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# Structural characterization of protein–polymer conjugates. I. Assessing heterogeneity of a small PEGylated protein and mapping conjugation sites using ion exchange chromatography and top-down tandem mass spectrometry

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ABSTRACT

PEGylated proteins are a rapidly growing class of biopharmaceutical products, but their analytical characterization remains a formidable problem due to the extreme heterogeneity of these species. While significant advances have been made in recent years in this field due to integration of mass spectrometry in the analytical workflow, quick identification of PEGylation sites remains an unmet goal, particularly if several isoforms of the protein–polymer conjugate are present in the sample. To achieve this objective, a new method is developed, which utilizes a combination of ion exchange chromatography and top-down mass spectrometry consisting of two consecutive fragmentation steps ($MS^3$) to identify various conjugates. The method is tested with a complex mixture of products of ubiquitin conjugation with 5 kDa PEG.



## 1. Introduction

PEGylated proteins are a rapidly growing class of biopharmaceutical products, which often demonstrate improved therapeutic properties due to their enhanced resistance to proteolytic degradation, lower rates of renal clearance resulting in increased circulation lifetimes, and at least in some cases lower incidence of immunogenic response leading to better safety profiles [1]. While it is widely expected that the number of PEGylated therapeutic proteins will continue to expand in the near future and beyond, rapid proliferation of these biopharmaceuticals poses significant challenges *vis-à-vis* analytical characterization. PEGylated proteins are highly complex species, where structural heterogeneity is present at different levels. Indeed, various protein isoforms may differ from each other by (i) the number of PEG chains attached to a single polypeptide chain, (ii) location of the conjugation sites, and (iii) lengths of PEG chains. Protein separation methods, particularly size exclusion chromatography (SEC), in most cases can easily resolve isoforms of the first type based on significant differences in their physical size in solution. The extent of heterogeneity associated with inhomogeneity of PEG chain length can be visualized and evaluated by MALDI MS. However, it is the heterogeneity of the second type (protein–polymer conjugates differing in position of PEGylation sites in the polypeptide sequence) that is usually most difficult to evaluate. While significant advances have been made in recent years to address this problem, quick identification of PEGylation sites frequently remains an unmet goal, particularly if several isoforms of the protein–polymer conjugate are present in the sample.

A pioneering work of Fenselau and co-authors nearly twenty years ago opened the way for mapping the PEGylation sites in therapeutic proteins by introducing an experimental strategy that combines chemical treatment of the PEG chain, proteolysis and peptide fragment identification using fast atom bombardment (FAB) MS [2]. In this approach base treatment of PEGylated protein superoxide dismutase (conjugated via a succinyl bridge) removed the PEG chain, but left a succinyl group on the polypeptide chain as a marker of the PEGylation site, which was then localized by examining the protein tryptic map. A similar approach to mapping the PEGylation sites was used by Veronese et al., who employed new PEG derivatives, which could be easily removed by cyanogen bromide treatment leaving “placeholder” tags on the polypeptide chain [3]. An extension of this strategy employs monodisperse PEG chains, whose position within the polypeptide side chain can be determined directly by MS [4]. Although in some cases peptide mapping alone may provide information on the PEGylation sites within protein–polymer conjugates [5,6], incorporation of peptide

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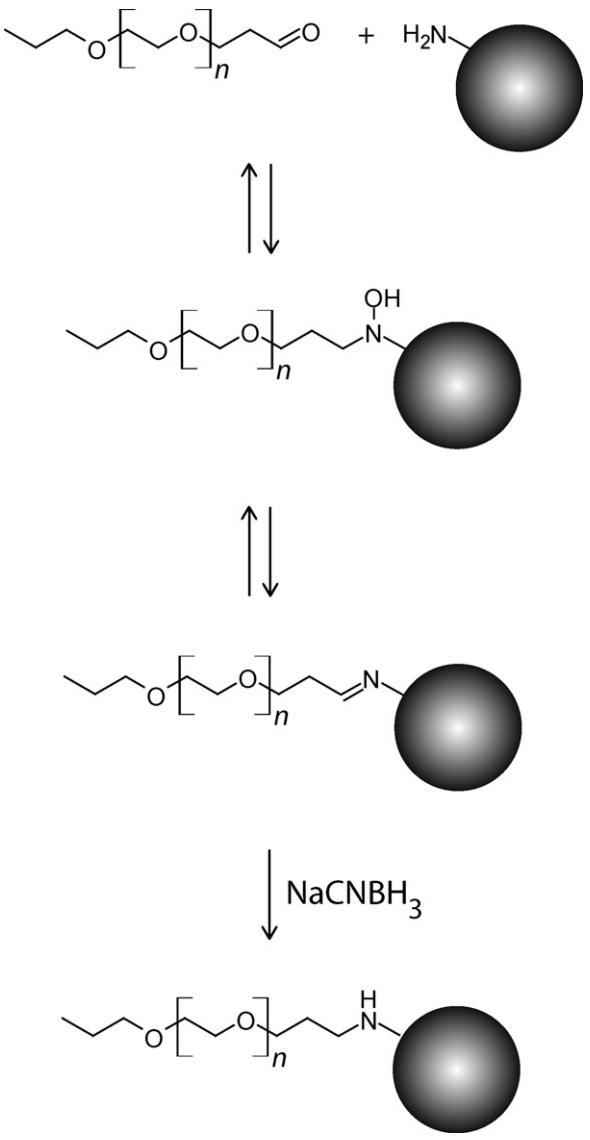
ion fragmentation into the analytical workflow [7] or selective enrichment of PEGylated peptides prior to MS analysis [8] have been shown to be advantageous.

