

## Title

Rapid Online Buffer Exchange: A Method for Screening of Proteins, Protein Complexes, and Cell Lysates by Native Mass Spectrometry

## Authors

Zachary L. VanAernum<sup>1,2,#</sup>, Florian Busch<sup>1,2,#</sup>, Benjamin J. Jones<sup>1,2</sup>, Mengxuan Jia<sup>1,2</sup>, Zibo Chen<sup>3,4,x</sup>, Scott E. Boyken<sup>3,4,†</sup>, Aniruddha Sahasrabudhe<sup>1,+</sup>, David Baker<sup>3,4,5</sup>, and Vicki H. Wysocki<sup>1,2,\*</sup>

<sup>1</sup>Department of Chemistry and Biochemistry, The Ohio State University, Columbus, OH 43210, USA.

<sup>2</sup>Resource for Native Mass Spectrometry Guided Structural Biology, The Ohio State University, Columbus, OH 43210, USA.

<sup>3</sup>Department of Biochemistry, University of Washington, Seattle, WA 98195, USA.

<sup>4</sup>Institute for Protein Design, University of Washington, Seattle, WA 98195, USA.

<sup>5</sup>Howard Hughes Medical Institute, University of Washington, Seattle, WA 98195, USA.

<sup>x</sup>Present address: California Institute of Technology, Pasadena, CA 91125, USA.

<sup>†</sup>Present address: Lyell Immunopharma, Inc., Seattle, WA 98109, USA.

<sup>+</sup>Present address: Amgen Inc., Thousand Oaks, CA 91320, USA.

<sup>#</sup>These authors contributed equally

\*Corresponding Author: [wysocki.11@osu.edu](mailto:wysocki.11@osu.edu), +1 614-292-8687  
<https://research.cbc.osu.edu/wysocki.11/>  
<https://nativems.osu.edu/>

**Keywords:** Native MS, online desalting, inline desalting, online buffer exchange, protein characterization, protein and protein complex screening.

## Abstract

It is important to assess the identity and purity of proteins and protein complexes during and after protein purification to ensure that samples are of sufficient quality for further biochemical and structural characterization, as well as for use in consumer products, chemical processes, and

therapeutics. Native mass spectrometry (nMS) has become an important tool in protein analysis due to its ability to retain non-covalent interactions during measurements, making it possible to obtain protein structural information with high sensitivity and at high speed. Interferences from the presence of non-volatiles are typically alleviated by offline buffer exchange, which is time-consuming and difficult to automate. We provide a protocol for rapid online buffer exchange (OBE) nMS to directly screen structural features of pre-purified proteins, protein complexes, or clarified cell lysates. Information obtained by OBE nMS can be used for fast (<5 min) quality control and can further guide protein expression and purification optimization.

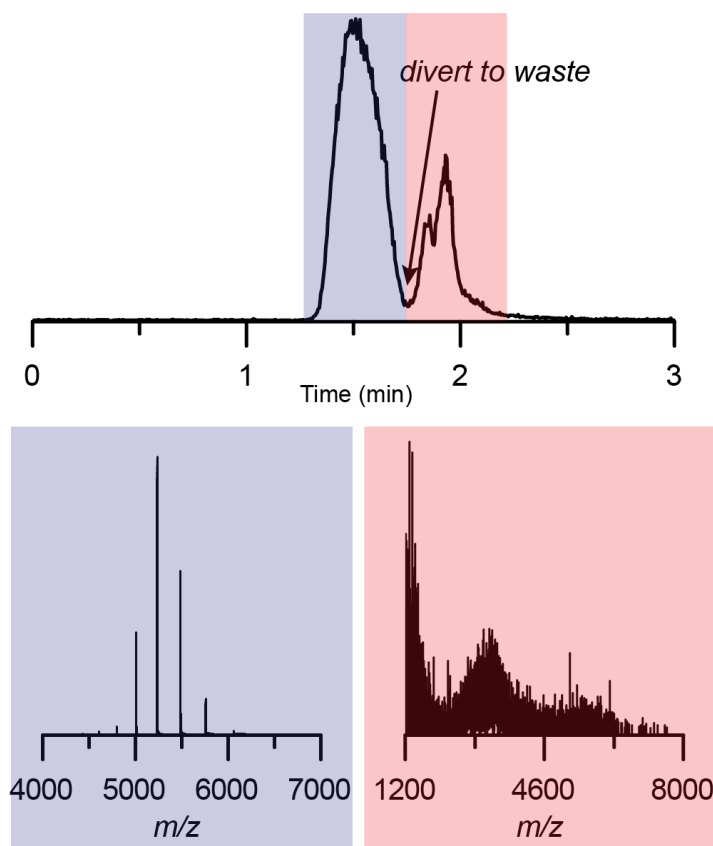
## Introduction

Protein production and purification have become increasingly accessible to researchers in all biomedical disciplines due to the rise of cost-efficient gene synthesis methods, standardized vectors and expression systems, and the routine use of protein purification tags.<sup>1–3</sup> Commonly, proteins are overexpressed with an affinity tag in a suitable host cell system, for instance a derivative of *Escherichia coli* BL21(DE3). Cells are subsequently lysed in well-buffered, high ionic strength solution to preserve the initial structure of the protein of interest.<sup>1</sup> Reducing agents, stabilizing agents, and ligands are frequently added to minimize protein oxidation and stabilize proteins, thereby also preventing them from aggregating.<sup>4,5</sup> Soluble proteins can be directly purified from the supernatant by affinity chromatography, typically resulting in a preparation with relatively low host cell protein contamination level.<sup>6</sup> It is often desirable to determine key protein properties at this point to make an informed decision on whether a sample is e.g. suitable for in-depth biophysical and structural analysis. Commonly, intact protein molecular weight and sample purity is estimated based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), or is assessed after denaturation by matrix-assisted laser desorption/ionization (MALDI-) or electrospray ionization (ESI-) mass spectrometry (MS).<sup>1</sup> As those measurements are performed under denaturing conditions, no information on protein quaternary structure, a key indicator of protein functionality, is obtained. Here, we demonstrate that online buffer exchange native mass spectrometry (OBE nMS) can be readily implemented to obtain information on tertiary and quaternary structure, thus rapidly assessing protein and protein complex integrity of large numbers of samples, in an automated fashion, using small sample quantities.

## Development of the protocol:

Online buffer exchange coupled to native mass spectrometry (OBE nMS) was first described by Cavanagh et al.,<sup>7</sup> with further development and potential use for drug discovery being reported by Waitt et al.<sup>8</sup> More recently OBE has been implemented as a fast desalting step after HIC separation coupled online with native MS.<sup>9</sup> The separation of proteins and non-volatile small molecules is accomplished by a short size exclusion column, typically PEEK tubing filled with a porous stationary phase. We have improved upon and implemented OBE nMS to accommodate aqueous mobile phases containing enough ammonium acetate to provide sufficient ionic strength to maintain native protein structure and prevent interactions between analytes and the stationary phase. A typical chromatogram from the OBE method is shown in [Figure 1](#) demonstrating the efficient removal of non-volatile salts from a protein complex and subsequent

MS detection. We have recently used this method for the high-throughput characterization of *de novo* designed proteins, allowing for unprecedented speed of native MS analysis to guide protein design and purification.<sup>10</sup> The procedure can be used for a variety of protein and protein complex samples and can help with efficient removal of non-volatiles prior to MS. In the following, we will provide information on: **1)** suitable columns for OBE nMS, **2)** typical samples, **3)** coupling to MS, and **4)** data analysis.

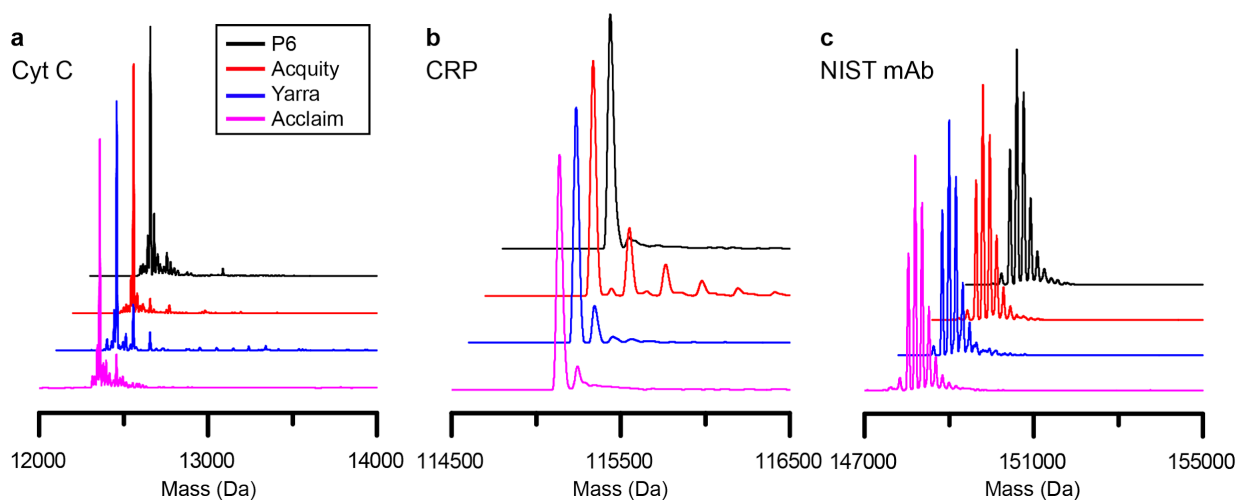


**Figure 1.** Total ion chromatogram and mass spectra of C-reactive protein (blue) separated from non-volatile PBS components (red) using the OBE nMS method. A mobile phase of 200 mM ammonium acetate was delivered at a flow rate of 100  $\mu$ L/min to a Yarra SEC-3000 column (290 Å pore size, 3.0  $\mu$ m, 2.1 mm x 50 mm). The y-axis of each spectrum represents relative intensity.

### Suitable columns for OBE nMS

The main purpose of the stationary phase in OBE is to separate proteins from small non-volatiles within a short amount of time at a given flow rate, thereby limiting sample dilution and the extent to which biomolecular interactions with high  $k_{\text{off}}$  rates dissociate. For optimal OBE performance, a column should be chosen that has an exclusion limit below the mass of the proteins to be buffer-exchanged. This allows the buffer-exchanged protein to be rapidly eluted in the void volume, followed by the non-volatile salts. We have found that Bio-Gel P6 material (Bio-Rad) can be easily packed in 0.03 inch I.D. PEEK tubing to manufacture disposable gel filtration columns at very low cost. The self-packed P6 columns efficiently separate proteins from non-volatile salts with favorably short elution times. Alternatively, short SEC columns are available from several

commercial manufacturers and can also be used for OBE nMS. A comparison of OBE nMS using commercial and self-packed columns is shown in **Figure 2**. Cytochrome C (12 kDa monomeric protein), C-reactive protein (CRP, 115 kDa pentameric protein complex) and NIST mAb (148 kDa) prepared in 1x phosphate-buffered saline (PBS) were buffer-exchanged using different columns. The desalting performance of each commercial column was comparable to that of the self-packed P6 column, with a few minor exceptions. The Yarra column resulted in less efficient non-volatile removal from cytochrome C (**Figure 2a**), as cytochrome C is close to the exclusion limit of this column and is not as well separated from the non-volatile salts compared to larger proteins. C-reactive protein retained noticeable ~213 Da mass adducts when buffer exchanged using the Acquity column. The origin of these adducts is not known and will require further investigation, however it may be responsible for the shift to the lower charge state distribution shown in **Figure S1**. The elution times of protein varied between the columns we investigated. The elution time of BSA was determined for each column by injecting 5  $\mu$ L of 4  $\mu$ M BSA with a mobile phase composition of 200 mM ammonium acetate and flow rate of 100  $\mu$ L/min. The self-packed P6 column had the shortest elution time of all the columns, while the Acclaim column had the longest (**Table S1**), demonstrating the advantage of using a column with an exclusion limit below the mass of the protein of interest. Each column generally exhibits efficient removal of non-volatile salts from the protein of interest, so the next most valuable figure of merit for a column used for sample screening is likely speed. Under these conditions, the self-packed P6 column would allow for the highest throughput. Although mobile phase flow rate can be modified to make up for the increased retention time for some of the columns, one should take caution in increasing the flow rate too much as too high flow rates and pressure can induce protein structure changes due to frictional heating.<sup>11</sup>



**Figure 2.** Comparison of OBE nMS using different size exclusion columns. An Acquity UPLC BEH SEC (Waters, 125 Å, 1.7  $\mu$ m, 4.6 mm x 30 mm), Acclaim SEC-300 (Thermo Scientific, 300 Å, 5  $\mu$ m, 4.6 mm x 33 mm), and Yarra SEC-3000 (Phenomenex 290 Å, 3.0  $\mu$ m, 2.1 mm x 50 mm) column were compared to the self-packed P6 Bio-Gel columns. Deconvoluted mass spectra of **a**) Cytochrome C, **b**) C-reactive protein, and **c**) NIST mAb exchanged from PBS into ammonium acetate using different columns (shown in legend). All spectra were acquired on an Exactive Plus EMR instrument and deconvoluted using Intact Mass software. The y-axis of each spectrum represents relative intensity.

