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# A Direct-Infusion- and HPLC-ESI-Orbitrap-MS Approach for the Characterization of Intact PEGylated Proteins

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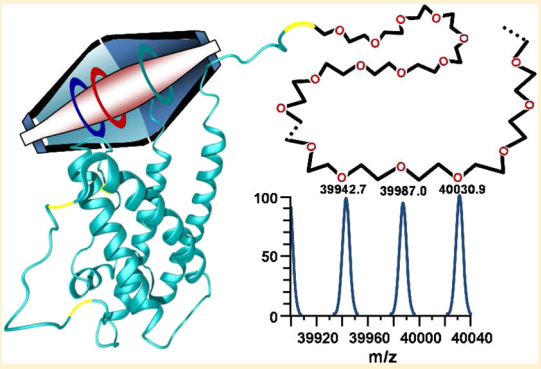
Supporting Information

**ABSTRACT:** The characterization of proteins modified with poly(ethylene glycol) (PEG), such as recombinant human granulocyte-colony stimulating factor (PEGylated rhG-CSF or pegfilgrastim), by electrospray ionization-mass spectrometry (ESI-MS) constitutes a challenge due to the overlapping protein charge state pattern and PEG polydispersity. In order to minimize spectral overlaps, charge reduction by means of the addition of amine was applied. Method development for direct-infusion measurements, carried out on an ESI-time-of-flight (ESI-TOF) instrument, demonstrated the potential of triethylamine (TEA) for shifting the charge state pattern toward lower-charged species and of formic acid (FA) for causing higher charging. After successful method transfer to the LTQ Orbitrap XL instrument, isotopically resolved mass spectra could be acquired. With a median mass accuracy of 1.26 ppm, a number-average monoisotopic molecular mass of 40074.64 Da was determined for pegfilgrastim. The direct comparison of three Orbitrap mass spectrometers, namely the LTQ Orbitrap XL, the Exactive, and the Q Exactive, demonstrated that online interfacing to high performance liquid chromatography (HPLC) was only feasible with the Q Exactive, which offers adequate spectral quality on a time scale compatible with chromatographic separation (i.e., 0.2 min acquisition time per chromatographic peak). Finally, the applicability of both direct-infusion Orbitrap MS and HPLC interfaced to Orbitrap MS was demonstrated for the detection of methionine oxidation in pegfilgrastim. Singly, doubly, and triply oxidized species were readily resolved in the chromatogram, while their oxidation status was easily determined from the mass shifts observed in the deconvoluted mass spectra.




Since their introduction in the 1980s, proteins and peptides have emerged as very efficient drugs in the treatment of many pathophysiological states. The first generation of biopharmaceuticals consisted of "simple replacement proteins" for their natural analogues.[1] However, due to their small molecular size, first generation biopharmaceuticals suffered from problems such as physicochemical instability, short circulating half-life, and immunogenicity.[2] Several strategies emerged in order to circumvent these problems, leading to the second generation of protein biopharmaceuticals, including proteins modified with poly(ethylene glycol) (PEG).[3] The covalent attachment of PEG (PEGylation) is a well-established approach to improve protein stability and solubility, to reduce renal clearance and proteolytic degradation, and to decrease immunogenicity and antigenicity, all of which contribute to an improved clinical efficiency and safety profile.[4] To provide a safe and efficient drug and to meet the regulatory criteria for human use, however, the PEGylated protein needs to be thoroughly characterized.[5]

Quality attributes that need to be monitored are protein sequence, protein integrity, PEG polydispersity, PEGylation site, average length of PEG, and number of attachment sites.[6] Additionally, analytical methods need to be able to characterize degradation products resulting from oxidation or deamidation.[7] Most of the published data available concerning mass spectrometric measurements of PEGylated peptides and proteins were conducted by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).[8] As MALDI-MS provides predominantly singly charged ions, the PEG heterogeneity does not represent a major problem.[9] However,

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the MALDI-MS technology suffers from two major limitations: (1) MALDI-MS measurements are routinely performed on time-of-flight (TOF) analyzers with a limited resolving power of ~60 000 (defined through full width at half-maximum, fwhm),[10] and analyte signals often fall into a higher mass range, in which resolving power of the instrument is lower. (2) MALDI cannot be directly interfaced with separation by high-performance liquid chromatography (HPLC). In electrospray ionization (ESI), on the other hand, a multiplicity of signals is generated because of the presence of ladders of multiply charged protein ions,[11] and the overlaying polydispersity of the attached PEG. This results in spectra containing overlapping signals that are difficult to deconvolute and interpret.[12]

DePEGylation followed by MS analysis[13] as well as charge reduction[14] have been previously described as strategies to tackle PEG polydispersity. DePEGylation is mainly mentioned in the context of PEGylation site mapping but also constitutes a simple measure for reducing the complexity due to the PEG heterogeneity.[15] Although dePEGylation approaches greatly simplify the mass spectrometric measurements, information about PEG chain length, total PEGylated protein mass, as well as PEG size distribution is lost under these conditions.

The spacing in between two neighboring charge states can be increased by shifting the charge-state pattern to a higher mass range through charge reduction, which allows a better resolution both of the charge state pattern and the PEG polydispersity. Several charge reduction methods are documented in the literature,[16,17] although only a few have been applied to PEGylated proteins.[14,18] Bagal et al. utilized superbases such as tetramethyl guanidine or diazabicyclo undecene, introduced via a second electrospray device, to reduce the charge states of PEGylated recombinant human granulocyte colony stimulating factor (rhG-CSF). Comparison of the Gaussian distributions of species related to PEGylated rhG-CSF and chemically oxidized PEGylated rhG-CSF revealed a difference in the intact molecular masses of 16 Da.[18] An easily applicable and promising approach is the postcolumn addition of amines developed by Huang and co-workers. This group investigated the addition of triethylamine (TEA) and diethylmethylamine as well as the effect of amine concentration on the ESI-TOF measurements of PEGylated peptides and proteins. Generally, charge state distributions were shifted to higher $m/z$ upon the addition of amines. Because of the mix of adducts present, it has been stated that deconvolution was not possible with commercially available software.[14]

In this study, we report on measurements of a PEGylated protein using three different mass spectrometers featuring the Orbitrap mass analyzer, specifically a linear ion trap Orbitrap hybrid mass spectrometer (LTQ Orbitrap XL), a single stage Orbitrap mass spectrometer (Exactive), and a quadrupole Orbitrap instrument (Q Exactive), which were previously postulated not to be capable of the measurement even of PEGylated peptides.[19] Using rhG-CSF as a model protein, we investigated the effect of organic solvents, amine and organic acid addition, and instrumental parameters on the charge state distribution, isotopic resolution, mass accuracy, and the representation of PEG polydispersity. The quality and information content of the obtained protein spectra were assessed after deconvolution using commercially available deconvolution software. Furthermore, based on its higher sensitivity and scan speed, the potential of the Q Exactive instrument was evaluated for online interfacing to HPLC. Finally, the performance of both direct-infusion-MS and HPLC-MS for the detection of protein oxidation was demonstrated.