Development of the top-down methods of protein ion fragmentation and their rapid proliferation during the past decade led to a dramatic expansion of the capabilities of analytical mass spectrometry in structural characterization of proteins [9]. Protein modification analysis was one of the areas where top-down methods made a significant impact [10], although until recently the scope of inquiry was limited to mapping sites of enzymatic post-translational modifications (PTM). The few examples of successful applications of top-down MS to localizing non-enzymatic chemical modifications have been focused on relatively small modifications of polypeptide chains, such as protein–drug conjugates [11] or oxidation [12].

The initial reports on utilizing top-down MS for mapping conjugation sites in PEGylated proteins used MALDI ionization followed by in-source fragmentation [13], and a combination of collision-activated dissociation (CAD) in ESI interface followed by a second stage fragmentation using electron-capture dissociation, ECD [14]. The latter approach gives an additional advantage of reducing the complexity of the ion population during the initial fragmentation stage by eliminating PEG chain from the protein while leaving a small "placeholder tag" at the conjugation site, which is identified by the second fragmentation step [14]. A similar approach was recently applied to identify the conjugation site on a relatively small polypeptide (glucagon), although its application to a larger protein system (light chain of a monoclonal antibody) still required utilization of a proteolytic step prior to MS/MS [15].

The straightforward application of the top-down MS methodology to mapping PEGylation sites within intact protein [15] failed due to the structural complexity (heterogeneity) of the protein–polymer conjugates. The abundance of lysine side chains (which are typically targeted by commonly used PEGylation strategies [16]) in most proteins leads to a significant heterogeneity of the products which differ from one another by the extent of PEGylation (*e.g.*, mono- and di-PEGylated species) and the position of the conjugation site. Separation of the protein–PEG conjugates based on the extent of PEGylation can be easily achieved in many cases using size exclusion chromatography (SEC), which also separates the conjugates from unreacted PEG and protein molecules [17]. However, this task can also be accomplished using ion exchange chromatography (IXC), which often provides an additional benefit of separating isomeric forms of PEGylated proteins differing from one another by the location of the conjugation site within the protein sequence [17]. In some favorable cases IXC can be used to separate a significant number of isomeric species [18], although complete separation may not always be possible.

MS has been used in the past to identify IXC fractions of PEGylated proteins based on the number of PEG chains attached to a single protein molecule [19]; however, positional isomers cannot be distinguished from each other based on mass measurement alone. In this work we introduce a new method of mapping PEGylation sites within a protein by combining the power of IXC and top-down tandem MS technologies. The method is tested with a complex mixture of an 8.6 kDa protein ubiquitin conjugated with a 5 kDa PEG chain at various locations. Even though IXC does not provide complete separation of several isomers of monoPEGylated ubiquitin, monitoring intensity changes of various fragment ion peaks in response to changes of fraction collection times for poorly resolved peaks allows conjugation sites to be confidently identified. Furthermore, combination of orthogonal ion fragmentation techniques in $MS^3$ experiments allows conjugation sites to be localized even in protein segments with high density of lysine residues.




