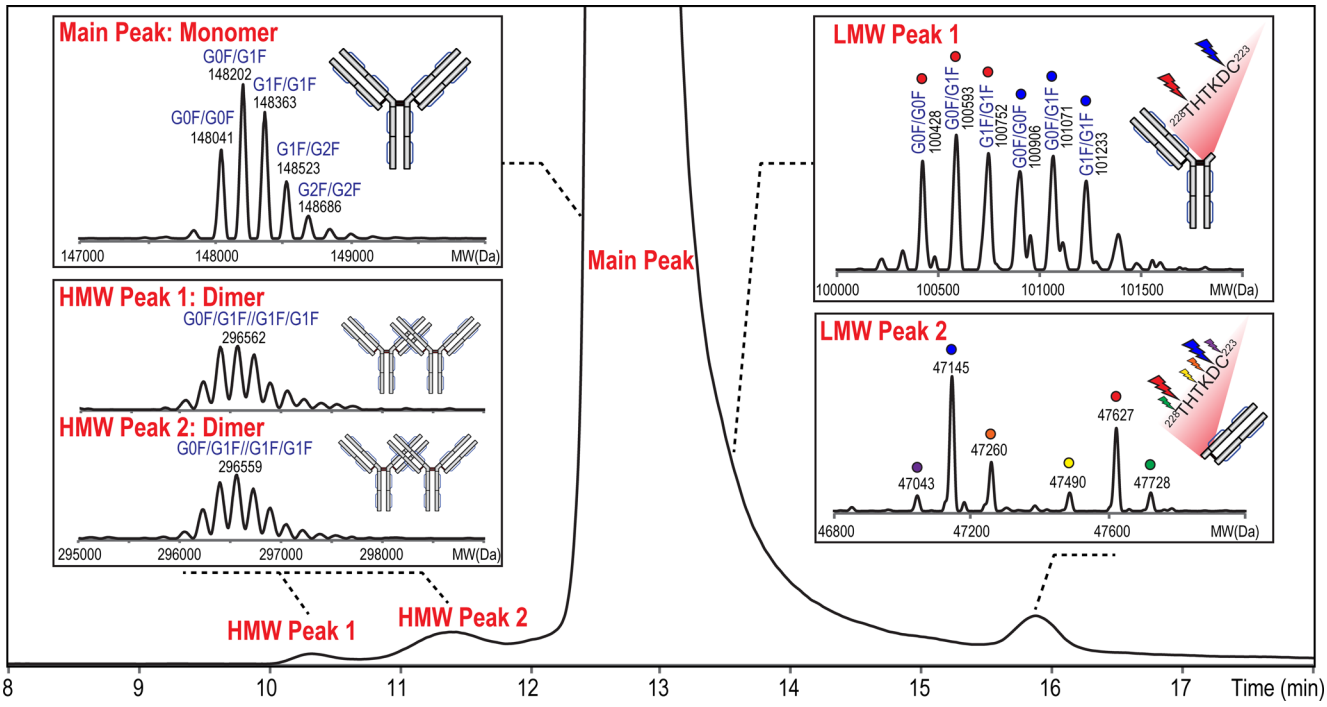

Figure 6. nSEC−MS analysis of NISTmAb reference standard with injection amount of 10 μg. The deconvoluted mass spectrum of each TIC peak were shown in insets. Note: G1F/G1F glycoform cannot be differentiated from G0F/G2F because of the same mass. The latter is not labeled in the figure due to space limitation.

be achieved by applying both nSEC−MS and nIEX−MS methods on this developed nLC−MS platform with an injection amount of 10 μg of NISTmAb. It is worth noting that, as 0.02% of variant species from a 10 μg injection represented an absolute quantity of 2 ng variant on the column, this platform was also considered highly sensitive and may be valuable for MS-based bioanalysis applications.