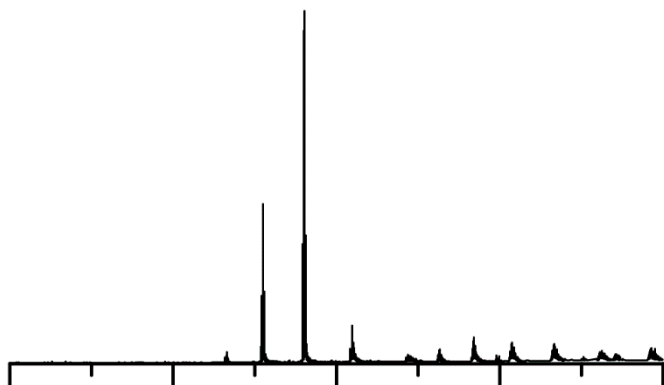
## Typical samples for OBE nMS

### A) Mass range

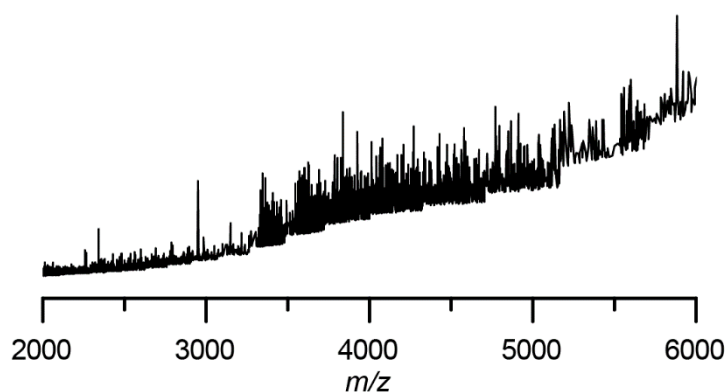
During the development of the OBE protocol, nine proteins and protein complexes ranging in size from 12-150 kDa were used to optimize MS tuning conditions (**Figure S2**). However, it should be noted that there is no reason that OBE cannot be used for larger analytes. For instance, we have recently successfully analyzed the 800 kDa tetradecamer bacterial chaperonin GroEL on a Q Exactive UHMR instrument without any changes to the OBE method aside from the MS tuning parameters. The proteins were dissolved or diluted in 1x PBS, desalted by OBE using a self-packed column with Bio-Rad P6 resin at an injection concentration of 4  $\mu$ M protein or protein complex, and recorded on a Thermo Scientific Exactive Plus EMR Orbitrap instrument. At a flow rate of 100  $\mu$ L/min, the buffer exchanged proteins are detected between 0.7-1.3 min, followed by the non-volatile salts between 1.3-2.3 min. The elution time was observed to shift by up to 0.05-0.1 min between different columns, presumably due to slight differences in column packing efficiency. Importantly, the elution time for an individual column remained constant over hundreds of runs. Because all proteins used here are above the exclusion limit of the resin (approximately 6 kDa), all proteins elute from the column in the void volume which allows for the development of a single LC-MS method regardless of the size of the protein or protein complex being analyzed. Desalting efficiency of all nine proteins via the online buffer exchange approach was comparable to, or better than offline buffer exchange via P6 spin columns with subsequent analysis by nanoESI (data not shown). In all cases, the most abundant signal corresponded to adduct-free protein with only minor adduction occurring in a few of the samples. Some samples also show multiple proteoforms present in minor abundances. A zoomed-in, deconvoluted spectrum of each buffer-exchanged protein and protein complex is available in **Figure S3**. The minor adducts present in each spectrum are due to non-volatile salts such as sodium (+21 Da) and phosphoric acid (+98 Da). Some of the peaks to high and low mass of the main peak are also due to proteoforms present in the sample, such as in the case of NIST mAb, which has multiple different glycoforms present, and streptavidin, which has the N-terminal methionine removed on a fraction of subunits present in each tetramer. In the cases where sodium adducts could not be resolved from the adduct-free ion at the resolution setting used (i.e. CRP and NIST mAb), the mass accuracy of the adduct-free signal was not sufficiently affected, indicating that only small amounts of sodium adduction are likely present. A comparison of streptavidin in PBS analyzed with and without the buffer exchange column is shown in **Figure 3** demonstrating the performance of the P6 column and

the necessity of non-volatile removal prior to MS analysis.

**a**



**b**

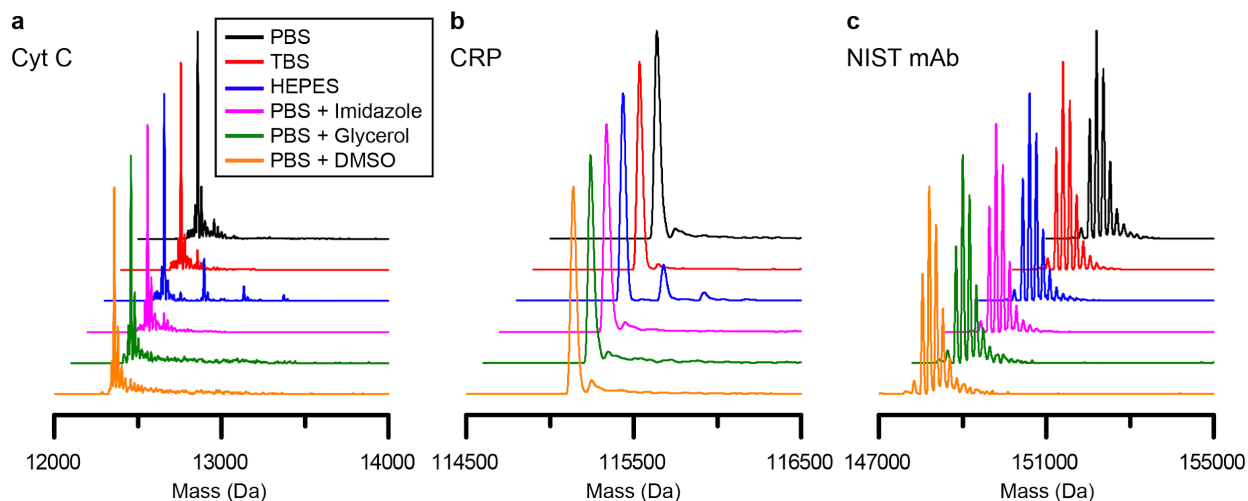


**Figure 3.** Effect of online buffer exchange on protein spectral quality. Mass spectra of streptavidin tetramer in PBS collected on a Solarix FT-ICR **a**) with a P6 online buffer exchange column and **b**) without the use of a buffer exchange column. The experimental setup and all variables (MS tune settings, LC settings etc.) were identical except that the P6 column for a) was replaced with tubing for b). The y-axis of each spectrum represents relative intensity.

#### B) Removal of non-volatiles

A variety of buffers are used during protein expression and purification. A buffer is generally chosen based on the pH range of interest, ionic strength, and chemical properties to stabilize the native structure of the protein or protein complex of interest. In addition to the wide range of buffers, solution additives such as preservatives, metal chelators and cryoprotectants are often included into the biomolecule purification workflow and storage process to further stabilize and protect the protein of interest. Here we demonstrate the removal efficiency by OBE of three different common buffers mimicking physiological conditions: phosphate-buffered saline (PBS), tris-buffered saline (TBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, and three different commonly used additives: glycerol, imidazole, and DMSO. Cytochrome C, CRP and NIST mAb were diluted or dissolved in PBS, TBS, or HEPES buffer, or in PBS with 200 mM imidazole, 20% glycerol or 20% DMSO added. The samples buffer exchanged online using a self-packed P6 column and data were acquired on an Exactive Plus EMR instrument ([Figure 4](#)). The dominant peak in each spectrum is the adduct free protein or protein complex, with only

minor signals due to small mass adducts such as sodium (+21 Da) and phosphoric acid (+98 Da). The extent of adducting on the samples prepared in TBS, as well as PBS with imidazole, glycerol, and DMSO is similar to the level of adducting present on the ions prepared in PBS only, and is comparable to what would be expected for samples prepared by offline buffer exchange. The main adducts from these buffers were also sodium and phosphoric acid. No distinct adducts corresponding to Tris, Imidazole, Glycerol, or DMSO were observed. The samples that contained 200 mM imidazole exhibit ions that are shifted to lower charge states (higher  $m/z$ ) which is consistent with imidazole having been previously reported as a charge reducing reagent in electrospray ionization.<sup>12–14</sup> Interestingly, the samples in HEPES buffer displayed +238 Da mass adducts indicating that HEPES is not as efficiently removed compared to the other buffers and additives. However, it should be noted that even in the case of HEPES, the adducted protein ions are in far lower abundance than the adduct-free protein ions, and sensitivity does not seem to be significantly lower. Mass spectra containing all charge states are shown in [Figure S4](#). Overall, these experiments demonstrate that the online buffer exchange method is useful for analyzing protein samples directly from common expression, purification, and storage buffer conditions.



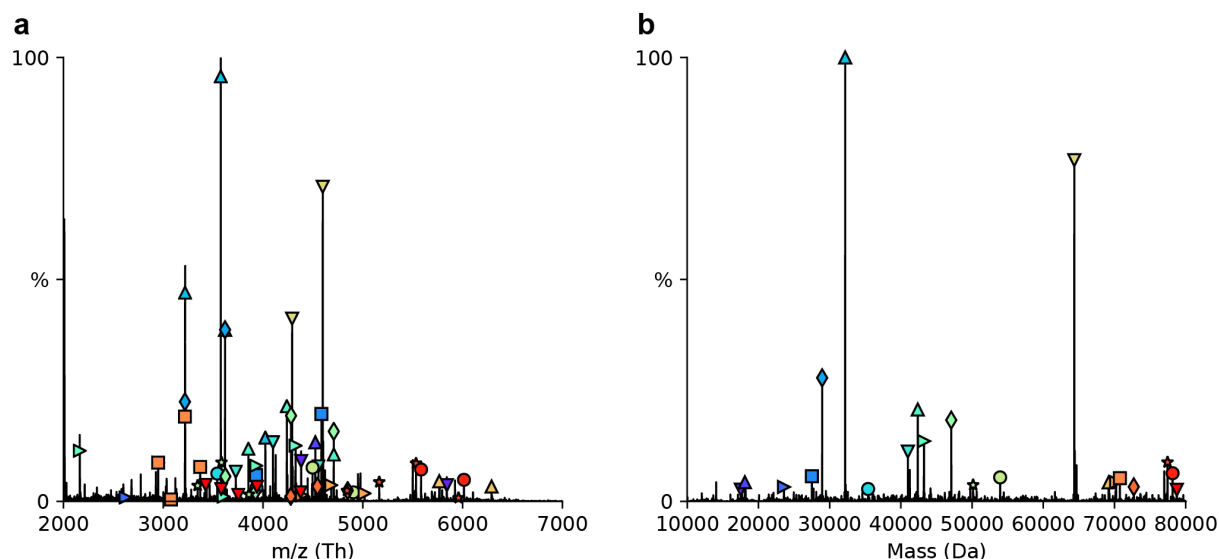
**Figure 4.** Deconvoluted mass spectra demonstrating the removal of non-volatile components from proteins in common biological buffers by OBE. Deconvoluted mass spectra of **a)** Cytochrome C, **b)** C-reactive protein, and **c)** NIST mAb exchanged from various non-volatile buffers into ammonium acetate. All spectra were acquired on an Exactive Plus EMR instrument after removal of small molecular weight non-volatiles using a self-packed P6 column. The heterogeneity in c) is due to the presence of various glycoforms. The y-axis of each spectrum represents relative intensity. Spectra were deconvoluted using Intact Mass software.