**Scheme 1.**

## 2. Experimental

### 2.1. Materials

5 kDa methoxy poly(ethylene glycol) propionaldehyde (mPEG-PA) was purchased from Dow Chemical Company (Midland, MI); ubiquitin was purchased from Sigma–Aldrich Chemical Company (St. Louis, MO), and used without further purification. All other chemicals and solvents were of analytical grade or higher. Ubiquitin PEGylation was carried out using reductive alkylation chemistry [20], which is a site directed method for conjugating proteins to PEG activated with aldehydes (mPEG-PA) that are facilitated by free amines from protein N-terminus or from lysine residues (Scheme 1). Briefly, 4 mg of mPEG-PA and 2.5 mg of ubiquitin were dissolved in 10 mM phosphate buffer at pH 5.0, and a 10 μL of 20 mM $NaCNBH_3$ reducing agent was added to a final volume of 300 μL in a micro centrifuge tube at room temperature.

### 2.2. Methods

Separation of PEGylated ubiquitin forms was carried out using cation exchange chromatography (IXC) implemented with Agi-

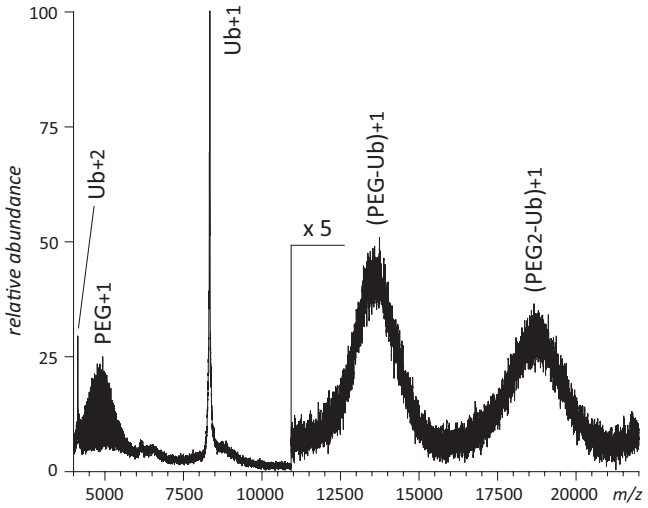



**Fig. 1.** MALDI TOF mass spectrum of unseparated products of ubiquitin PEGylation.

lent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA) equipped with a PolyCAT A column (Western Analytical, Murrieta, CA). The column was equilibrated with 20 mM ammonium acetate at pH 4.5, followed by an injection of a 40 μL PEGylated ubiquitin previously filtered through a 0.2 mm Acrodisc syringe filter (Pall Life Sciences, Ann Arbor, MI) to remove particulate matter. Di-PEGylated and mono-PEGylated ubiquitin samples with several isomers were eluted using 20 mM ammonium acetate at pH 5.0 with 200 mM NaCl (5–65% of 200 mM NaCl at pH 5.0 within 40 min as a gradient). Mass profiles of various forms of PEGylated ubiquitin were obtained with a Reflex III (Bruker Daltonics, Billerica, MA) time-of-flight (TOF) MS with MALDI ionization prior to IXC separation, and with a QStar-XL (ABI Sciex, Toronto, Canada) hybrid quadrupole/TOF MS with electrospray ionization (ESI) following IXC separation. MS/MS and $MS^3$ experiments were carried out using an APEX III (Bruker Daltonics, Billerica, MA) Fourier transform ion cyclotron resonance (FT ICR) MS with an ESI source. Ion fragmentation was induced in the ESI interface (CAD without mass-separation of precursor ions) and/or the ICR cell (ECD following separating the precursor ion by mass).

## 3. Results and discussion

### 3.1. IXC and MS analyses of the PEGylation reaction products

Ubiquitin has eight primary amines (seven lysine residues and the N-terminus), and its conjugation with activated 5 kDa PEG using reductive alkylation chemistry generates a range of