## EXPERIMENTAL SECTION

**Materials.** Methanol (MeOH, LC-MS Chromasolv grade), acetonitrile (ACN ≥ 99.9%), isopropanol (LC-MS Chromasolv grade), formic acid (FA, 98−100%), triethylamine (≥99.5%), hydrogen peroxide ($H_2O_2$, 35.0−36.5%), and ammonium hexafluorophosphate (99.99%) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Millipore water was produced in house by the Milli-Q System (Millipore Corporation, Billerica, USA). The PEGylated rhG-CSF (drug substance, 16.2 mg $mL^{-1}$ solution in 10 mmol $L^{-1}$ acetate, 50 mg $mL^{-1}$ sorbitol, pH 3.8−4.2) was provided by Sandoz GmbH (Kundl, Austria).

**Sample Preparation.** The PEGylated rhG-CSF samples prepared for method optimization on the TOF instrument were dissolved in the indicated MeOH, TEA, and FA concentrations to a final protein concentration of 100 $\mu$g $mL^{-1}$. The Orbitrap MS measurements were conducted with the sample dissolved in 25/75% (v/v) water/MeOH containing 10 mmol $L^{-1}$ TEA and 30 mmol $L^{-1}$ FA to a final protein concentration of 100 $\mu$g $mL^{-1}$.

For the HPLC-postcolumn addition of TEA-ESI-Orbitrap MS measurements, the PEGylated rhG-CSF was dissolved in deionized water to a final concentration of 1 mg $mL^{-1}$. Oxidation was forced by the addition of $H_2O_2$ to a final concentration of 0.5% for 15 min at room temperature. The oxidation process was slowed by storing the sample at 5 °C. For the postcolumn-addition of amines approach, TEA was dissolved in a concentration of 400 mmol $L^{-1}$ in Millipore water. A total of 1 mg $mL^{-1}$ ammonium hexafluorophosphate was prepared in 50% MeOH with the addition of 0.1% FA.

**HPLC Settings.** The separation of oxidation variants was carried out on a capillary/nano HPLC system (UltiMate 3000, Thermo Fisher Scientific, Germering, Germany) using a Discovery BIO wide pore C18 column (150 × 2.1 mm, 3 $\mu$m particle size, Supelco, Bellefonte, PA, USA) at a flow rate of 200 $\mu$L $min^{-1}$ and a column temperature of 50 °C. The gradient of solutions A (50% ACN + 0.1% TFA) and B (95% ACN + 0.1% TFA) was performed as follows: 28% B for 1 min, 28−31% B for 10 min, 31−100% B for 1 min, 100% B for 2 min, 100−28% B for 1 min, and 28% B for 5 min. A partial loop injection of 0.5 $\mu$L with a loop volume of 5 $\mu$L was performed.

**Mass Spectrometry.** For the direct-infusion experiments and for the postcolumn addition, a flow of 5 $\mu$L $min^{-1}$ was provided by a syringe pump (11 Plus, Harvard Apparatus, Holliston, MA, USA) using a 500 $\mu$L syringe (Hamilton, Reno, NV, USA). The TOF mass spectrometer (micrOTOF, Bruker Daltonics, Bremen, Germany) was operated with an ESI source for the direct-infusion method development. The following settings were applied: capillary voltage of −4.5 kV, end plate offset of −500 V, capillary exit of 250.0 V, mass range of $m/z$ 1000 to 8000, dry gas of 3.0 L $min^{-1}$, and drying temperature of 200 °C.

The three different Orbitrap instruments used in this study were a linear ion trap Orbitrap hybrid mass spectrometer (LTQ Orbitrap XL), a single stage Orbitrap mass spectrometer (Exactive), and a quadrupole Orbitrap instrument (Q Exactive), all from Thermo Fisher Scientific in Bremen, Germany. All direct-infusion Orbitrap measurements were carried out by using the Ion Max source from Thermo Fisher Scientific and applying the following parameters: sheath gas flow 15 arbitrary


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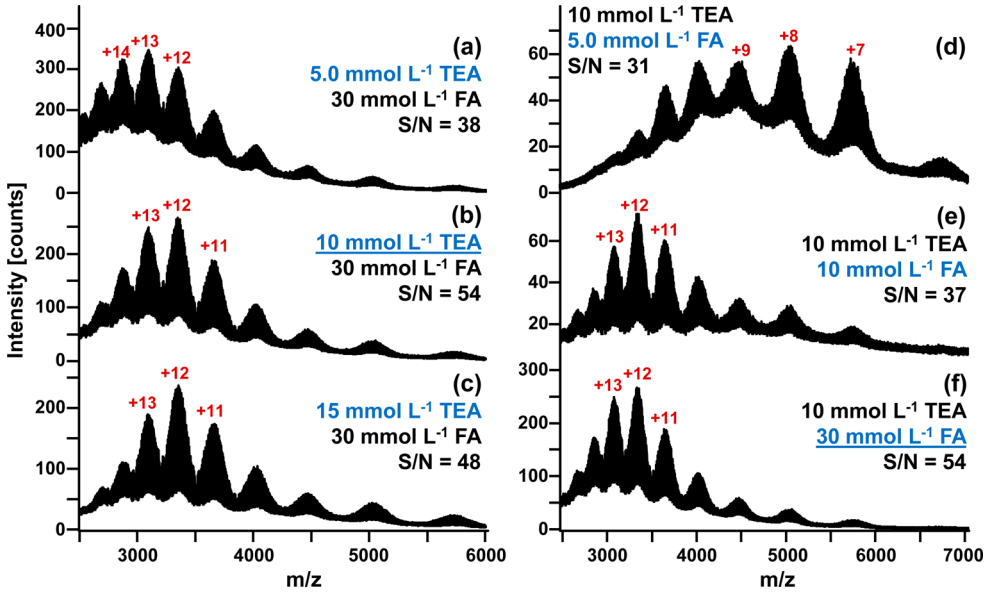


**Figure 1.** Effect of triethylamine and formic acid concentration on charge state distribution and signal intensity. Instrument: micrOTOF; spray solvent, 5.0 mmol $L^{-1}$ TEA/30 mmol $L^{-1}$ FA (a), 10 mmol $L^{-1}$ TEA/30 mmol $L^{-1}$ FA (b), 15 mmol $L^{-1}$ TEA/30 mmol $L^{-1}$ FA (c), 10 mmol $L^{-1}$ TEA/5.0 mmol $L^{-1}$ FA (d), 10 mmol $L^{-1}$ TEA/10 mmol $L^{-1}$ FA (e), 10 mmol $L^{-1}$ TEA/30 mmol $L^{-1}$ FA (f). All samples (100 $\mu$g $mL^{-1}$ rhG-CSF) were dissolved in 75:25 MeOH/water (v/v). The three charge states of the highest intensities are labeled with their charges. The optimized TEA and FA concentrations are underlined. The given S/N were extracted from the deconvoluted spectra.

units, auxiliary gas flow 5 arbitrary units, and capillary temperature of 300 °C. The AGC target was set to 1e6 and the maximum injection time to 50 ms. In the measurements with low resolution settings of 15 000, 10 000, and 17 500, 10 microscans were averaged per scan. In the high resolution measurements with settings of 100 000 and 140 000, one microscan was recorded. Moreover, the detect delay was set to 5 ms on the LTQ Orbitrap XL. For the LTQ Orbitrap XL and the Exactive, a spray voltage of 2.50 kV, a tube lens voltage of 230 V, an in-source fragmentation of 5.0 eV, and a $m/z$ range of 2000 to 4000 were selected. With the Q Exactive system, the spray voltage could be operated at 3.5 kV due to less fragmentation occurring, and the in-source fragmentation needed to be increased to 70.0 eV for proper desolvation. The S-lens RF level was set to 80.0 and the scan range to $m/z$ 2000−6000. The HPLC-postcolumn addition of TEA-ESI-Orbitrap MS measurements was carried out on a Thermo Scientific Q Exactive mass spectrometer that was operated with an ESI source with a heater temperature of 70 °C.