### C) Analysis of cell lysates

While the previous results have demonstrated the OBE method's utility for pre-purified proteins and protein complexes, in the case where a protein of interest is overexpressed, we have found that it is not necessary to carry out pre-purification steps such as affinity, size exclusion or ion exchange chromatography. Here we have directly analyzed a clarified cell lysate of a protein of interest using the OBE method ([Figure 5](#)). The results show the protein of interest in both the monomeric (32 kDa) and dimeric (64 kDa) form as the most abundant signals in the spectrum. In



this case, it is clear that the protein of interest was overexpressed and is a good candidate to be screened by OBE nMS without prior purifications steps. This method allows the determination of molecular weight, proteoforms, and oligomeric state in less than 5 minutes after clarification of the cell lysate and could even be extended to use top-down MS/MS to determine sequence information of the protein of interest.



**Figure 5.** Detection of over-expressed proteins from a clarified cell-lysate after online buffer exchange with a self-packed P6 column. **a)** Mass spectrum of a clarified cell lysate directly analyzed after online exchange to 200 mM ammonium acetate and recorded on an Exactive Plus EMR instrument. **b)** Deconvoluted (zero-charge) mass spectrum. The overexpressed protein of interest is labeled by a blue up triangle at 32 kDa (monomer) and a yellow down triangle at 64 kDa (dimer). The spectrum in a) was deconvoluted using UniDec to produce the mass domain spectrum in b).

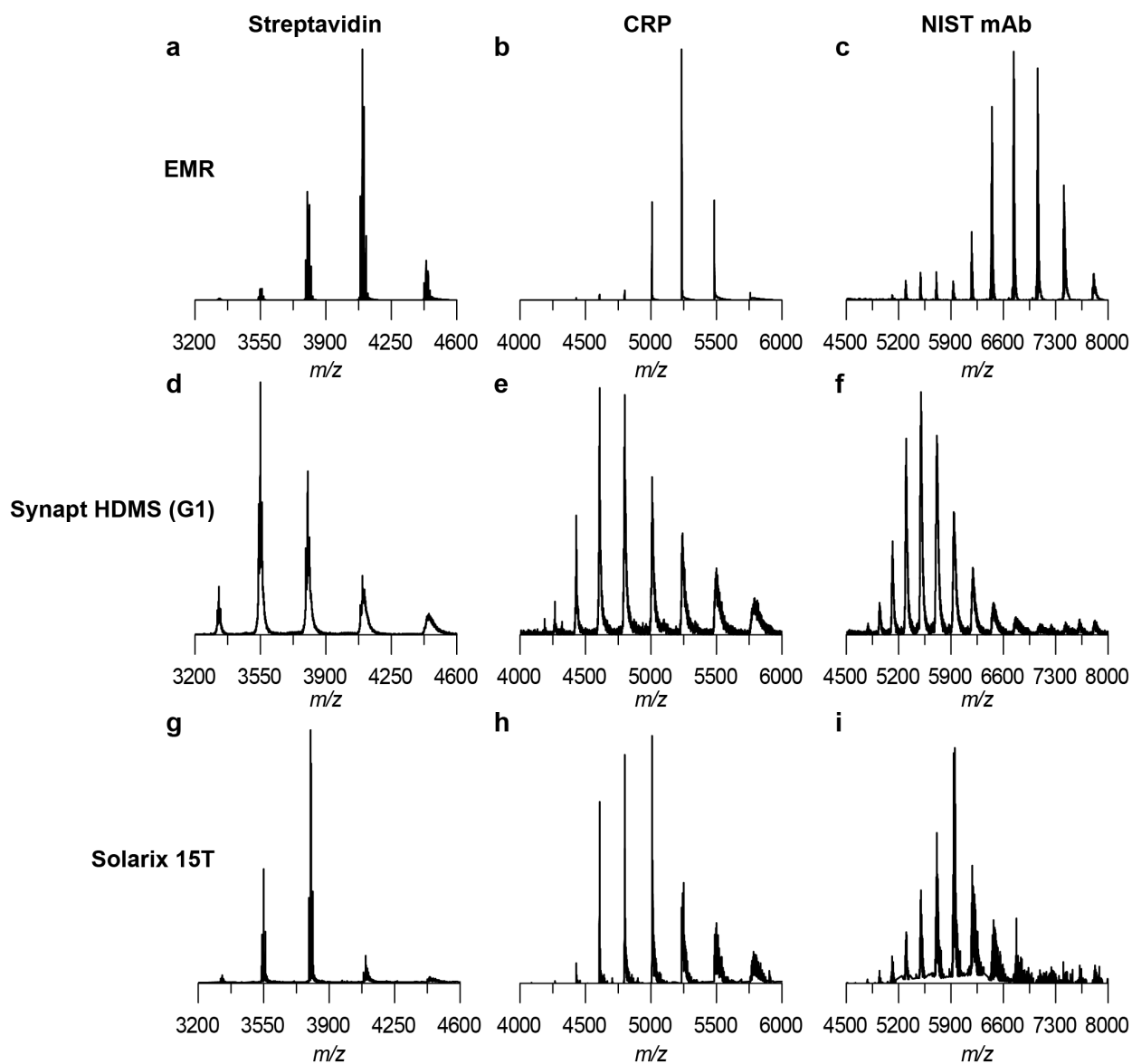
### Coupling of OBE to MS

In an effort to establish the transferability of the OBE method to different mass spectrometry platforms, we also analyzed three different proteins and protein complexes on a Bruker Solarix XR 15T FT-ICR instrument and a Waters Synapt "G1" HDMS Q-IM-TOF instrument. Streptavidin tetramer, CRP pentamer and NIST mAb prepared in PBS were buffer-exchanged using a self-packed P6 column, and the results were compared to the experiments performed on the Thermo Exactive Plus EMR instrument (**Figure 6**). All experiments that were recorded on the Q-IM-TOF and FT-ICR platform produced spectra with good signal and easily resolvable charge states, however the spectra obtained on these instruments resulted in protein ions with more adducting present than the spectra obtained on the Exactive Plus EMR platform. These results are consistent with the general trend observed when analyzing offline-desalted proteins and protein complexes by nanoESI on these instruments which indicates that the lower amount of adducting present in spectra collected on the Exactive instrument is likely a result of more efficient desolvation and declustering of the ions in the source region of the Exactive instrument relative to the Solarix and Synapt instruments.

We do note that the extra adducting present in the spectra obtained on the Synapt and Solarix instruments does not mean that OBE should not be implemented on these instruments. We



encourage the OBE method to be used on all three instrument platforms, especially with the high-resolution and ion mobility capabilities of the Solarix and Synapt instruments, respectively. Interestingly the charge state distributions shifted slightly depending on which instrument was used. We generally observed lower charge states than with nanoESI and believe that the change in charge state distributions between instruments is due to different ESI probe diameters, flow rates, probe positions, and desolvation gas flow rates used on each instrument.<sup>15</sup> We have also obtained MS/MS and MS/IM/MS data using the OBE method on a Q Exactive UHMR instrument and the Synapt instrument respectively (data not shown). The tandem MS experiments can be automated to incorporate oligomeric state, subunit connectivity and amino acid sequence information into the buffer exchange/screening workflow.

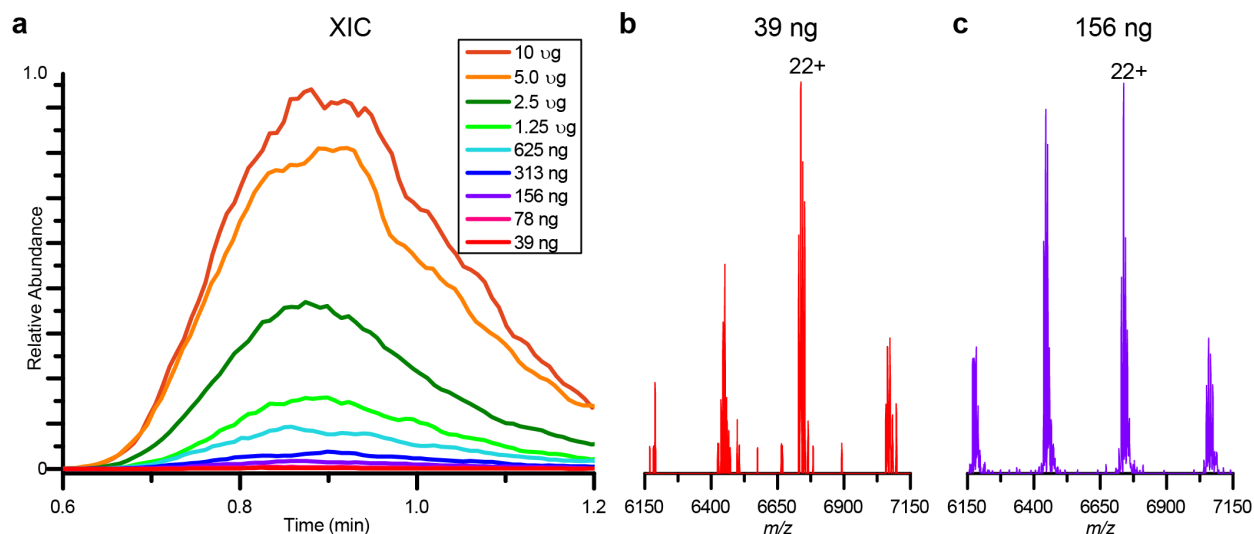


**Figure 6.** Online buffer exchange coupled to different mass spectrometers. Mass spectra of streptavidin tetramer, CRP pentamer, and NIST mAb were acquired on a Thermo Exactive Plus EMR mass spectrometer **a-c**), a Waters Synapt HDMS mass spectrometer **d-f**); and a Bruker Solarix XR 15T FT-ICR

mass spectrometer **g-j**) after online exchange from PBS into ammonium acetate. Ion source temperature and collision voltage were tuned for optimal desolvation without causing dissociation or fragmentation. All proteins were present in phosphate-buffered saline before being buffer-exchanged online with a self-packed P6 column. Differences in charge state distributions likely result from differences in ESI probe positions, and/or desolvation gas flow rates and are not indicative of structural changes of the analyte. The y-axis of each spectrum represents relative intensity.

### Limit of detection of the OBE method.

Throughout the development of the OBE method, we have found that injecting samples of roughly 4  $\mu\text{M}$  (5  $\mu\text{L}$  injection) protein or protein complex results in favorable data regardless of a protein's ionization efficiency or which mass spectrometer is being used. However, we recognize that some samples are precious and difficult to obtain in such large quantities. Under these circumstances it is often desirable to use the least amount of sample possible for screening purposes as the remainder of the sample may be needed for additional experiments. In an effort to establish a reasonable lower concentration limit that can be analyzed using the OBE method, we conducted a set of dilution experiments with NIST mAb, online buffer exchanged with a P6 column and acquired on an Exactive Plus EMR instrument. **Figure 7a** shows the extracted ion chromatogram of NIST mAb recorded at concentrations of 13  $\mu\text{M}$  down to 53 nM (10  $\mu\text{g}$  - 39 ng loaded onto the column with 5  $\mu\text{L}$  injection volume). The charge states of NIST mAb are still well observable above the noise for the 39 ng injection (**Figure 7b**), with a S/N ratio of  $\sim 8$ . However, we feel that a more reasonable lower bound is approximately 156 ng, which results in a S/N of  $>50$  (**Figure 7c**).



**Figure 7.** Limit of detection for OBE-MS on an EMR mass spectrometer. A dilution series from 10  $\mu\text{g}$  to 39 ng NIST mAb in PBS were injected onto a self-packed P6 column and eluted with 200 mM ammonium acetate. Extracted ion chromatograms (6,400-6,800  $m/z$ ) are shown in **a**). Mass spectra corresponding to 39 ng **b**) and 156 ng **c**) injected NIST mAb demonstrate acceptable signal to noise for OBE-MS even when low ng quantities are analyzed. The y-axis of each spectrum represents relative intensity.