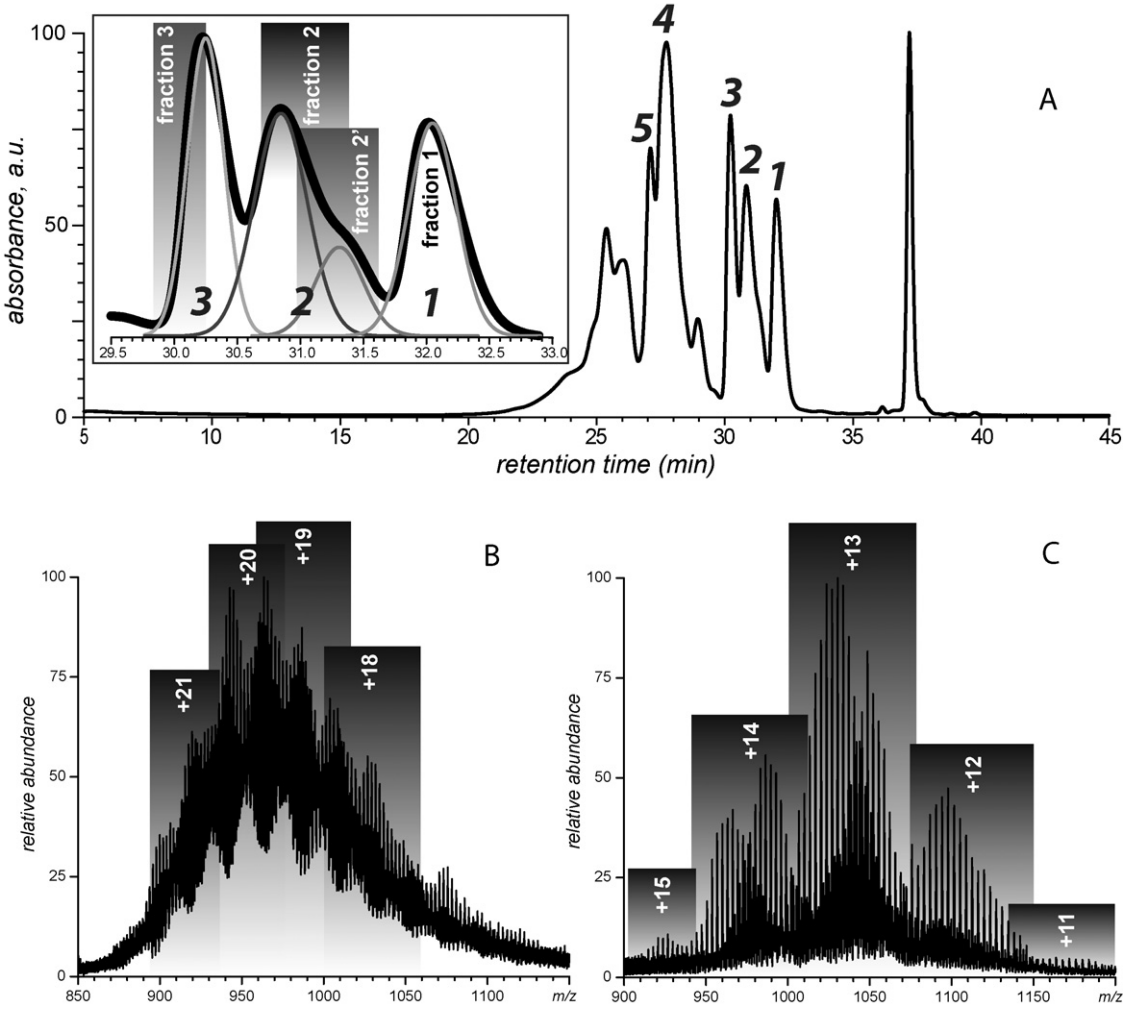



**Fig. 2.** IXC profile of PEGylated ubiquitin (A) and ESI mass spectra of pooled fractions representing IXC peaks 4 and 5 (B) and 1, 2, and 3 (C). The magnified view of the chromatogram (inset in panel A) shows the elution profiles of mono-PEGylated product (black trace), deconvoluted contributions of individual isomers (gray traces) and fractions analyzed by MS and MS/MS.