High mass calibration on all instruments was performed using ammonium hexafluorophosphate. High mass calibration was done for the mass range from $m/z$ 2000 to 4000 on the LTQ Orbitrap XL and the Exactive and, on the Q Exactive, up to $m/z$ 6000. The following masses were used for calibration: $m/z$ 2137.01504, 2626.01071, 3115.00637, 3604.00204, and 3929.99915. Additional masses for the Q Exactive mass calibration were $m/z$ 4092.99770, 4581.99337, and 5070.98903.

**Data Analysis.** Spectra recorded with the micrOTOF were deconvoluted with the Bruker Daltonics Data Analysis software, version 4.0 SP 1, using the Maximum Entropy option. The protein mass spectra obtained with the Orbitrap based instruments were deconvoluted by the Thermo Fisher Scientific software Protein Deconvolution 2.0, using the ReSpect algorithm. The following main parameters were applied: charge carrier, $H^+$; $m/z$ range, min. 2000 to max. 6000; output mass range, min. 30 000 to max. 50 000; mass tolerance, 0.05 Da; target mass, 40 000 Da; charge state range, 5 to 20. The advanced parameters applied were the following: minimum peak significance, 2 standard deviations; noise rejection, 68% confidence, no baseline correction; number of iterations, 4; noise compensation, on; minimum adjacent charges, 3 to 5; number of peak models, 1; left/right peak shape, left 2, right 2.

The isotopically resolved data from the LTQ Orbitrap XL measurements were additionally deconvoluted with the Xtract algorithm also implemented in the Protein Deconvolution software. The main parameters applied were the following: resolution at 400 $m/z$, 100 000; S/N threshold, 18; rel. abundance threshold, 0%; $m/z$ range, min. 2000 to max. 4000; charge carrier, $H^+$; minimum number detected charge, 3. The advanced parameters applied were as follows: fit factor, 80%; remainder threshold, 25%, overlaps were considered; charge range, low 5 to high 20; minimum intensity, 1; expected intensity error, 3.

## RESULTS AND DISCUSSION

**Method Development for the Direct-Infusion Approach.** Conventional ESI-MS measurements of PEGylated peptides or proteins result in complex and uninterpretable spectra consisting of the protein charge state pattern with an overlaying PEG polydispersity. For resolving the pattern, the charge states need to be shifted to a higher mass range, thus increasing the distance between two neighboring charge states. All published PEGylated protein spectra acquired on ESI-TOF instruments were within the $m/z$ window of 4000 to 14 000 $m/z$.[14,18] Charge state reduction is also important with Orbitrap instruments in order to shift the charge states into an available mass range of $m/z$ 2000−4000 (LTQ Orbitrap XL, Exactive) and $m/z$ 2000−6000 (Q Exactive). As the Orbitrap-based instruments used in this study have a more limited mass range than TOF instruments, optimization needed to be carried out on a TOF system to monitor the mass shift to the desired $m/z$ range.


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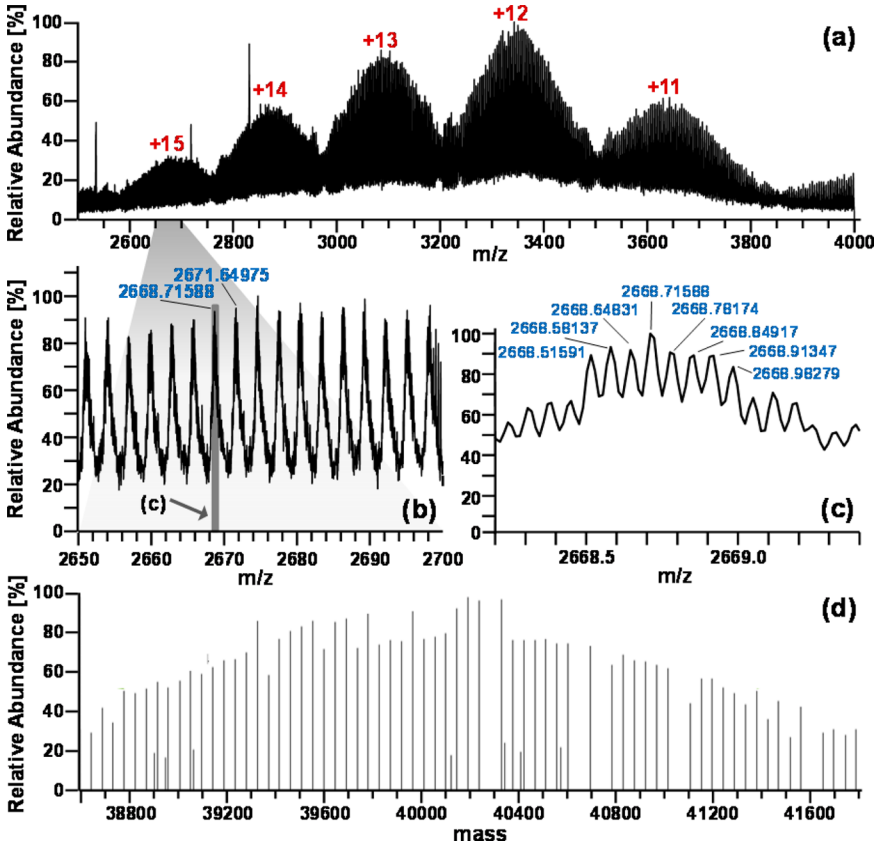


**Figure 2.** Intact measurement of PEGylated rhG-CSF on the LTQ Orbitrap XL. Charge state pattern of the intact PEGylated rhG-CSF (a); zoomed spectrum within charge state +15 (b); further zoom (indicated in b) showing isotopically resolved spectrum (c); deconvoluted spectrum (d). Deconvolution was carried out by applying the Xtract algorithm. Instrument, LTQ Orbitrap XL operated at $R$ = 100 000 at $m/z$ 400; acquisition time, 10 min; sample, 100 $\mu$g mL$^{-1}$ rhG-CSF in 10 mmol L$^{-1}$ TEA/30 mmol L$^{-1}$ FA, 75:25 MeOH/water (v/v).