## Data analysis

With a routine data acquisition rate of < 5 min, it is feasible to acquire mass spectra of > 250 samples per 24h instrument run time. Consequently, data analysis often becomes the rate-limiting step for OBE nMS. Many software options are available for deconvolution, analysis, and reporting of data collected using the OBE method. We provide a summary below of the three most commonly used software packages in our laboratory. All three packages allow deconvolution and mass matching of detected species, making them a great option for reporting the protein identify, relative abundance, oligomeric state, heterogeneity, etc. of samples analyzed using the OBE method. A general guidance of their use is given in the **Procedure** section.

Intact Mass by Protein Metrics: Intact Mass is a commercial software that is used for the spectral deconvolution and reporting of intact proteins as well as protein complexes, based on a parsimonious algorithm.<sup>16</sup> We find it particularly suitable for batch deconvolution and reporting of spectra produced by OBE screening. Additionally, Intact Mass can be used with data collected on mass spectrometers from various vendors.

UniDec by the laboratory of Professor Michael Marty: UniDec is a free and open source software suite based on a Bayesian deconvolution algorithm.<sup>17</sup> Deconvolution by UniDec is fast and easily implemented for mass and ion mobility spectra, with a focus on native mass spectrometry data. A recently incorporated module “MetaUniDec” also allows for high-throughput batch deconvolution of mass spectra.<sup>18</sup> UniDec is directly compatible with data collected on Thermo and Waters mass spectrometers, and indirectly compatible with other mass spectrometer brands by first converting the raw data to mzML, or .txt file format.

BioPharma Finder by Thermo Scientific: BioPharma Finder is a software used for the analysis of protein MS data for the characterization of proteins and biotherapeutics. When OBE data is acquired on a Thermo Scientific mass spectrometer, BioPharma Finder can be readily used for deconvolution and reporting of detected species.

## Application of the method:

OBE nMS is particularly suitable for soluble protein and protein complex samples with masses ranging from roughly 10 kDa to 800 kDa (we have not encountered an upper mass limit, but 800 kDa is the largest we have analyzed in our laboratory so far). The main purpose is to allow for rapid buffer exchange of sample aliquots and to obtain information on sample purity and quaternary structure, during or after the protein expression and purification process. Tandem MS and IM can be implemented for complex-down/top-down and collision cross section determination. The rather short time-scale for buffer exchange bears potential for measuring weak biomolecular interactions that would not be retained during size exclusion chromatography.<sup>19</sup> Broader applications may include, but are not limited to, the analysis of RNA, DNA, (metal) cofactor-protein interactions, ligand-protein interactions, protein-nucleic acid interactions and protein-protein interactions. As protein samples in various buffers can be used for OBE nMS, this

method is also useful for testing the effect of small molecules on protein and protein complex (long-term) stability.

### **Comparison with other methods:**

Information on oligomeric state and biomolecular interactions can to some extent be obtained by size exclusion chromatography coupled with either UV detection (SEC-UV) or multiangle light scattering (SEC-MALS).<sup>20</sup> Whereas SEC-UV provides only relative molecular weight information based on the apparent hydrodynamic radius, absolute molecular weights can be determined by SEC-MALS - albeit with relatively low accuracy and at low speed. Furthermore, a main disadvantage of this approach is the inability to determine distinct molecular weights of co-eluting species.

Native mass spectrometry is advantageous due to its ability to differentiate coeluting species and resolve subtle mass differences such as post translational modifications or small ligands.<sup>21</sup> Although several methods have been demonstrated that allow the native MS analysis of samples present in non-volatile buffers, we believe that OBE has advantages in speed, simplicity, and robustness. Whereas proteins can be directly ionized from non-volatile buffers via nano ESI when small diameter tips are used,<sup>22–24</sup> this procedure requires significant expertise and time to pull the proper tips, making it difficult to use as a routine method of analyzing dozens or even hundreds of samples. Additives,<sup>25</sup> electrolytes,<sup>26,27</sup> and supercharging reagents<sup>28</sup> can also help to counteract the effect of non-volatile buffer components on protein spectral quality, but their capability is generally limited to non-volatile concentrations lower than what would be used during protein purification, and the lack of non-volatile removal prior to ionization can increase the frequency of required instrument maintenance. Electrophoresis and dialysis can in principle also be used to remove small ions and small molecules, respectively.<sup>29–32</sup> Compared to OBE-MS, these methods have the clear advantage of a limited dilution of proteins during removal of small molecule non-volatiles. However, incomplete removal of non-volatiles and/or a more challenging technical setup might hamper the widespread use of these methods for online salt/ small molecule removal prior to MS.

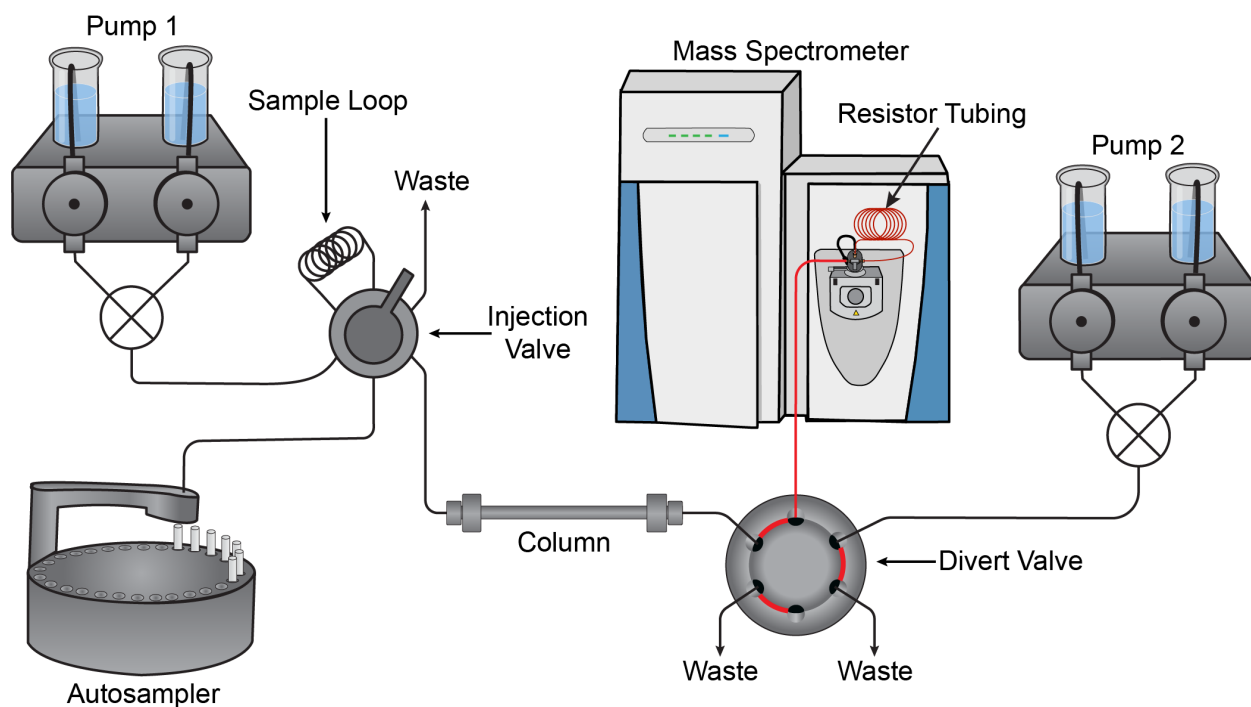
Excellent work has recently demonstrated that intracellular and secreted proteins can be analyzed by native MS after overexpression via a so-called “direct MS” method if nonvolatile molecules are excluded in the resuspension solution and are first removed by washing the cell pellets.<sup>33–36</sup> The direct MS method is tailored for the analysis of cell lysates and supernatants, making it suitable for monitoring protein overexpression. In case additional purification steps are required due to low expression or weak ionization, this method typically cannot be used without a buffer exchange step due to the necessity of introducing non-volatiles (i.e. affinity chromatography requires the elution with a small, non-volatile competitor). The direct MS approach is thus complementary to OBE nMS. The focus of direct MS is on monitoring proteins during expression, whereas OBE nMS is mainly used for pre-purified proteins (albeit it is also feasible to analyze cell lysates as outlined above). In our lab we often use OBE nMS for analysis of pre-purified samples. As after screening by OBE nMS, those samples can subsequently also be used in complementary biophysical characterization experiments as well as more extensive native MS measurements. As an example, we have recently shown that OBE nMS can be used to determine the quality of

samples prior to their usage in mixing and subunit exchange experiments to determine the specificity of protein-protein interactions in complex mixtures by native MS.<sup>10</sup>

### Experimental design:

OBE nMS can be used subsequent or in parallel to additional protein characterization methods. For example, OBE can serve as a rapid method to assess protein identity, purity, oligomeric state, heterogeneity etc. in parallel with techniques such as SDS-PAGE analysis, intact mass analysis, but prior to time consuming techniques or techniques that require a large amount of sample such as NMR spectroscopy or X-ray crystallography (see anticipated results section). OBE can be implemented at the protein expression level to monitor the production of the protein of interest, or it can be used post protein purification to assess protein quality.

In general, protein samples in common biological buffers are centrifuged to remove aggregates and are subsequently transferred into HPLC autosampler vials. Samples can be injected onto either a self-packed or a commercial short SEC column. Analytes are eluted with aqueous ammonium acetate solution. Proteins are directed to the MS, whereas subsequently eluting non-volatile small molecules are diverted to waste (**Figure 8**).



**Figure 8.** Experimental setup for OBE MS. The sample is injected and separated from non-volatile salts by a size exclusion column. The switching valve is used to divert salt to waste and to deliver the analyte toward the MS via a second pump. Note that the initial position of the divert valve is designated by the red lines. The valve is switched to the second position for diversion of non-volatiles to waste.

## Expertise needed to implement the protocol:

Throughout this protocol, it will be assumed that the reader has a general understanding and expertise in mass spectrometry as well as biological and chemical sample handling. Specifically, it is necessary to have expertise operating and tuning a mass spectrometer capable of performing native MS. Basic HPLC experience such as proper care, setup and troubleshooting is also assumed (i.e. degassing mobile phases, purging lines, flushing system post use, etc.). Basic experience in solution preparation, sample handling, compressed gas cylinder handling and safety, and interpretation of protein mass spectra is also assumed. In our experience, a knowledgeable undergraduate or graduate student can successfully and routinely perform this method. The robustness of the method makes it ideal for integration into core facilities as well as analytical divisions in pharmaceutical companies given the availability of a mass spectrometer capable of transmitting and detecting high  $m/z$  ions. OBE can be easily connected and disconnected. We frequently change between OBE and direct infusion nano ESI, requiring only a few minutes for changing the source.

## Limitations:

This protocol is specifically intended for the analysis of soluble proteins and protein complexes. Although they are areas of interest to us, we have not yet developed OBE nMS for the analysis of membrane proteins or nucleotide-protein complexes which would require high amounts of non-volatile detergents and bivalent cations, respectively. It should be noted that the mass spectra obtained by OBE are comparable to those obtained by nanoESI after manual buffer exchange. In other words, OBE is specifically designed to be an automated, fast and efficient way of buffer-exchanging, that will improve spectra quality of samples, where heterogeneity is due to the presence of salt adducts. In contrast, OBE won't improve the spectral quality for samples where heterogeneity is due to the presence of an excess of proteoforms. However, OBE can help to readily identify protein heterogeneity and partial proteolysis and thus provide feedback to guide further optimization of protein expression and purification. Additionally, because OBE does not typically provide separation between proteins present in the sample, ion suppression can become a problem with complex or heterogeneous samples. In such cases, an SEC column that provides separation between proteins would be more beneficial.