Platform Robustness. Robustness is another major consideration when developing analytical methods for routine applications in industrial laboratories. Particularly, nLC−MS methods continuously introduce aqueous salt solutions into the MS ion source, leading to difficulties in maintaining spray stability and signal intensity over an extended analysis time. To evaluate if the developed nLC−MS platform can be applied to continuous analysis and if the spray stability and method sensitivity can be maintained over a long period of time, both nSEC−MS and nIEX−MS analyses of mAb molecules were repeatedly performed over 24 h, generating more than 50 runs each. As shown in Figure 7, good MS intensity and stability were maintained over 24 h, with CV values at 6.0% and 4.1% for nSEC−MS and nIEX−MS analyses, respectively. The robustness achieved by this nLC−MS platform is likely attributed to both the application of the multinozzle emitter and the IPA-assisted native MS. The former not only reduces the size of droplets introduced to NSI but also mitigates the risk of clogging from protein precipitation and accumulation. The IPA-assisted native MS further improved the desolvation efficiency, likely by reducing the surface tension of the droplets. Finally, it is worth mentioning that, although the individual nozzles will gradually become clogged over extended usage, it is unlikely to interrupt the ongoing analysis until a majority of nozzles become clogged. This further improves the robustness of this nLC−MS platform.

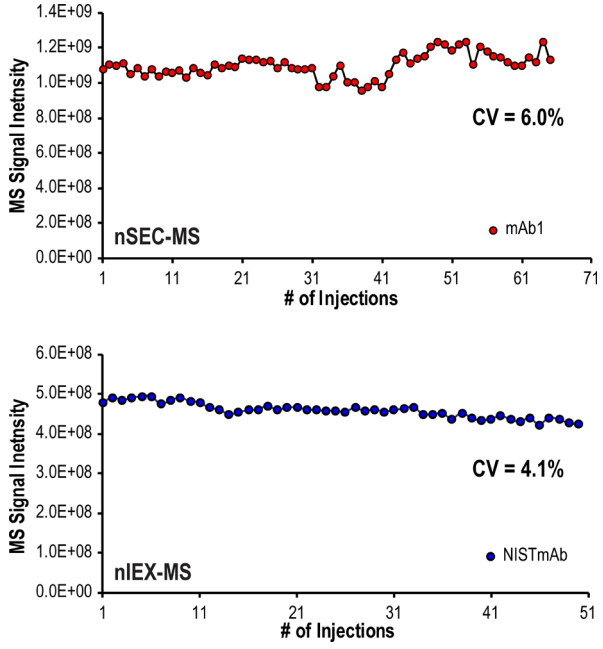

Figure 7. Evaluation of the robustness of the nLC−MS platform by performing continuous analysis using nSEC−MS (top, 24 min duty cycle) or nIEX−MS (bottom, 30 min duty cycle) for >24 h each. The MS signal intensity was based on the integrated peak area from the extracted ion chromatogram of the main species (including all charge states).

CONCLUSION

Developing a nLC−MS platform suitable for industrial laboratory applications is of great importance to the biopharmaceutical community, due to the increasing interest and demand in applying various nLC−MS methods for protein

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drug characterization. In this study, we described the development of a versatile, sensitive, and robust nLC−MS platform that can be easily integrated with various nLC methods. We demonstrated that the developed platform could handle a wide range of LC flow rates (0.1−0.8 mL/min) and could tolerate high salt concentrations (up to 600 mM in mobile phase), which together contributed to the achieved versatility in accommodating various nLC methods. We then explored different dopant-modified desolvation gases to achieve online charge-reduction native MS on this platform. Subsequently, a case study of a Cys-linked ADC Mimic showcased the suitability and advantages of applying charge-reduction native MS for characterizing labile and/or heterogeneous protein molecules. In addition, in-depth characterization of the size and charge heterogeneity of NISTmAb was achieved by both nSEC−MS and nIEX−MS analyses on this platform, which demonstrated a large dynamic range and high sensitivity. Furthermore, this nLC−MS platform proved to be highly robust and thus suitable for continuous analysis over extended periods of time (>24 h). Finally, it is worth noting that the new platform was built with commercially available parts and can be readily implemented by other laboratories for consistent performance.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jasms.0c00277.

Figures of native mass spectra, total ion chromatograms, raw mass spectra, and nIEX−MS analysis, and table of summary of size and charge variants (PDF)

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Notes

The authors declare the following competing financial interest(s): Y.Y., T.X., S.W., and N.L. are full-time employees and shareholders of Regeneron Pharmaceuticals Inc.

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