For this reason, different triethylamine and formic acid concentrations were optimized to shift the charge states of the PEGylated rhG-CSF into the desired range. Acetonitrile, methanol, and isopropanol were tested as potential solvents, and methanol was determined to be the optimal solvent based on mass ranges and signal-to-noise ratios (S/N, data available as Supporting Information, Figure S-1). Zero percent MeOH resulted in an uninterpretable spectrum. The signal intensity of the protein charge state pattern as well as of the deconvoluted spectrum was highest for 25% MeOH and continuously decreased with the lowest values for 100% MeOH, while the noise was also lowest for 100% MeOH (see Figure S-2). A concentration of 75% MeOH was found to be the optimal balance between signal intensity, the $m/z$ 4000 limit, and S/N in the deconvoluted spectrum.

With regard to organic, we observed that an increasing amount of MeOH shifted the charge state pattern toward lower-charged species and at the same time decreased the signal intensity and the noise. This solvent-dependent charge state shift corroborates the previous data of Wang and Cole, who found that solvents of higher polarity were more capable of stabilizing multiply charged species.[20]

The addition of TEA has already been described to be a potent tool for shifting the charge state pattern toward a higher mass range.[14,17] We optimized this method by varying the TEA and FA content in order to shift the charge state pattern into the desired mass range with an $m/z$ cutoff at 4000. Figure 1 shows representative mass spectra which indicate that an increasing amount of TEA results in lower charge states, while an increase in FA concentration yields higher charge states. The optimal TEA concentration was determined by evaluating the S/N in the deconvoluted spectra. The best results were obtained with 10 mmol L$^{-1}$ TEA (see Figure 1b). For the TEA concentration optimization, a constant FA concentration of 30 mmol L$^{-1}$ was used, and for the FA concentration optimization, a constant TEA concentration of 10 mmol L$^{-1}$ was applied. Concentrations of 5, 10, 20, 30, 40, and 50 mmol L$^{-1}$ were evaluated for FA optimization. The addition of 30, 40, and 50 mmol L$^{-1}$ of FA resulted in comparable mass spectra, and no further shift toward higher charge states could be detected (data not shown). The best S/N ratio was achieved with 30 mmol L$^{-1}$ FA (see Figure 1f).

**Method Transfer to Orbitrap Mass Spectrometers.** So far, no intact PEGylated protein measurements acquired on Orbitrap mass spectrometers have been reported in the literature. In fact, suggestions that the C-trap prevents PEGylated proteins from passing through to the Orbitrap mass analyzer can be found.[19] Method transfer from the TOF instrument to the LTQ Orbitrap XL was hampered by the low spectral quality obtained in single scans, which required a preliminary tuning of instrument parameters in the linear ion trap before fine-tuning was accomplished with the Orbitrap mass analyzer. Figure 2a illustrates the protein charge state pattern with the underlying PEG heterogeneity obtained after averaging spectra over an acquisition time of 10 min. As already observed with the TOF instrument, charge states of +15 to +11 were predominant. The different ethylene glycol units can be distinguished in the zoomed spectrum depicted in Figure 2b.


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**Table 1. Instrument Comparison of LTQ Orbitrap XL, Exactive, and Q Exactive at Low and High Resolution Settings for the Specification of PEGylation Polydispersity**

| | | | number of detected PEG variants[c] | | | | | |
|---|---|---|---|---|---|---|---|---|
| | resolution setting[a] | scan rate[b] [Hz] | 0.2 [min] | 0.5 [min] | 1.0 [min] | 5.0 [min] | 10 [min] | 30 [min] |
| LTQ Orbitrap XL | 15 000 (at $m/z$ 400) | 3.3 | 5 | 9 | 21 | 54 | 47 | 36 |
| | 100 000 (at $m/z$ 400) | 0.6 | 44 | 54 | 68 | 75 | 75 | 79 |
| Exactive | 10 000 (at $m/z$ 200) | 10.7 | 31 | 32 | 28 | 28 | 29 | 28 |
| | 10 000 (at $m/z$ 200) | 10.7 | 52[d] | 61[d] | 61[d] | 62[d] | 59[d] | 59[d] |
| | 100 000 (at $m/z$ 200) | 1.2 | 57 | 67 | 73 | 81 | 87 | 83 |
| Q Exactive | 17 500 (at $m/z$ 200) | 12.7 | **78** | 79 | 79 | 79 | 79 | 79 |
| | 140 000 (at $m/z$ 200) | 1.6 | 31 | 43 | 55 | 77 | 67 | **89** |

[a]A resolution setting of 17 500 at $m/z$ 200 is equivalent to 12 500 at $m/z$ 400, one of 140 000 is corresponding to 100 000 at $m/z$ 400.[23] [b]Experimentally evaluated scan rates. [c]Scans were averaged over the given acquisition times and deconvoluted by applying the ReSpect algorithm. The numbers in the table represent the numbers of detected signals in the deconvoluted spectrum (spectra for 5.0 min acquisition are shown in Figure S-6). [d]Low resolution Exactive deconvolution with noise reduction set to off. The visual inspection of the deconvoluted spectrum (Figures S-5 and S-6) confirms that a noise reduction setting of 68% for low resolution Exactive data results in an artificially narrow signal distribution.

By multiplying the spacing in between two neighboring signals with its charge state, the value of one ethylene glycol monomer can be calculated as 44.00805, which compares well to the theoretical monoisotopic mass of 44.02621 Da for $C_2H_4O$. A more detailed view (indicated in Figure 2b) allows determination of the average spacing of the isotope peaks, which resulted in $\Delta m/z$ 0.06669. This value reflects the theoretical spacing of an isotopic cluster in charge state +15, which is $m/z$ 0.06667 (Figure 2c). The drawback of this measurement is the long acquisition time that is needed to obtain good quality spectra. As shown in Figure 2d, some signals of PEGylated protein species are absent in the deconvoluted spectrum, although signals for the corresponding species were clearly visible in the raw spectrum (see Figure 2a and b). This is most probably due to the deconvolution algorithm, for which it is quite difficult to distinguish between real signals and noise because of the very high number of observable signals in very close $m/z$ distances. The deconvolution result strongly depends on the setting of the "S/N threshold" parameter in the program. For this investigation, we were seeking a compromise that yielded the highest number of signals related to PEG variants while keeping noise signals at a minimum, which was a setting of S/N of 18 in our analysis. Therefore, taking the fact into account that chromatographic peaks are approximately 0.2 to 0.5 min in width, the coupling of LTQ Orbitrap XL measurements with chromatographic separation is not feasible at this stage of optimization with this instrument.

For further optimization and interfacing to chromatographic separation, newer generation Orbitrap mass spectrometers, the Exactive and the Q Exactive, which provide higher spectral quality in a single scan and higher scan speed, were evaluated. For a comparison, a 100 $\mu$g mL$^{-1}$ sample of rhG-CSF was directly infused into the mass spectrometers at a flow rate of 5 $\mu$L min$^{-1}$. Spectra were continuously recorded for 30 min, and mass spectral information was evaluated upon averaging spectra over defined time windows.