## Materials

### Reagents

- Ultrapure water (type 1) generated from a Sartorius Arium Pro water system (or suitable alternative), hereby referred to as "water".
- Ammonium acetate  $\geq 99.99$  trace metals basis (Sigma-Aldrich, cat. No. 431311).
- Methanol, LC-MS grade (Fisher Scientific, cat. No. A456)
- Bovine serum albumin (BSA)  $\geq 96\%$  (Sigma-Aldrich, cat. No. A2153)
- Bio-Rad P6 resin as spin columns or bulk resin (Bio-Rad cat. No. 7326221 or 1504130)

- Cesium iodide (Sigma-Aldrich cat. No. 203033)
- Isopropanol LC-MS grade (Fisher Scientific cat. No. A461)
- Sodium phosphate, dibasic (Sigma-Aldrich cat. No. 04272)
- Potassium phosphate, monobasic (Sigma-Aldrich cat. No. P9791)
- Sodium chloride (Sigma-Aldrich cat. No. S3014)
- Potassium chloride (Sigma-Aldrich cat. No. 60130)
- Hydrochloric acid (Sigma-Aldrich cat. No. H1758)
- Perfluoroheptanoic acid (PFHA) (Sigma-Aldrich cat. No. 342041)
- Sodium bicarbonate (Sigma-Aldrich cat. No. S6014)
- Acetonitrile LC-MS grade (Fisher Scientific cat. No. A955)
- Bio-Rad Protein assay (Bradford reagent; Bio-Rad cat. No. 5000001)
- Pierce BCA Protein Assay Kit (Thermo Fisher cat. No. 23225)
- Qubit Protein Assay Kit (Invitrogen cat. No. Q33211)

## Equipment

- Micropipettes (Eppendorf Research Plus, or similar) and appropriate tips
- Microcentrifuge tubes 1.5 ml (Thermo Scientific cat. No. 3448)
- Microcentrifuge capable of 21,000 xG (Thermo Scientific Sorvall Legend Micro 21 or similar alternative. Refrigerated models are recommended)
- Assortment of volumetric flasks for solution preparation
- Glass bottles for buffers and mobile phases
- Nanodrop 2000c spectrophotometer (Thermo Scientific)
- Qubit fluorometer (Thermo Fisher)
- Glass funnel and filter flask
- PTFE membrane filters 0.2  $\mu$ m (Millipore cat. No. JGWP04700)
- Ultrasonicator for degassing of mobile phases
- PEEK tubing 0.005 in. I.D. (Sigma-Aldrich cat. No. Z227307)
- PEEK tubing 0.03 in. I.D. (Sigma-Aldrich cat. No. Z226955)
- Tubing cutter (Sigma-Aldrich cat. No. 57665-U)
- PEEK finger tight fittings (Upchurch Scientific cat. No. F-120x)
- Precolumn filters (Sigma-Aldrich cat. No. 55215-U)
- Column packing station (Proxeon Biosystems cat. No. SP036) or similar alternative
- Micro stir bar (Fisher Scientific cat. No. 14-513-63SIX)
- Compressed nitrogen cylinder with appropriate gas regulator capable of providing several hundred psi of pressure
- Dual pump HPLC system (Dionex/Thermo Scientific Ultimate 3000 RSLC series or similar)
- Short SEC columns (optional, choose 1): Acclaim SEC-300 4.6 x 33 mm (Thermo Scientific cat. No. 01425030), Acquity UPLC BEH125 4.6 x 30 mm (Waters cat. No. 186006504), or Yarra SEC-3000 2.1 x 50 mm (Phenomenex prototype column).
- Autosampler vials (Waters cat. No. 186000384c, or similar)



- Mass spectrometer capable of high mass-range transmission and detection: we used an Exactive Plus EMR Orbitrap instrument (Thermo Scientific), a Synapt HDMS Q-IM-TOF instrument (Waters) and a Bruker Solarix XR 15T FT-ICR instrument (Bruker).
- 6 port switching valve (Idex part number MXT715)
- pH meter
- Analytical balance

## Software

- Xcalibur Version 3.0 (Thermo Scientific): Used to analyze data recorded on the Exactive Plus EMR instrument.
- MassLynx version 4.1 (Waters): Used to analyze data recorded on the Synapt HDMS instrument.
- Bruker Compass Data analysis version 5.0 (Bruker Daltonics): Used to analyze data recorded on the Solarix XR instrument.
- UniDec version 3.2.0 or newer (<https://github.com/michaelmarty/UniDec/releases>): Deconvolution, data analysis and batch processing of MS data.
- Intact Mass Version 3.1-19 (Protein Metrics): Deconvolution, data analysis and batch processing of MS data.
- BioPharma Finder Version 3.0 (Thermo Scientific): Deconvolution, data analysis and batch processing of MS data.

## Reagent setup

**Ammonium acetate mobile phase** To make 500 mL of a 200 mM ammonium acetate solution, add 7.7 g of ammonium acetate to ~300 mL of water, dissolve, and then bring the final volume to 500 mL with water. Filter into a clean filter flask using a 0.2  $\mu$ m PTFE membrane filter to remove any solids. Store at 4 °C in glass mobile phase bottles for up to two weeks. Degas the mobile phase solution by sonicating uncapped for 15 minutes prior to use.

**Phosphate-buffered saline** To make 1 L of 1x PBS, combine 800 mL water, 8.0 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of sodium phosphate dibasic, and 0.24 g of potassium phosphate monobasic. Adjust to pH 7.4 at room temperature with hydrochloric acid. Adjust to a final volume of 1,000 mL. Store at 4 °C for up to one month.

**CsI calibration solution** To make 5 mL of a 2 mg/mL CsI calibration solution, combine 2.5 mL of isopropanol with 2.5 mL of water. Dissolve 10 mg of CsI in the isopropanol:water solution. For best results the calibration solution should be made fresh daily, or as needed for calibration.

**Perfluoroheptanoic acid (PFHA) calibration solution** To make a 10x stock solution, heat PFHA above its melting point of 54.3 °C and combine 1  $\mu$ L of PFHA with 500  $\mu$ L isopropanol and 300  $\mu$ L of 16.7 mM sodium bicarbonate. The stock solution can be stored at -20 °C for up to a year. To make the PFHA calibration solution, dilute the stock solution 10-fold in a 1:1 isopropanol:acetonitrile solution. The calibration solution should be prepared fresh daily or as needed for calibration.

**Bovine serum albumin (BSA) stock solution** To prepare 1 mL of a 5 mg/mL BSA stock solution, combine 5 mg of BSA with 1 mL of PBS and dissolve. Aliquot and store at -80 °C for up to one year. Prior to use, thaw an aliquot and centrifuge at high speed (~21,000 x G, 4 °C) for 15 minutes to pellet any solids as to avoid column clogging.

***E. coli* Cell lysate sample** Can be prepared by mechanical cell lysis in a physiological buffer after induction and protein (over)-expression. It is advantageous to perform all steps on ice to minimize proteolytic degradation of the proteins and/or protein complexes of interest. Protease inhibitors (i.e. Halt™ Protease and Phosphatase Inhibitor Cocktail, Thermo Fisher) can be added, but care must be taken that those don't lead to artifact formation due to protein binding or covalent protein modification. If the cell lysates are not measured immediately, it is advantageous to flash freeze in liquid N<sub>2</sub> and store at -80 °C.

Cell debris can be removed by centrifugation (21,000 x G, 4 °C) for 15 minutes and the clarified cell lysate can be directly used for OBE nMS.

**Pre-purified protein or protein complex** To prepare a protein or protein complex sample for analysis by OBE nMS, the sample should be centrifuged at high speed to precipitate any solids, and the concentration of the sample should be measured. First centrifuge the sample at high speed (21,000 x G, 4 °C) for 15 minutes and - being careful not to disturb any pelleted precipitate - transfer the supernatant to a clean tube. Next, measure the concentration of the sample using a Nanodrop 2000 spectrophotometer or similar instrument. The concentration of the sample should be adjusted to 1-20 µM protein or protein complex. Higher concentrations might result in partial retention of protein on the column, making it necessary to increase the regeneration time before applying the next sample. Store the sample on ice during preparation and before adding to the autosampler.

## Equipment setup

### Column packing station setup

Fit a high-pressure helium or nitrogen gas cylinder with an appropriate regulator capable of delivering 100-200 psi. Connect the gas regulator to the column packing station ensuring that all valves are safely closed. Clean the glass vial in the column packing station that is used to hold the slurry. Fit the swage fitting on the column packing station lid with an appropriately sized ferrule to fit the outer diameter of the PEEK tubing that will be used for column packing (usually 1/16 inch).

**!Caution** This method uses high pressure gas, we recommend wearing safety glasses, and performing the column packing steps inside of a hood or behind an impact-resistant barrier.

### HPLC setup

This study used a Dionex Ultimate 3000 HPLC equipped with a 5 µL sample loop to deliver sample and mobile phase to the buffer exchange column. Filtered and degassed 200 mM ammonium acetate was used as the mobile phase with a flow rate of 50-100 µL/min. The sample to be

analyzed was loaded into the sample loop and injected using an autosampler by a full-loop method with an overfill factor of 1.2, or via a manual injection valve.

### **Coupling of the buffer exchange column, secondary pump and switching valve to the mass spectrometer**

Connect the buffer exchange column to the switching valve so that flow from the column is directed to the mass spectrometer in position 1 and waste in position 2 (**Figure 8**). Connect a secondary HPLC pump to the switching valve so that its flow of 200 mM ammonium acetate is directed to waste in position 1, and to the mass spectrometer in position 2. This configuration allows the protein of interest eluting from the column to be directed to the mass spectrometer in position 1, and the non-volatile salts eluting from the column to be sent to waste in position 2. Simultaneously, the secondary pump continues delivering the protein of interest through the divert valve to the mass spectrometer in position 2 while the non-volatile salts are being diverted to waste. Note that if a dual pump HPLC is not available, a syringe pump with an appropriately large syringe can be used as the second pump because the pressure requirements are low.

### **Mass spectrometer**

In this study, we used three different mass spectrometers: an Exactive Plus EMR Orbitrap instrument modified with a selection quadrupole and a surface-induced dissociation device,<sup>37</sup> a Synapt HDMS Q-IM-TOF instrument, and a Solarix XR 15T FT-ICR instrument. We chose to use three instruments from different vendors to demonstrate that the OBE method is suitable for coupling with instruments from multiple vendors such as these or others. In each case, the instrument was tuned to maximize desolvation and transmission of the ions of interest. Tune settings for the Exactive Plus EMR instrument are provided in **Table 1**, and tune settings for the Synapt and Solarix instrument can be found in **Table S2**. The Synapt instrument was fitted with a Speedivalve to increase the backing pressure and assist in desolvation and transmission of large  $m/z$  ions as described by Sobott et al.<sup>38</sup> The source regions of all three instruments were tuned to assist with desolvation by adjusting the source temperature, ESI gas, and the in-source collision voltage.

Both the EMR and the Synapt instrument were fitted with a 10 ft x 0.005 in “resistor” tube between the ESI probe and ground to reduce the electrospray current and make it possible to electrospray mobile phases with high ionic strength (**Figure 8**, **Figure S5**).

**Critical:** If a resistor tubing is not used and ammonium acetate levels greater than 20 mM are used as mobile phase, the electrospray current will likely exceed the maximum limit set in the instrument software, resulting in reduced sensitivity or loss of electrospray. The electrospray current as a function of mobile phase ionic strength recorded on an Exactive Plus EMR instrument is shown in **Table S3** for mobile phases up to 2 M ammonium acetate. It should be noted that a 10 ft resistor tubing is generally not necessary and, in most cases, (mobile phase ionic strength < 300 mM) a resistor tube of 2-3 feet should be enough to keep the ESI current below the maximum limit while also reducing the post column dead volume of the system. The Solarix instrument does not require the resistor tubing as the electrospray voltage is applied to the MS inlet rather than the ESI probe.

**Caution** This method uses mass spectrometers with high-voltage electrospray sources. Ensure that the electrospray source is properly grounded. An improperly grounded electrospray

source can result in high voltage being floated on the LC instrument, resulting in an electrical shock.

Table 1. Tune settings for the Exactive Plus EMR	
Setting	Value
Scan range (m/z)	1000-15000
Resolution (at 200 m/z)	17,500
Microscans	2
AGC target	5.00E+05
Max inject (ms)	100
Sheath Gas (psi)	50
Aux Gas (psi)	0
Sweep Gas (psi)	0
Spray Voltage (kV)	3.8
Capillary Temp (°C)	350
S-Lens RF Level (V)	200
In-source dissociation (V)	10
HCD Direct eV (V)	10
AGC Mode	Prescan
Source DC Offset (V)	40
Injection Flatapole DC (V)	13
Inter Flatapole Lens (V)	13
Bent Flatapole DC (V)	4
Trapping Gas Pressure Setting	4

### LC-MS method setup

The LC-MS method timing and acquisition parameters for an OBE experiment using a 12 cm long P6 column are given in [Table 2](#). Note that these parameters and times may need to be optimized depending on the individual equipment setup and column used.