The instrumental scan rate is an important parameter determining, among other factors, the extent of data obtainable for a complex sample mixture. Because of different physical designs of the ion optics and mass analyzers and changes in the electronics, experimentally observed scan rates differ significantly among instruments, leading to different levels of spectral quality obtainable in a given time window.

The experimentally evaluated scan rates for the LTQ Orbitrap XL range from 0.6 Hz at 100 000 resolution to 3.3 Hz at a 15 000 resolution setting. For the Exactive instrument, scan rates between 1.2 Hz at 100 000 resolution and 10.7 Hz at 10 000 resolution can be obtained, compared to the Q Exactive, which provides scan rates between 1.6 Hz at 140 000 resolution and 12.7 Hz at 17 500 resolution (see Table 1). The different scan rates result from differences in hardware, instrument design, and also software. Hardware differences relate to the design as an ion trap−Orbitrap hybrid instrument (LTQ Orbitrap XL) versus nonhybrid systems (Exactive and Q Exactive) resulting in different places where ions are accumulated in the first instance and differences in length of ion path. In addition, the Q Exactive contains an improved ion optic device in the source region (S-lens), which provides improved ion capture efficiency translating into significantly shorter injection times. In addition, a new preamplifier has been implemented in the Q Exactive allowing for a significant reduction of the FT detect delay, the delay time required for the preamplifier to stabilize before a noise-free signal can be acquired. Thus, spectral quality is greatly improved by allowing the capture of the very first part of the transient[21] that features the highest signal intensity for protein oscillations.[22] In addition, the reduced detect delay allows for the use of a new and improved Fourier-Transformation algorithm, also referred to as Advanced Signal Processing, providing improved resolution by a factor 1.8 to 2. Lastly, ion accumulation in the C-trap is performed in parallel to ion detection in the Orbitrap.[23] On the LTQ Orbitrap XL, a detection delay of 20 ms is implemented as the default setting in the acquisition software. The reduction of the FT detect delay to 5 ms is recommended for intact protein analysis as a good compromise to capture the early onset of the transient and allowing the hardware some time for stabilization. Thus, for all experiments carried out in this study on the LTQ Orbitrap XL instrument, a reduced FT detect delay of 5 ms was used.

Table 1 summarizes the number of PEG variants obtained from the different instruments with data acquisition times and subsequent averaging over a period of time varying from 0.2 to 30 min. Within the time range of 0.2 min, equivalent to an average chromatographic peak width, most information was obtained with the Q Exactive at a resolution of 17 500. Seventy-eight signals were obtained after deconvolution of the spectrum obtained after averaging over 0.2 min, only one short of the 79 signals obtained on the LTQ Orbitrap XL after 30 min. Thus, the Q Exactive instrument operated at a resolution setting of


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**Table 2. Molecular Mass Data of rhG-CSF Including Statistical Data Evaluation**

| parameter[a] | LTQ Orbitrap XL | | | Exactive | | Q Exactive | |
|---|---|---|---|---|---|---|---|
| resolution setting | 15 000 (at $m/z$ 400) | 100 000 (at $m/z$ 400) | 100 000[b] (at $m/z$ 400) | 10 000 (at $m/z$ 200) | 100 000 (at $m/z$ 200) | 17 500 (at $m/z$ 200) | 140 000 (at $m/z$ 200) |
| $M_{\text{average or mono}}$ | 40147.42 | 40147.23 | 40123.53[b] | 40147.32 | 40148.31 | 40147.65 | 40148.01 |
| median $\Delta m/m$ [ppm] | −28.6 | −42.5 | 1.26[b] | −36.9 | −36.5 | −32.2 | −49.6 |
| $M_n$ [Da] | 40116.46 | 40088.26 | 40074.64[b] | 40107.07 | 40038.29 | 40204.96 | 40147.27 |
| $M_m$ [Da] | 40126.85 | 40106.23 | 40087.91[b] | 40117.45 | 40054.40 | 40220.27 | 40164.49 |
| PD | 1.0003 | 1.0004 | 1.0003[b] | 1.0003 | 1.0004 | 1.0004 | 1.0004 |
| $\sigma$ [Da] | 645.42 | 848.87 | 729.25[b] | 343.69 | 803.18 | 784.35 | 831.46 |

[a]Statistical data evaluation was carried out as described elsewhere[27] for the spectra averaged over 5 min. Parameters: $R$, resolution, $M_{\text{average or mono}}$, average or monoisotopic. [b]Molecular mass of the protein molecule comprising the sequence of 175 amino acids (DB00019)[28] plus PEG containing $n$ = 483 ethylene glycol units (theoretical average molecular mass 40 148.7594 Da, theoretical monoisotopic molecular mass 40123.3859 Da); $\Delta m/m$, median mass accuracy; $M_n$, number-average molecular mass; $M_m$, mass-average molecular mass; PD, polydispersity; and $\sigma$, width of Gaussian distribution. For the deconvolution of the Exactive $R$ = 10 000 spectrum, no noise reduction was applied, as discussed with Table 1 and demonstrated in Figure S-6.

17 500 represents the most powerful setup for online interfacing with liquid chromatography.

Visual comparison of the spectra demonstrates the moderate quality of the measurement at a resolution setting of 15 000 with the LTQ Orbitrap XL instrument, while both the Exactive and Q Exactive instruments yielded high-quality spectra at resolution settings of 10 000 and 17 500, respectively, featuring series of multiply charged protein species with fully resolved, underlying PEG heterogeneity (see Figure S-3). Overall, the most detailed picture of the PEG length distribution was accomplished within a 30 min acquisition on the Q Exactive when applying a resolution of 140 000. Unexpectedly, the high resolution acquisitions from the LTQ Orbitrap XL and from the Exactive mass spectrometers yielded more signals representing PEGylated protein species than those acquired at low resolution with the same instruments.

Molecular mass data of intact PEGylated rhG-CSF gained with different instrumental configurations are summarized in Table 2. As an example for one of the most abundant of the investigated PEGylated protein species, we specified the intact molecular mass of the molecule that contains a PEG modification having a degree of polymerization of $n$ = 483. Resolving power of the LTQ Orbitrap XL operated at a resolution setting of 15 000 and of the Exactive and Q Exactive employing both high (100 000 and 140 000) and low resolution (10 000 and 17 500) was insufficient to resolve the isotopic clusters of the protein ions (spectra shown as Figure S-4). Therefore, most of the molecular mass calculations were based on average molecular masses obtained from the envelopes of the isotopic clusters, which led to a systematic underestimation of the average molecular mass,[24] which is also corroborated by the mass deviations listed in Table 2. Nevertheless, resolution of the isotopic clusters with the LTQ Orbitrap XL operated at R = 100 000 (spectrum shown as Figure S-4) facilitated calculation of the monoisotopic mass using the Xtract algorithm as described in ref 25, such that data corresponding to monoisotopic masses can be presented in Table 2 (labeled with an asterisk).