Table 2. LC-MS method timing and parameters (P6 100 µL/min)	
Time (min)	Steps
	0 Start MS acquisition upon injection by LC
1.7	End acquisition (column flushes)
3	End method
Parameter	Value

Flow rate (pump 1 and 2)	100 $\mu$ L/min
Injection volume	5 $\mu$ L
Scan Range	1,000-8,000 or as appropriate for the analyte of interest.

## Procedure

### (Optional) preparation of buffer exchange columns **Timing ~60 min**

1. Obtain a P6 spin column and mix well to obtain a uniform slurry. Alternatively, if using dry P6 resin, add a small amount (approximately 250 mg) to 1.3 mL of water and mix into a uniform slurry.
2. Add 500  $\mu$ L of the P6 slurry to 1.5 mL water in the vial that came with the column packing station (usually a standard HPLC vial).
3. Add a clean micro stir bar to the vial and place the vial in the chamber of the column packing station. Set the stirrer to a medium speed.
4. Cut a piece of 0.03 inch I.D. PEEK tubing to approximately 14 cm and fit it with a finger tight 1/16" male connector and pre-column filter on one end. Ensure that the filter is sufficiently tight that it will not move during the packing process.
5. Place the PEEK tubing (open end first) through the lid of the packing station. Assemble the lid onto the packing station and push the open end of the PEEK tubing down into the vial containing the slurry until it is approximately 3 mm from the bottom (making sure that the stir bar can move freely) **Figure S6a**
6. Tighten the lid to the column packing station and tighten the swage nut to firmly hold the PEEK tubing in place. **Figure S6b**  
**!Caution** ensure that the packing station lid and swage nut are securely tightened prior to opening the gas valve. Failure to securely tighten either part could result in a dangerous release of pressure.  
**Critical step** it is easy to crush thin wall PEEK tubing if the nut is overtightened. Tighten the swage nut so that the tubing cannot be easily removed by hand, but not so tight that the tubing is crushed.
7. Set the pressure regulator to 100-200 psi and slowly open the valve on the column packing station being careful to keep your body and eyes clear of the packing station. Listen and visually inspect for leaks. Proper function will be indicated by a slow drip of solution (about one drop every 5 seconds) from the end of the column.  
**Troubleshooting?**
8. After approximately ten minutes, slowly relieve the pressure and inspect the column and slurry.  
**Critical step** If the slurry has gone dry, you can reform it by adding 1.5 mL of water. The packing process can then be repeated 1-2 more times to ensure that the column is sufficiently

packed. Narrower tubing may take longer packing times, higher packing pressure, or multiple rounds of packing.

9. Trim the open end of the PEEK tubing to approximately 12 cm (length can be adjusted to your preference or application) and fit the open end with a finger tight fitting and precolumn filter.
10. Attach the column to an HPLC and flush with 200 mM ammonium acetate at 50-100  $\mu\text{L}/\text{min}$  for at least 30 minutes. Ensure that the HPLC pressure is stable (likely between 100-200 psi depending on length of column) and not increasing over time.

#### **Troubleshooting?**

11. Inject 5  $\mu\text{L}$  of a 1 mg/mL BSA solution several times onto the column to passivate any sites that may adsorb protein. Flush the column with ammonium acetate for 30 more minutes.
12. When not in use, cap the column ends and store at 4  $^{\circ}\text{C}$ .

### **Estimation of protein concentration (pre-purified protein or protein complex)**

#### **Timing < 5 min per sample**

13. Determine the molecular weight and estimated molar extinction coefficient from the sequence of the protein to be analyzed. Tools such as the ExPaSy ProtParam (<https://web.expasy.org/protparam/>)<sup>39</sup> are useful for determining both of these values.
14. Select the "Protein A280" option on the Nanodrop spectrophotometer and then select the "Other protein (E & MW)" measurement type. Enter the molecular weight and molar extinction coefficient information on the screen.
15. Clean the sample pedestal by applying 2  $\mu\text{L}$  of water to the lower pedestal and lowering the upper pedestal. Ensure that a liquid column forms between the pedestal and let sit for 2-3 minutes. Wipe with a lint free lab wipe.
16. To blank the spectrophotometer, apply 1-2  $\mu\text{L}$  of the sample buffer, lower the pedestal and select the blank option on the screen. After blanking is complete, wipe the pedestal and apply a fresh drop of buffer. Analyze the buffer drop as if it were a sample by choosing the measure button on the screen. If the resulting spectrum has minimal absorbance ( $< 0.04 \text{ A}$ ), the blank was successful. If higher absorbance is observed, re-clean the pedestal and repeat the blanking procedure.
17. To measure the protein concentration of your sample, pipette 1-2  $\mu\text{L}$  of sample onto the lower pedestal and lower the upper pedestal. Ensure that a liquid column has formed successfully between the pedestals. Click the measure button and wait for the measurement to be completed. The resulting concentration value can be converted from mg/ml to molar concentration using the protein molecular weight information. Note: if the monomer molecular weight was used for the measurement of a protein complex, be sure to divide the resulting molar concentration by the oligomeric state of the protein complex to obtain the concentration of protein complex in the sample.

### **Estimation of protein concentration (clarified cell lysate) Timing ~45 min total**

18. The protein concentration of a clarified cell lysate should be determined by a colorimetric assay to prevent interference from other biomolecules (DNA/RNA). Several  $\mu\text{L}$  of clarified cell lysate will be consumed for the measurements.

19. Choose the colorimetric assay based on requirements (sensitivity; compatibility); **Table 3** shows three commonly used assays that are commercially available
20. Prepare a dilution series of BSA or other standard protein like IgG for a working range matching the quantification limit range.
21. Add reagent; dependent on the specific assay used, different incubation times are required for color development. The protein concentration can be determined based on the absorbance relative to that of a standard dilution series.

<b>Table 3: Common colorimetric assays for protein concentration determination</b>		
<b>Assay</b>	<b>Quantitation limit</b>	<b>Main advantage</b>
Bradford	20- 2000 µg/mL	Compatible with reducing agents
BCA	20- 2000 µg/mL	Compatible with detergents
Qubit	0.25 - 5 µg	High sensitivity

## Online Buffer Exchange

Determining switching valve trigger time **Timing 20-30 min**

22. The switching valve method in **Table 4** will serve as a good starting point, however the precise trigger time for the switching valve to divert non-volatile salts to waste will depend on the dead volume of the system, flow rate, as well as the column length and specific retention times. Start by connecting the HPLC, column, switching valve and mass spectrometer as shown in **Figure 8**.
23. With the mass spectrometer set to start acquisition upon injection, inject 5 µL of a 5 µM BSA solution.
24. Observe as the BSA elutes into the mass spectrometer. Stop the acquisition and turn off the electrospray voltage as the salt starts to elute to avoid spraying non-volatile small molecules into the mass spectrometer.
25. Set up a new LC-MS method with the switching valve set to trigger  $\frac{2}{3}$  of the way through the BSA peak from step 3. Note: the precise timing of the switching valve relative to the detection of the BSA peak will depend on the dead volume in the system between the switching valve and the ESI source. With longer “resistor” tubing, the switching valve will need to be triggered earlier relative to the detection time of the BSA peak.
26. Repeat steps 23-25 until the switching time of the divert valve is optimized such that the BSA peak elutes without any non-volatile salt entering the mass spectrometer. Note: if a P6 column is used, the timing of the switching valve should not need to be further modified for different samples as there is no significant separation between different sized proteins (all proteins above 6 kDa are above the exclusion limit). However, if a different column is used, the timing of the divert valve may need to be slightly modified relative to this test with BSA due to differences in protein elution time.

**Critical step** It is important to divert all non-volatiles away from the mass spectrometer (to waste). If non-volatiles enter the mass spectrometer, it can lead to reduced sensitivity, spectral contamination, and extensive down time for instrument cleaning.



## Troubleshooting?

Table 4. Switching valve timing (P6 100 $\mu$ L/min)	
Time (min)	Steps
0	Pump 1: 100 $\mu$ L/min, DV position 1-2 (column to MS), Inject, Start acquisition
0.85	Start Pump 2: 100 $\mu$ L/min
0.9	Switch DV to position 1-6 (column to waste)
1.7	End acquisition
1.8	Stop Pump 2
3	DV to position 1-2 (column to MS), end method

## Screening of proteins, protein complexes and clarified lysates **Timing ~5 min per sample**

27. Adjust all samples to 1-20  $\mu$ M protein or protein complex using the mobile phase buffer, or the buffer that the sample is already in. The lower the concentration that is used, the less carryover and the shorter the amount of time needed for flushing the column between runs.
28. Ensure that the mass range and tune parameters in **Table 1** and **Table S2** are amenable to the samples that will be injected and, if not, adjust.
29. Load samples into LC vials and place in autosampler. If possible, cool the autosampler to 4-8°C whenever samples are present.
30. Setup LC-MS method and switching valve method as in **Table 2** and **Table 4**, add time for flushing of salt to waste between runs (adjust the total method time to be longer if samples are concentrated and more extensive flushing is needed between samples.)
31. Setup the sample sequence and vial position for each sample that needs to be analyzed and run the sequence. Observe the first couple of runs to ensure that the signal is appropriate, the switching valve is diverting salt to waste, and the column is adequately flushed between runs.

## Troubleshooting?

## Data analysis **Timing 10-90 minutes**

### Intact Mass by Protein Metrics

32. Open Intact Mass and select “New Reference Project”.
33. Select and drag the acquired raw files into the sample input screen.
34. If sequences are available for each sample, they can be added under the “protein input screen” by browsing for FASTA files, or by adding a row and importing each sequence manually. Alternatively, if the mass of each sample is known, they can be imported as a csv file along with the protein name under the “sample-protein input” screen (see csv template in **Table S4**). Importing sequences or masses will allow for automatic mass matching and assignment of the deconvoluted signals.

35. Set deconvolution parameters under the “Deconvolution” tab. Specific parameter values will depend on the types of samples being analyzed (mass, charge, resolution, etc.) but a good starting point for all parameters can be found in [Table 5](#).
36. If protein masses or sequences were included, make sure that “reference” is checked under the “Mass Matching” so that deconvolved peaks will be matched to theoretical masses.
37. Check or uncheck common PTMs if you would like them to be considered in the mass matching process.
38. Set the match tolerance to your preferred value. 6 Da is a good starting point for native spectra on a high-resolution instrument, but a larger value may need to be used for data collected on lower resolution instruments.
39. If you wish to calculate the areas of each deconvoluted species, check “compute areas of mass peaks” and set the integration width.
40. If a P6 column was used for the online buffer exchange, all data should have the same elution time. To speed up the deconvolution calculations, under the “sample input” click the TIC button and under “peak smoothing width” choose “disable (single peak)”. This tells the software not to look for multiple peaks in the TIC. Next, under the “Advanced” menu, type the following:  
 [ElutionPeaks]  
 ConstraintStartTime = X.X  
 ConstraintEndTime = X.X  
 where X.X is replaced with the start time and end time of the elution peak in the TIC of all acquisitions. This tells the software to only calculate the data within the specific elution profile selected.
41. Save the reference project by selecting “save preset” and then start the deconvolution by selecting “create”.

### [Troubleshooting?](#)

Table 5. Deconvolution parameters for Intact Mass		
Deconvolution "Basic" Parameters		
Parameter	Value	Notes
Mass Range	10,000-160,000	Adjust to mass range of your samples
<i>m/z</i> range	600-9,000	Adjust to <i>m/z</i> range of your acquisition
Min difference between mass peaks	15 (Da)	
Maximum number of mass peaks	10	Increase if multiple species or proteoforms are present in one spectrum.
Deconvolution "Advanced" Parameters		
Parameter	Value	Notes
Charge vector spacing	0.2	A larger value (1-2) may work better for native MS with broad <i>m/z</i> peaks.
Baseline Radius ( <i>m/z</i> )	15	Controls the stiffness of the baseline. Larger values (100 or more) may be needed for native MS with broad <i>m/z</i>

		peaks.
Smoothing Sigma ( $m/z$ )	0.02	
Spacing ( $m/z$ )	0.04	For native MS, higher values (0.05-0.1) can generally be used and will speed processing time.
Mass Spacing (0.5)	0.5	Controls the spacing of points in the neutral mass spectrum. For spectra without isotopic resolution, a value of 0.2-1 is best for target molecules below 200 kDa. Spacing of 10 or more is best for targets above 300 kDa.
Iteration max	10	
Charge range	3-35	Adjust to include the general charge range of species of interest.