Figure S-7 illustrates boxplots representing the mass accuracies derived from measurements performed with the different instrumental setups. The plotted mass deviations are based on the multiple signals representing PEG polydispersity created upon deconvolution by applying the ReSpect algorithm for calculating average molecular masses or the Xtract algorithm for getting monoisotopic masses, respectively. The obtained medians were in the range of −52.6 to −30.3 ppm, which is a characteristic molecular mass deviation involving average masses, while a significantly smaller median for the mass accuracy (1.26 ppm) was achieved with measurements involving monoisotopic masses. In conclusion, the observed figures of merit demonstrate that the molecular mass of PEGylated proteins can be measured with high confidence. Finally, both the resolution of the isotopic clusters with the LTQ Orbitrap XL and the high mass accuracy achieved in the measurements are clear proof of the absence of amine adducts in the measurements, which contrasts with the findings reported by Huang et al.[14]

Additionally, Table 2 also shows a number of parameters that characterize the polydispersity of the attached PEG molecules, including number- and mass-average molecular mass (for definition of those parameters, see ref 26), polydispersity (PD), and the width of Gaussian distribution of the molecular weights ($\sigma$). The latter parameter shows that the broadest protein molecular mass distribution was revealed with the Q Exactive operated at a resolution setting of 140 000, wherein a standard deviation of 831.46 Da translates into a range of ±19 ethylene glycol units in the attached PEG.

**Direct-Infusion Measurements with a More Complex Sample.** In order to evaluate the performance of direct-infusion Orbitrap MS on a more complex sample, a rhG-CSF solution was chemically oxidized for 15 min at room temperature with hydrogen peroxide at a final concentration of 0.50% and measured on the Q Exactive instrument. Oxidation is a major shelf life limiting factor that is described for rhG-CSF and other biopharmaceuticals. Therefore, the development of analytical methods for monitoring the oxidation state and assigning oxidation sites on the PEGylated rhG-CSF is of high relevance.[29]

The comparison of an unstressed with a stressed sample (Figure 3) allowed the determination of the number of oxidations occurring on the basis of the deconvoluted data. The unstressed sample mainly contained nonoxidized species (see Figure 3a). Close inspection of both the raw and the deconvoluted spectra from the unstressed sample revealed that the smaller signals of 10−20% relative intensity were most probably artifacts of deconvolution, as signals in the raw spectrum were only poorly defined and the signals could not be assigned to any known modified or truncated protein sequence. The most abundant signal in the stressed sample was observed for the singly oxidized variant, but doubly oxidized variants


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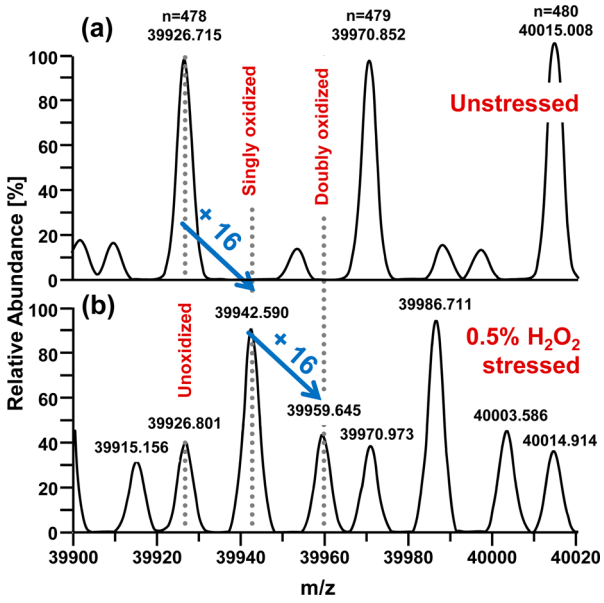


**Figure 3.** Direct-infusion ESI-Q Exactive Orbitrap MS for distinguishing oxidation variants of PEGylated rhG-CSF. Zoomed deconvoluted mass spectrum of nonstressed PEGylated rhG-CSF (a) and zoomed deconvoluted mass spectrum of 0.5% $H_2O_2$ stressed PEGylated rhG-CSF (b). Measurements were conducted on the Q Exactive with a resolution of 70 000. For data evaluation, spectra acquired over a period of 5 min of acquisition were averaged, and deconvolution was carried out as described in the Experimental Section.

were also observed (see Figure 3b). Nevertheless, this measurement is not capable of distinguishing the exact position of oxidation but is a very powerful tool for rough protein characterization and estimation of the degree of oxidation. For obtaining information about the sites of oxidation, either gas-phase fragmentation or chromatographic or electrophoretic separations have to be performed before mass spectrometric investigation.

**Chromatographic Separation of Oxidized Variants and Mass Spectrometric Determination of the Degree of Oxidation in Intact PEGylated rhG-CSF.** Having developed a suitable method for the mass spectrometric detection of PEGylated rhG-CSF, the final logical step toward in-depth characterization of the target product and its variants or impurities was the interfacing of MS to an efficient HPLC-based separation system. Because of its high resolving power for proteins differing in their hydrophobicity, ion-pair reversed-phase HPLC employing a nonpolar octadecyl stationary phase and a gradient of acetonitrile in aqueous trifluoroacetic acid were considered appropriate for the separation of oxidized variants. As optimized previously for direct infusion experiments, we utilized postcolumn addition of TEA through a T-piece for decharging and shifting the charge state pattern into an appropriate mass range.

Figure 4 shows the chromatographic separation of the protein species derived from rhG-CSF upon oxidative stress. It can be seen that four different variants are efficiently separated almost to the baseline. The spectrum of the singly oxidized variant eluting at the end of the chromatogram is presented in Figure 4b, while Figure 4c shows zoom-ins of the +10 charge states of all observed protein species.

Figure 5 illustrates further steps in data analysis upon deconvolution of the raw spectra, and Figure 5b–d show zoom-ins into the deconvoluted spectra extracted for each of the four peaks in the chromatogram. The multiple signals (three variants are seen in the zoomed spectra) clearly reflect the PEG polydispersity, while mass shifts between the spectra are due to