## UniDec

42. Unzip the downloaded UniDec release and open the folder. No installation is needed.
43. Open the UniDec launcher by clicking on GUI UniDec.exe and selecting the UniDec module.
44. Individual spectra can be imported by selecting “open” under the file dropdown menu (x y list, mzML or Thermo Raw format), by selecting “open waters raw file” for Waters data, or by selecting “get spectrum from clipboard” if you have copied the spectrum list.
45. Select “presets” from the file dropdown menu and choose the preset that best matches your collected data (low-resolution native, high-resolution native, isotopic resolution, etc.).
46. Set the  $m/z$  range of interest and select “process data”. Note that additional options (baseline subtraction, and smoothing are available under the data processing tab but generally do not need to be adjusted if the appropriate preset option (step 3) is used.
47. Set the appropriate charge range of all species present in the data (an estimate is okay, just make sure all species fall within the range i.e. make the range wider than you expect).
48. Set the mass range to include the mass of all species possibly present in the data.
49. Select “Run UniDec” to start the deconvolution process. After deconvolution has finished, a mass domain (zero-charge) spectrum is produced, as well as a charge vs.  $m/z$  and charge vs. mass plot. Ensure that the fitted data (shown as red in the original mass spectrum) aligns well with the original data. If it does not, the Peak Width under “Additional Deconvolution Parameters” may need to be adjusted to better fit the data. Alternatively, the peak width tool under the “tools” drop down menu can be used. Inspect the mass domain spectrum and ensure that all species seem reasonable.
50. Set an appropriate peak detection range (width between labeled peaks) and peak detection threshold (the threshold for labeling of peaks as a fraction of the most intense peak), and then select “Peak Detection” to label the calculated species onto the original mass spectrum. Check

that the assignments look appropriate. If the assignments do not look appropriate, it may be necessary to adjust some of the additional deconvolution parameters, however in our experience this is often not necessary.

51. Additional information on peaks can be gathered by clicking the “plot peaks” button, by right clicking peaks in the list, and through various tools in the Analysis menu.
52. Save the processed data figures by selecting “save figure presets” from the file drop down menu.
53. UniDec can also be used for batch processing of spectra by using the import wizard to convert Raw files to HDF5 format and then running UniDec. First open the HDF5 Import Wizard on the UniDec Launcher page.
54. Browse for a folder containing all of your Raw files and select the files to convert by clicking the top file, holding shift, and clicking the bottom file.
55. Select “add” to add all of the files to the bottom screen.
56. Select “Load All to HDF5” and write to an appropriate file location.
57. Open MetaUniDec from the UniDec launcher screen.
58. Select “open” from the file drop down and select the HDF5 file saved in step 13.
59. Repeat steps 45-52 to process the data for all spectra.
60. The deconvoluted data can be saved as figures by selecting “save figure presets” from the file drop down menu.

## BioPharma Finder

61. Open BioPharma Finder and select the Protein Sequence Manager.
62. Select “New” to create a new protein sequence.
63. Provide a name and description for the sequence and select the experiment category it will be used for.
64. The sequence can be manually copied and pasted into the “Manual Input Protein Sequence” section, or it can be imported from a FASTA file by selecting “Import Protein Sequence”.
65. Set any variable modifications that may be present.
66. Save the protein sequence to the sequence manager.
67. Under the Home tab, select “Intact Protein Analysis”.
68. Provide an experiment name, and load one or more Thermo raw data files. If multiple files are loaded, check “batch processing” as the result format.
69. Select the protein sequence(s) that should be considered for identification under the protein sequence menu.
70. Under processing method, select the “Default Native” method and select “Edit Method”.
71. Under chromatogram parameters, set the time, scan range, m/z range, and chromatogram type to be used for the deconvolution.
72. If a P6 column was used for OBE (no separation between proteins), select “Average Over Selected Retention Time” under the Source Spectra Method window, and input the start and end time of the elution peak. If a different column was used that does result in separation between different proteins, the “Sliding Windows” option should be used.
73. Unless all peaks are isotopically resolved, select the ReSpect algorithm.
74. Set the output mass range to an appropriate range for your data.

75. Check “Show Advanced Parameters” and ensure that the “Model Mass Range” and “Charge State Range” are wide enough to contain all species in the data.
76. The Rel. Abundance Threshold and Quality Threshold can be used to clean up noisy data, but by default these are set to 0 and can be adjusted later.
77. Select the “Identification” tab and set the sequence matching mass tolerance if you wish to match sequences to the deconvoluted results.
78. Select the “Report” tab and select the parameters that you wish to be included in the report. For example, figures of the deconvoluted data can be automatically saved in the reports.
79. Select the “Save Method” and name the modified method. Select the Finish button.
80. Navigate back to the “Intact Protein Analysis” tab and with the newly saved method selected, select “Add to Queue” to start the data analysis. Reports will be generated automatically as the data is processed.
81. Results can be viewed by selecting the “Load Results” tab. Each identified species can be viewed and evaluated for each raw file.
82. The results can be saved by selecting “Save Result File As”.

## Troubleshooting

Table 6. Troubleshooting table			
Step	Problem	Possible Reason	Solution
	Column drips too slow or too fast during packing.	Pressure used for column packing is inappropriate for the tubing size of the slurry viscosity.	Adjust pressure until the column drips about once every 5 seconds.
	Pressure on the newly packed column increases over time or is unstable.	Possible that the column bed has not settled, a frit is clogged or that the tubing was crushed during packing.	Reverse the column on the HPLC and pump at a low flow rate. Slowly increase the flow rate and observe if pressure is stable. Although uncommon, it may be necessary to repack the column.
		Possible that the P6 resin has compressed and become unstable.	Repack a column using a lower gas pressure ~100 psi.

	No protein signal on mass spectrometer	Electrospray current has exceeded maximum limit and stopped electrospray.	Most mass spectrometers have a maximum electrospray current programmed into the system. If a high ionic strength mobile phase is used without a proper resistor tubing, the ESI current may exceed the upper limit. Stop the experiment and add a longer piece of resistor tubing to reduce the ESI current.
	Low protein signal on mass spectrometer	High electrospray current leading to reduced sensitivity.	Depending on the ionic strength of the mobile phase, the length of the resistor tubing may need to be optimized for best sensitivity. We have found that a resistor tubing long enough to keep the ESI current below 50 $\mu$ Amps seems to give the best sensitivity.
	High electrospray current	Resistor tube is too short, or not connected properly.	Ensure that the resistor tube is connected between the ESI probe and ground. Use a longer resistor tube.
		Salt buildup on ESI probe tip.	Sometimes if the ESI probe is not flushed sufficiently after use, salts can build up on the tip causing high ESI currents and/or corona discharge. Ensure that the probe is flushed thoroughly with water and then methanol after every use.
	High HPLC pressure	Precipitate from a sample has entered the column, or sample has aggregated on the column.	Sometimes it is possible to resolve this problem by disconnecting the column, reversing the direction and restarting flow at a low flow rate, letting the column exit drip into waste. After sufficient flushing time, reverse the column back to the original direction and continue the experiment. If the problem is not resolved a new column may be necessary.

	Deconvolution artifacts	Analyte does not fall within the set charge or mass range, or advanced parameters are not appropriate for experimental data.	Adjust the charge state range and mass range to include the analyte(s) present in the data. Although uncommon, if artifacts are still present, it may be necessary to change the advanced parameters.
--	-------------------------	--	---

## Timing

- Steps 1-12, (optional, does not need to be performed each time) column packing: 60 min.
- Steps 13-17, estimation of protein concentration (purified proteins): < 5 min. per sample.
- Steps 18-21, estimation of protein concentration (cell lysates): ~ 45 min.
- Steps 22-26, determination of switching valve trigger time: 20-30 min.
- Steps 27-31, screening of proteins: ~ 5 min. per sample.
- Steps 32-82, data analysis (note: not all three software platforms are needed): 10-90 min.

## Anticipated Results

OBE nMS can be readily setup and configured given the availability of an analytical flow LC system and any MS capable of transmitting and detecting high  $m/z$  ions as shown in [Figure 6](#) and [Figure 8](#). Columns for OBE are commercially available but can also be self-packed for a fraction of the cost as shown in the Procedure. High flow rates, relatively inert stationary phases, and ESI provide sufficient robustness to allow for automated, routine measurement of hundreds of samples by native MS. Data generated by OBE nMS can be easily deconvoluted and annotated with software outlined above. OBE nMS can be used for analyzing cell lysates as well as purified proteins and protein complexes.

### Cell lysates

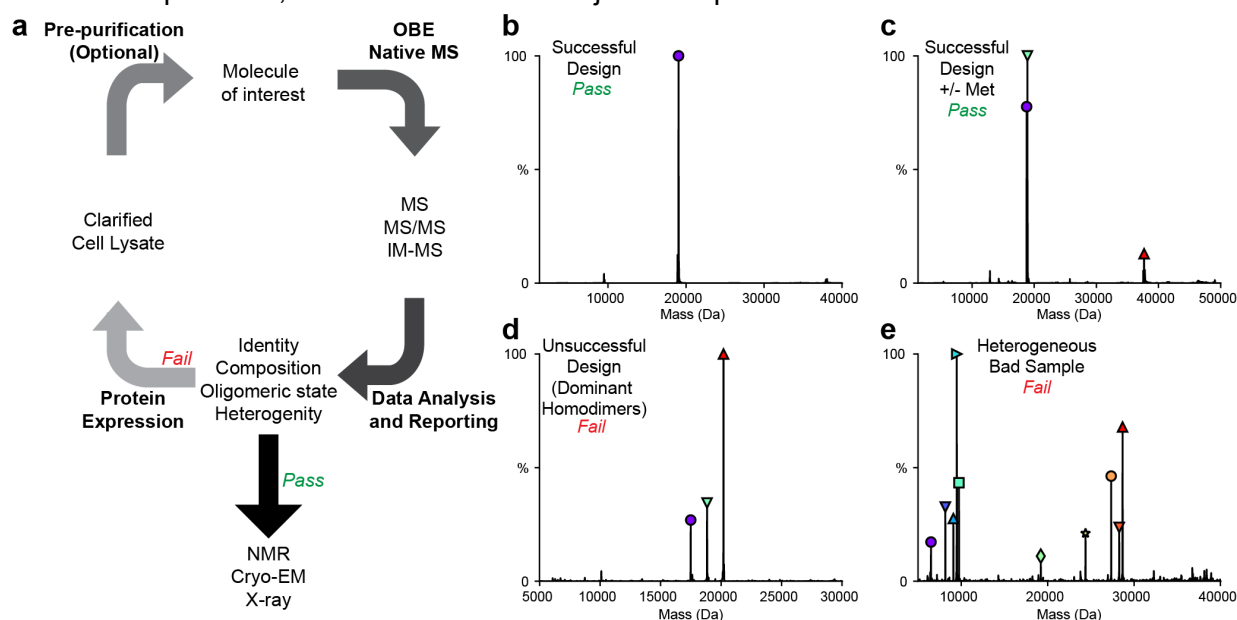
Recent work has demonstrated the use of native MS to directly analyze cell lysates or supernatants to monitor protein expression and biomolecular interactions.<sup>33,34,36</sup> Generally, these methods require washing or buffer exchange steps prior to analysis of the sample by nano ESI. We therefore envision that these “direct MS” methods are complementary to the OBE nMS method as OBE will allow for automated buffer exchange of the cell lysate, bypassing the offline washing and/or buffer exchange steps. The LC ESI-MS based OBE approach is generally less prone to clogging relative to static nanoESI due to the wide tubing and ESI needle diameter as well as the ability to increase the pressure to flush out minor aggregates. If the protein is overexpressed sufficiently, it is reasonable to determine protein identity, molecular weight, stoichiometry, modifications and even top-down sequence information using the OBE nMS method. It should be noted however, that OBE only provides sufficient separation between small molecules and proteins, whereas different proteins within a sample are typically not separated.



Consequently, host cell proteins can interfere with the detection of proteins of interest, if the proteins of interest are not sufficiently overexpressed or do not ionize well. Furthermore, routinely applying complex protein mixtures can decrease the column life due to some extent of protein aggregation and precipitation during exchange to MS compatible solvent. We thus recommend using self-packed columns for this work, if budget is a concern, as they can be easily prepared in larger quantity and changed at very low cost. It might also be necessary to increase the column regeneration time to remove smaller amounts of aggregated protein between runs.

### Purified proteins and protein complexes

Commonly, proteins are expressed and purified in large quantities for subsequent usage, i.e. enzymatic and structural characterization. Frequently used buffers and additives are compatible with OBE nMS, making it possible to measure small sample aliquots without the need of prior buffer exchange. The acceleration in sample analysis can provide valuable feedback that can be used to set up a corresponding workflow (Figure 9). In the illustrated case, we have used OBE nMS to determine the purity and oligomeric state for proteins which were designed to exclusively form heterodimers.<sup>10</sup> Guiding expression optimization and rapidly identifying complex formation and determining oligomeric state resulted in the identification of 94 out of 114 designs that successfully formed the anticipated heterodimer. Importantly, OBE nMS also helped to reevaluate samples just immediately prior to further experiments to ensure that samples were not altered due to storage (i.e. by partial proteolysis). We also used OBE nMS for quality control purposes prior to mixing experiments to determine the specificity of the designed protein-protein interactions and now routinely use this method prior to more time-consuming experiments. We thus also consider OBE nMS to be a very useful tool to help rule out any artifact formation or degradation due to sample storage. In addition to full MS experiments, OBE can be used with MS/MS type experiments as well. In general, completing an MS/MS experiment will involve the same setup as a full MS experiment, with the MS method adjusted to perform MS/MS.



**Figure 9. a)** Flow chart showing the position of rapid OBE native MS in a proposed workflow to accelerate the process from protein expression to structure determination. Online buffer exchange native MS can be

implemented to provide feedback on planning and execution of protein expression to optimize for more time-consuming structural biology characterization methods. **b-e**) Deconvoluted (zero-charge) mass spectra of computationally designed heterodimers screened using OBE. b) and c) are examples of successfully designed heterodimers which display the expected molecular weight except for partial N-terminal methionine cleavage for one of the subunits in c). d) is an example of an unsuccessful design which forms homooligomers as the dominant species. e) is an example of a heterogeneous sample where the expected heterodimer is low abundance relative to the contaminants in the sample. Spectra were deconvoluted using UniDec.

## References

1. Structural Genomics Consortium *et al.* Protein production and purification. *Nat. Methods* **5**, 135–146 (2008).
2. Arnau, J., Lauritzen, C. & Pedersen, J. Cloning strategy, production and purification of proteins with exopeptidase-cleavable His-tags. *Nat. Protoc.* **1**, 2326 (2006).
3. Nallamsetty, S. & Waugh, D. S. A generic protocol for the expression and purification of recombinant proteins in *Escherichia coli* using a combinatorial His<sub>6</sub>-maltose binding protein fusion tag. *Nat. Protoc.* **2**, 383–391 (2007).
4. Bondos, S. E. & Bicknell, A. Detection and prevention of protein aggregation before, during, and after purification. *Anal. Biochem.* **316**, 223–231 (2003).
5. Vagenende, V., Yap, M. G. S. & Trout, B. L. Mechanisms of Protein Stabilization and Prevention of Protein Aggregation by Glycerol. *Biochemistry* **48**, 11084–11096 (2009).
6. Kimple, M. E., Brill, A. L. & Pasker, R. L. Overview of Affinity Tags for Protein Purification. *Curr. Protoc. Protein Sci.* **73**, 9.9.1-9.9.23 (2013).
7. Cavanagh, J., Benson, L. M., Thompson, R. & Naylor, S. In-Line Desalting Mass Spectrometry for the Study of Noncovalent Biological Complexes. *Anal. Chem.* **75**, 3281–3286 (2003).
8. Waitt, G. M., Xu, R., Wisely, G. B. & Williams, J. D. Automated In-Line Gel Filtration for Native State Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **19**, 239–245 (2008).
9. Ehkirch, A. *et al.* An Online Four-Dimensional HIC×SEC-IM×MS Methodology for Proof-of-

- Concept Characterization of Antibody Drug Conjugates. *Anal. Chem.* **90**, 1578–1586 (2018).
10. Chen, Z. *et al.* Programmable design of orthogonal protein heterodimers. *Nature* **565**, 106 (2019).
  11. Fekete, S., Ganzler, K. & Guilleme, D. Critical evaluation of fast size exclusion chromatographic separations of protein aggregates, applying sub-2µm particles. *J. Pharm. Biomed. Anal.* **78–79**, 141–149 (2013).
  12. Muddiman, D. C., Cheng, X., Udseth, H. R. & Smith, R. D. Charge-state reduction with improved signal intensity of oligonucleotides in electrospray ionization mass spectrometry. *J. Am. Soc. Mass Spectrom.* **7**, 697–706 (1996).
  13. Mehmood, S. *et al.* Charge Reduction Stabilizes Intact Membrane Protein Complexes for Mass Spectrometry. *J. Am. Chem. Soc.* **136**, 17010–17012 (2014).
  14. Marcoux, J. *et al.* Native mass spectrometry and ion mobility characterization of trastuzumab emtansine, a lysine-linked antibody drug conjugate. *Protein Sci.* **24**, 1210–1223 (2015).
  15. Li, Y. & Cole, R. B. Shifts in Peptide and Protein Charge State Distributions with Varying Spray Tip Orifice Diameter in Nanoelectrospray Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. *Anal. Chem.* **75**, 5739–5746 (2003).
  16. Bern, M. *et al.* Parsimonious Charge Deconvolution for Native Mass Spectrometry. *J. Proteome Res.* **17**, 1216–1226 (2018).
  17. Marty, M. T. *et al.* Bayesian Deconvolution of Mass and Ion Mobility Spectra: From Binary Interactions to Polydisperse Ensembles. *Anal. Chem.* **87**, 4370–4376 (2015).
  18. Reid, D. J. *et al.* MetaUniDec: High-Throughput Deconvolution of Native Mass Spectra. *J. Am. Soc. Mass Spectrom.* (2018). doi:10.1007/s13361-018-1951-9
  19. Ren, C. *et al.* Quantitative Determination of Protein–Ligand Affinity by Size Exclusion Chromatography Directly Coupled to High-Resolution Native Mass Spectrometry. *Anal.*

*Chem.* **91**, 903–911 (2019).

20. Folta-Stogniew, E. Oligomeric States of Proteins Determined by Size-Exclusion Chromatography Coupled With Light Scattering, Absorbance, and Refractive Index Detectors. in *New and Emerging Proteomic Techniques* (eds. Nedelkov, D. & Nelson, R. W.) 97–112 (Humana Press, 2006). doi:10.1385/1-59745-026-X:97
21. Lössl, P., van de Waterbeemd, M. & Heck, A. J. The diverse and expanding role of mass spectrometry in structural and molecular biology. *EMBO J.* e201694818 (2016). doi:10.15252/embj.201694818
22. Susa, A. C., Xia, Z. & Williams, E. R. Native Mass Spectrometry from Common Buffers with Salts That Mimic the Extracellular Environment. *Angew. Chem. Int. Ed.* (2017). doi:10.1002/anie.201702330
23. Susa, A. C., Xia, Z. & Williams, E. R. Small Emitter Tips for Native Mass Spectrometry of Proteins and Protein Complexes from Nonvolatile Buffers That Mimic the Intracellular Environment. *Anal. Chem.* **89**, 3116–3122 (2017).
24. Nguyen, G. T. H. *et al.* Nanoscale Ion Emitters in Native Mass Spectrometry for Measuring Ligand–Protein Binding Affinities. *ACS Cent. Sci.* (2019). doi:10.1021/acscentsci.8b00787
25. J. Clarke, D. & J. Campopiano, D. Desalting large protein complexes during native electrospray mass spectrometry by addition of amino acids to the working solution. *Analyst* **140**, 2679–2686 (2015).
26. Iavarone, A. T., Udekwu, O. A. & Williams, E. R. Buffer Loading for Counteracting Metal Salt-Induced Signal Suppression in Electrospray Ionization. *Anal. Chem.* **76**, 3944–3950 (2004).
27. Flick, T. G., Cassou, C. A., Chang, T. M. & Williams, E. R. Solution Additives that Desalt Protein Ions in Native Mass Spectrometry. *Anal. Chem.* **84**, 7511–7517 (2012).
28. Cassou, C. A. & Williams, E. R. Desalting protein ions in native mass spectrometry using supercharging reagents. *Analyst* **139**, 4810–4819 (2014).

29. Chen, Y., Mori, M., Pastusek, A. C., Schug, K. A. & Dasgupta, P. K. On-Line Electrodialytic Salt Removal in Electrospray Ionization Mass Spectrometry of Proteins. *Anal. Chem.* **83**, 1015–1021 (2011).
30. Wilson, D. J. & Konermann, L. Ultrarapid Desalting of Protein Solutions for Electrospray Mass Spectrometry in a Microchannel Laminar Flow Device. *Anal. Chem.* **77**, 6887–6894 (2005).
31. Xu, N. *et al.* A Microfabricated Dialysis Device for Sample Cleanup in Electrospray Ionization Mass Spectrometry. *Anal. Chem.* **70**, 3553–3556 (1998).
32. Xiang, F., Lin, Y., Wen, J., Matson, D. W. & Smith, R. D. An Integrated Microfabricated Device for Dual Microdialysis and On-Line ESI-Ion Trap Mass Spectrometry for Analysis of Complex Biological Samples. *Anal. Chem.* **71**, 1485–1490 (1999).
33. Catcott Kalli C., Yan Jing, Qu Wanlu, Wysocki Vicki H. & Zhou Zhaohui Sunny. Identifying Unknown Enzyme–Substrate Pairs from the Cellular Milieu with Native Mass Spectrometry. *ChemBioChem* **18**, 613–617 (2017).
34. Gan, J. *et al.* Native Mass Spectrometry of Recombinant Proteins from Crude Cell Lysates. *Anal. Chem.* **89**, 4398–4404 (2017).
35. Cveticanin, J. *et al.* Estimating Interprotein Pairwise Interaction Energies in Cell Lysates from a Single Native Mass Spectrum. *Anal. Chem.* **90**, 10090–10094 (2018).
36. Ben-Nissan, G. *et al.* Rapid characterization of secreted recombinant proteins by native mass spectrometry. *Commun. Biol.* **1**, 213 (2018).
37. VanAernum, Z. L. *et al.* Surface-Induced Dissociation of Noncovalent Protein Complexes in an Extended Mass Range Orbitrap Mass Spectrometer. *Anal. Chem.* **91**, 3611–3618 (2019).
38. Sobott, F., Hernández, H., McCammon, M. G., Tito, M. A. & Robinson, C. V. A Tandem Mass Spectrometer for Improved Transmission and Analysis of Large Macromolecular Assemblies. *Anal. Chem.* **74**, 1402–1407 (2002).

39. Gasteiger, E. *et al.* ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res.* **31**, 3784–3788 (2003).

## Acknowledgements

The authors wish to thank R. Viner, A. Bailey and T. Zhang (Thermo Fisher Scientific) for assistance with the non-denaturing separations, M. Marty (University of Arizona) for assistance with UniDec, M. Bern and S.J. Skilton (Protein Metrics Inc.) for assistance with Intact Mass software, B. Rivera (Phenomenex) for helpful discussions and prototype Yarra columns, S. Thornton for assistance with figure production and S. Lai for careful proofing. The VP40 plasmid was a generous gift from the Ollmann Sapphire research group (Scripps Research Institute). Work in the Wysocki lab was supported by National Institutes of Health Grant P41 GM128577, Ohio Eminent Scholar funds, and a subaward from University of Washington, Baker lab. Design and preparation of proteins and protein complexes in the Baker lab was supported by the Howard Hughes Medical Institute, and the generosity of Eric and Wendy Schmidt by recommendation of the Schmidt Futures program.

## Author contributions

Z.L.V, F.B, M.J., and V.H.W designed and technically developed the protocol. Z.C., S.E.B. and D.B. provided inspiration for the conception of the protocol and provided valuable ideas and feedback throughout the development and optimization of the protocol. Z.L.V., F.B., B.J.J., M.J. and A.S. performed experiments. Z.L.V. and F.B. wrote the manuscript with assistance from V.H.W. All authors discussed the results and commented on the manuscript. Z.L.V. and F.B. contributed equally to this work.

## Competing interests

The authors declare that they have no competing financial interests.

## Additional information

### Related link

Key references using this protocol:

Chen, Z. *et al.* Programmable design of orthogonal protein heterodimers. *Nature* **565**, 106 (2019).