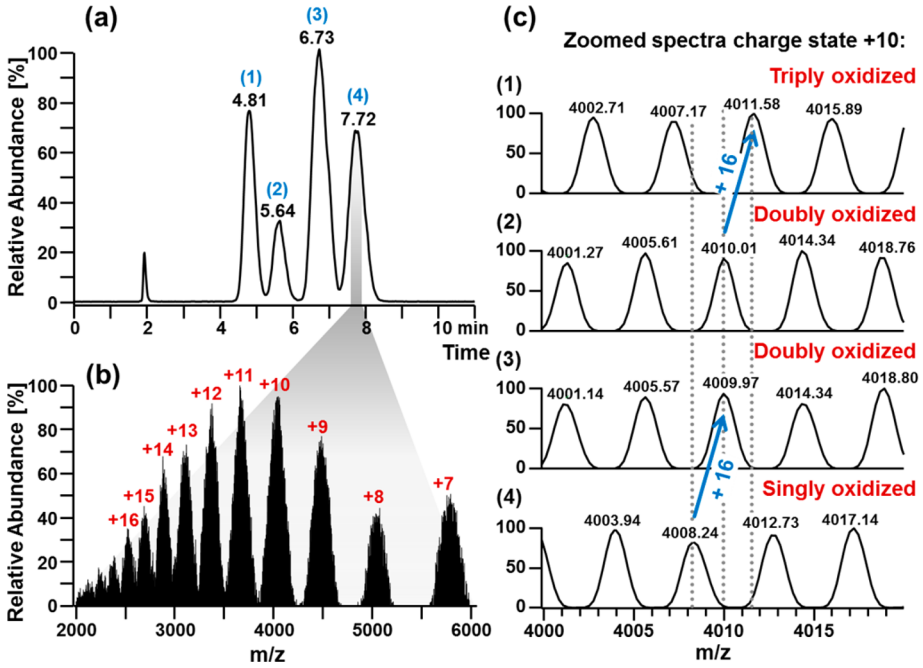


**Figure 4.** Ion-pair reversed-phase HPLC separation Q Exactive MS analysis of PEGylated and oxidized protein variants. Reconstructed total ion current chromatogram (a), extracted mass spectrum of peak 4 (b), zoomed mass spectra of peaks 1–4 showing the +10 charge state (c). Column, Discovery BIO wide pore C18 (150 × 2.1 mm); gradient, 62.6% acetonitrile for 1 min, 62.6–64% acetonitrile in 0.10% TFA in 10 min, 200 $\mu$L $min^{-1}$; temperature, 50 °C; postcolumn solvent, 400 mmol $L^{-1}$ TEA in water, 5 $\mu$L $min^{-1}$; sample, 1 mg $mL^{-1}$ PEGylated rhG-CSF stressed with 0.5% $H_2O_2$ for 15 min at room temperature; injection volume, 0.50 $\mu$L; detection, Q Exactive MS at $R$ = 17 500.


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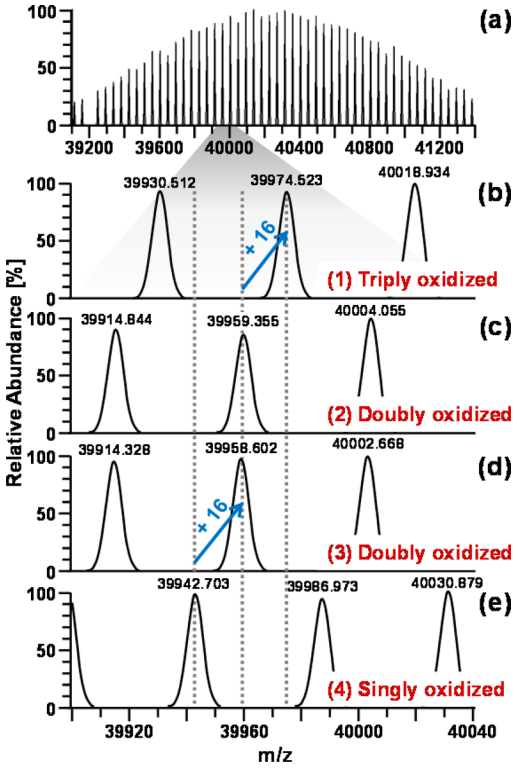


**Figure 5.** Deconvoluted spectra of oxidized and PEGylated rhG-CSF. Deconvoluted spectrum that corresponds to peak 1 (a) and zoomed deconvoluted spectra corresponding to peak 1 (b), peak 2 (c), peak 3 (d), and peak 4 (e) of the chromatographic run shown in Figure 4a. Mass differences between the peaks correspond to one ethylene glycol unit. Experimental conditions as in Figure 4.

oxidation in the protein sequence. The assignment of the oxidation state was carried out by comparison with the theoretical intact mass and by monitoring the mass shift of the different variants with respect to the unstressed PEGylated rhG-CSF. The discrimination of oxidation sites can be accomplished with both the nondeconvoluted (see Figure 4c) and the deconvoluted (see Figure 5b–e) spectra. Both spectra reveal that peak 1 consists of a triply oxidized variant, peaks 2 and 3 are comprised of doubly oxidized variants, and peak 4 represents the singly oxidized variant. Additionally, separation within the peaks occurred on the basis of the length of the PEG moiety attached (see Figure S-8). This elution order is fully consistent with the expected decrease in hydrophobicity of a protein upon oxidation.

Oxidation predominantly occurred on the sulfur-containing amino acids, whereas for rhG-CSF only methionine oxidation has been described.[7] rhG-CSF consists of 175 amino acids, of which four are methionines. The PEG chain is attached via reductive amination at the N-terminal methionine (Met 1),[30] which is known to be the most prone to oxidation. Met 122 is located in one of the four helices within the hydrophobic core of the protein and is described as the least accessible. Both Met 127 and Met 138 are located inside one of the loops and are considered to be moderately accessible.[31,32] Holzmann and co-workers performed direct oxidation site mapping on the intact non-PEGylated rhG-CSF via top-down MS using an Exactive mass spectrometer. Using a system very similar to that described in this work, they obtained a comparable chromatographic pattern to that shown in Figure 4 upon $H_2O_2$ treatment of rhG-CSF and subsequent separation and oxidation site mapping.[29] Therefore, we may argue that the location of oxidations in our sample is analogous to the oxidation sites determined in the non-PEGylated protein. This comparison leads us to the conclusion that single oxidation occurred at Met 1 (peak 4 in Figure 4), double oxidation at Met 1 and Met 138 or at Met 1 and Met 127 (peaks 3 and 2), and triple oxidation took place at Met 1, Met 127, and Met 138 (peak 1). The feasibility of oxidation site mapping of PEGylated rhG-CSF by top-down analysis using online HPLC and Q Exactive MS is beyond the scope of this work and will be the topic of a future study.

## CONCLUSION

In this study, we present for the first time a practical approach for the characterization of PEG heterogeneity of a therapeutic protein, usable with Orbitrap mass analyzers. As already demonstrated for TOF analyzers, the addition of triethylamine is highly useful for reducing the charge states of the analyzed proteins and for shifting the charge state pattern into an $m/z$ range applicable with the Orbitrap mass analyzers. The performance of different instruments employing Orbitrap mass analyzers was found to differ significantly with respect to scan speed, detectability of PEGylated variants, capability to resolve isotope clusters in the 40 kDa protein, and the feasibility of interfacing to HPLC. The heterogeneity introduced upon modification of the protein with PEG is readily revealed by the multiplicity of signals observable in the deconvoluted spectra.

Isotopic clusters of the +11 to +15 charge states of proteins can be well resolved on a LTQ Orbitrap XL mass spectrometer, which allows the determination of intact protein molecular mass with mass accuracies in the lower ppm range. Because of its slow scan speed, however, LTQ Orbitrap XL measurement is incompatible for intact PEGylated protein mass measurements with preceding chromatographic separation. Because of a lack of isotope resolution, only average molecular masses were obtained from measurements of the 40 kDa PEGylated protein on the Exactive and Q Exactive at the cost of a significant loss in mass accuracy. Owing to its faster scan speed and improved data processing, measurements with the Q Exactive can be efficiently interfaced with online chromatographic separation using ion-pair reversed-phase HPLC. Both direct-infusion MS and online coupled HPLC-MS are shown to facilitate the analysis and distinction of oxidized protein variants.

## ASSOCIATED CONTENT

### Supporting Information

Figures covering method development at the micrOTOF instruments as well as additional figures supporting the Orbitrap instrument comparison. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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## ■ REFERENCES

(1) Walsh, G. *Eur. J. Pharm. Biopharm.* **2004**, *58*, 185−96.
(2) Roberts, M. J.; Bentley, M. D.; Harris, J. M. *Adv. Drug Delivery Rev.* **2002**, *54*, 459−76.
(3) Harris, J. M.; Chess, R. B. *Nat. Rev. Drug Discovery* **2003**, *2*, 214−21.
(4) Caliceti, P.; Veronese, F. M. *Adv. Drug Delivery Rev.* **2003**, *55*, 1261−77.
(5) International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Topic Q 6 B. *Fed. Regist.* **1999**, *64*, 44928−35.
(6) Abzalimov, R. R.; Frimpong, A.; Kaltashov, I. A. *Int. J. Mass Spectrom.* **2012**, *312*, 135−143.
(7) Wang, W. *Int. J. Pharm.* **1999**, *185*, 129−88.
(8) Foser, S.; Schacher, A.; Weyer, K. A.; Brugger, D.; Dietel, E.; Marti, S.; Schreitmüller, T. *Protein Expr. Purif.* **2003**, *30*, 78−87. Lee, H.; Park, G. *J. Pharm. Sci.* **2002**, *92*, 97−103. Lee, K. C.; Moon, S. C.; Park, M. O.; Lee, J. T.; Na, D. H.; Yoo, S. D.; Lee, H. S.; DeLuca, P. P. *Pharm. Res.* **1999**, *16*, 813−8.
(9) Nielen, M. W. F. *Mass Spectrom. Rev.* **1999**, *18*, 35.
(10) Gelpi, E. *J. Mass Spectrom.* **2009**, *44*, 1137−61. Darwin, A. Bruker Drives Ultrahigh-Resolution Qq-TOF Mass Spectrometry to New Performance Levels with Major Innovations in maxis(TM) Platform to be Showcased at ASMS 2011; Bruker Corporation: Madison, WI, 2011.
(11) Mann, M.; Meng, C. K.; Fenn, J. B. *Anal. Chem.* **1989**, *61*, 1702−1708.
(12) Trimpin, S.; Plasencia, M.; Isailovic, D.; Clemmer, D. E. *Anal. Chem.* **2007**, *79*, 7965−74.
(13) Vestling, M. M.; Murphy, C. M.; Keller, D. A.; Fenselau, C.; Dedinas, J.; Ladd, D. L.; Olsen, M. A. *Drug Metab. Dispos.* **1993**, *21*, 911−7.
(14) Huang, L. H.; Gough, P. C.; DeFelippis, M. R. *Anal. Chem.* **2009**, *81*, 567−577.
(15) Veronese, F. M.; Sacca, B.; Polverino de Laureto, P.; Sergi, M.; Caliceti, P.; Schiavon, O.; Orsolini, P. *Bioconjugate Chem.* **2001**, *12*, 62−70. Lu, X. J.; Gough, P. C.; DeFelippis, M. R.; Huang, L. H. *J. Am. Soc. Mass Spectrom.* **2010**, *21*, 810−818.
(16) Ebeling, D. D.; Westphall, M. S.; Scalf, M.; Smith, L. M. *Anal. Chem.* **2000**, *72*, 5158−61. Lennon, J. D., 3rd; Cole, S. P.; Glish, G. L. *Anal. Chem.* **2006**, *78*, 8472−6. Malmstrom, J. *Anal. Bioanal. Chem.* **2012**, *403*, 1167−1177. Chakraborty, A. B.; Chen, W.; Gebler, J. C. *Pharm. Technol.* **2008**, *32*, 80−87.
(17) Nasioudis, A.; van Velde, J. W.; Heeren, R. M.; van den Brink, O. F. *Int. J. Mass Spectrom.* **2011**, *303*, 6.
(18) Bagal, D.; Zhang, H.; Schnier, P. D. *Anal. Chem.* **2008**, *80*, 2408−2418.
(19) Yoo, C.; Suckau, D.; Sauerland, V.; Ronk, M.; Ma, M. H. *J. Am. Soc. Mass Spectrom.* **2009**, *20*, 326−333.
(20) Wang, G.; Cole, R. B. *Am. Soc. Mass Spectrom.* **1996**, *7*, 9.
(21) Michalski, A.; Damoc, E.; Lange, O.; Denisov, E.; Nolting, D.; Müller, M.; Viner, R.; Schwartz, J.; Remes, P.; Belford, M.; Dunyach, J. J.; Cox, J.; Horning, S.; Mann, M.; Makarov, A. *Mol. Cell Proteomics* **2012**, *11*, O111 013698.
(22) Makarov, A.; Denisov, E. *J. Am. Soc. Mass Spectrom.* **2009**, *20*, 1486−95.
(23) Michalski, A.; Damoc, E.; Hauschild, J. P.; Lange, O.; Wieghaus, A.; Makarov, A.; Nagaraj, N.; Cox, J.; Mann, M.; Horning, S. *Mol. Cell Proteomics* **2011**, *10*, M111 011015.
(24) Zubarev, R. A.; Demirev, P. A.; Hakansson, P.; Sundqvist, B. U. R. *Anal. Chem.* **1995**, *67*, 3793−3798.
(25) Senko, M. W.; Beu, S. C.; McLafferty, F. W. *J. Am. Soc. Mass Spectrom.* **1995**, *6*, 229−233.
(26) Poole, C. F. *The Essence of Chromatography*, 1st ed.; Elsevier: Amsterdam, 2003, pp ix, 925.
(27) Robinson, E. W.; Garcia, D. E.; Leib, R. D.; Williams, E. R. *Anal. Chem.* **2006**, *78*, 2190−8.
(28) Wishart, D. S.; Knox, C.; Guo, A. C.; Cheng, D.; Shrivastava, S.; Tzur, D.; Gautam, B.; Hassanali, M. *Nucleic Acids Res.* **2008**, *36*, D901−6.
(29) Holzmann, J.; Hausberger, A.; Rupprechter, A.; Toll, H. *Anal. Bioanal. Chem.* **2013**, *405*, 6667−6674.
(30) Kinstler, O. B.; Brems, D. N.; Lauren, S. L.; Paige, A. G.; Hamburger, J. B.; Treuheit, M. J. *Pharm. Res.* **1996**, *13*, 996−1002.
(31) Pan, B.; Abel, J.; Ricci, M. S.; Brems, D. N.; Wang, D. I.; Trout, B. L. *Biochemistry (Moscow)* **2006**, *45*, 15430−43.
(32) Lu, H. S.; Fausset, P. R.; Narhi, L. O.; Horan, T.; Shinagawa, K.; Shimamoto, G.; Boone, T. C. *Arch. Biochem. Biophys.* **1999**, *362*, 1−11.