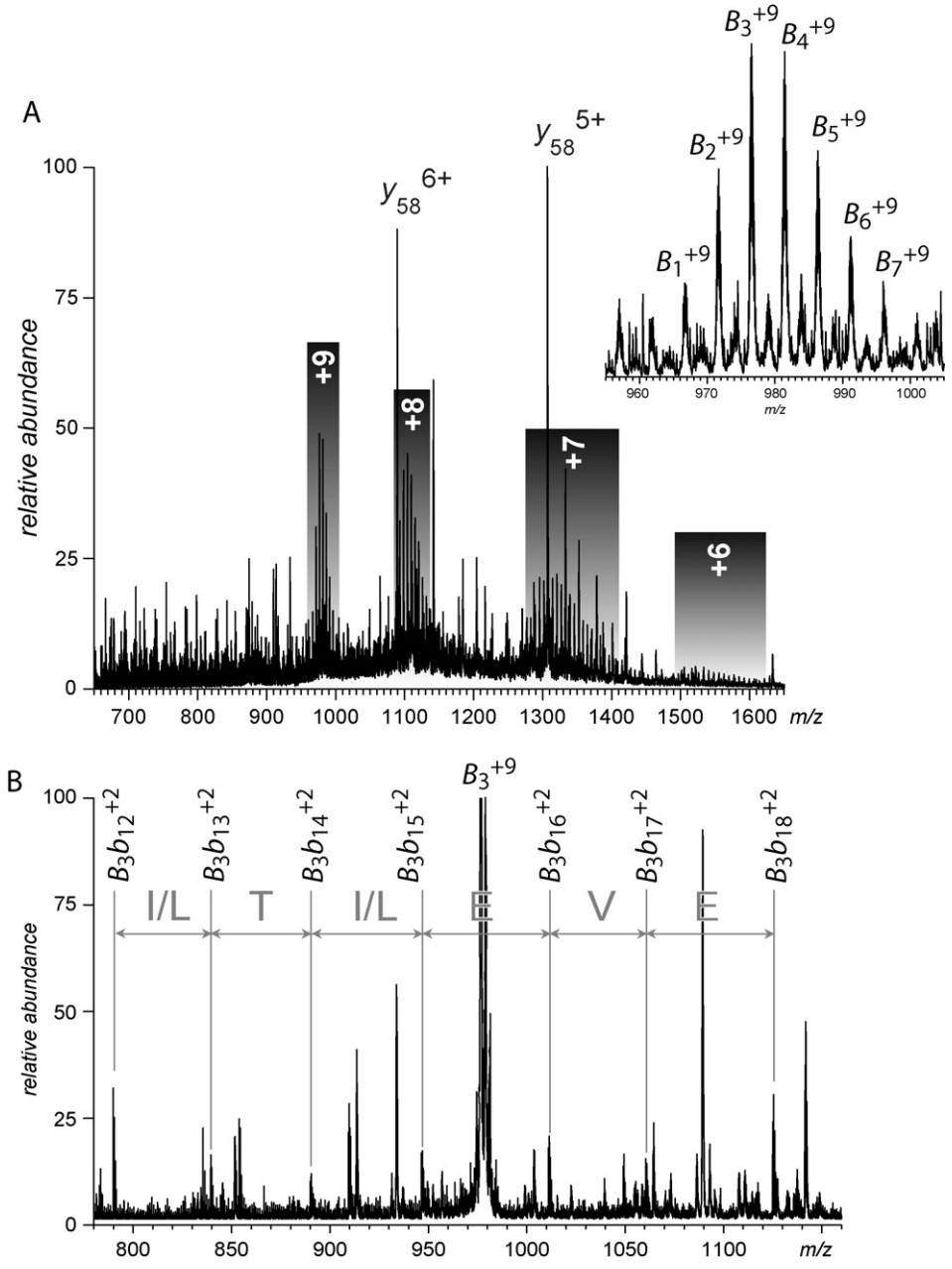
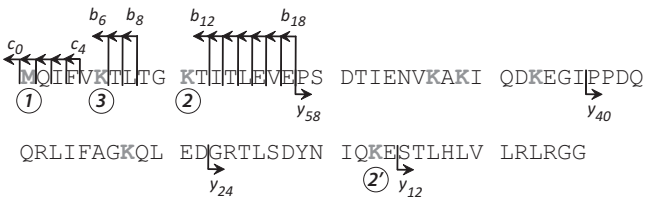



**Fig. 3.** Mass spectrum of fragment ions produced by collisional activation of PEGylated ubiquitin ions in the ESI interface region of a hybrid quadrupole/TOF MS (A). Clusters of fragment ions with truncated PEG chains are shaded and their charge states are indicated. The inset shows a zoomed view of one such cluster (charge state +9), where $B_3^{9+}$ fragment ion was used for the second-stage fragmentation (B). Refer to Scheme 3 for the explanation of the fragmentation nomenclature.

products as revealed by MALDI MS analysis (Fig. 1). Both mono- and di-PEGylated ubiquitin ions are detected alongside ions representing unconjugated protein molecules (charge states +1 and +2) and PEG chains. Analysis of the conjugation reaction products with IXC reveals even greater heterogeneity, with at least five major peaks being present in the chromatogram besides the peak representing unconjugated ubiquitin (Fig. 2, top). These five major peaks comprise two distinct clusters in the IXC chromatogram, which correspond to the elution time ranges 25–29 min and 30–33 min.




**Scheme 2.**

ESI MS analysis of the IXC fraction representing the first cluster of peaks reveals a very convoluted ionic signal (Fig. 2B). While the deconvolution of this mass spectrum cannot be carried out in a straightforward manner, charge state assignment for all groups of ion peaks can be easily done by measuring the distances between the adjacent peaks. Since the mass of a single repeat unit in PEG is 44 *u*, dividing this number by the measured inter-peak distance readily produces the number of charges carried by the ions of PEGylated ubiquitin. Charge state assignment carried this way produces

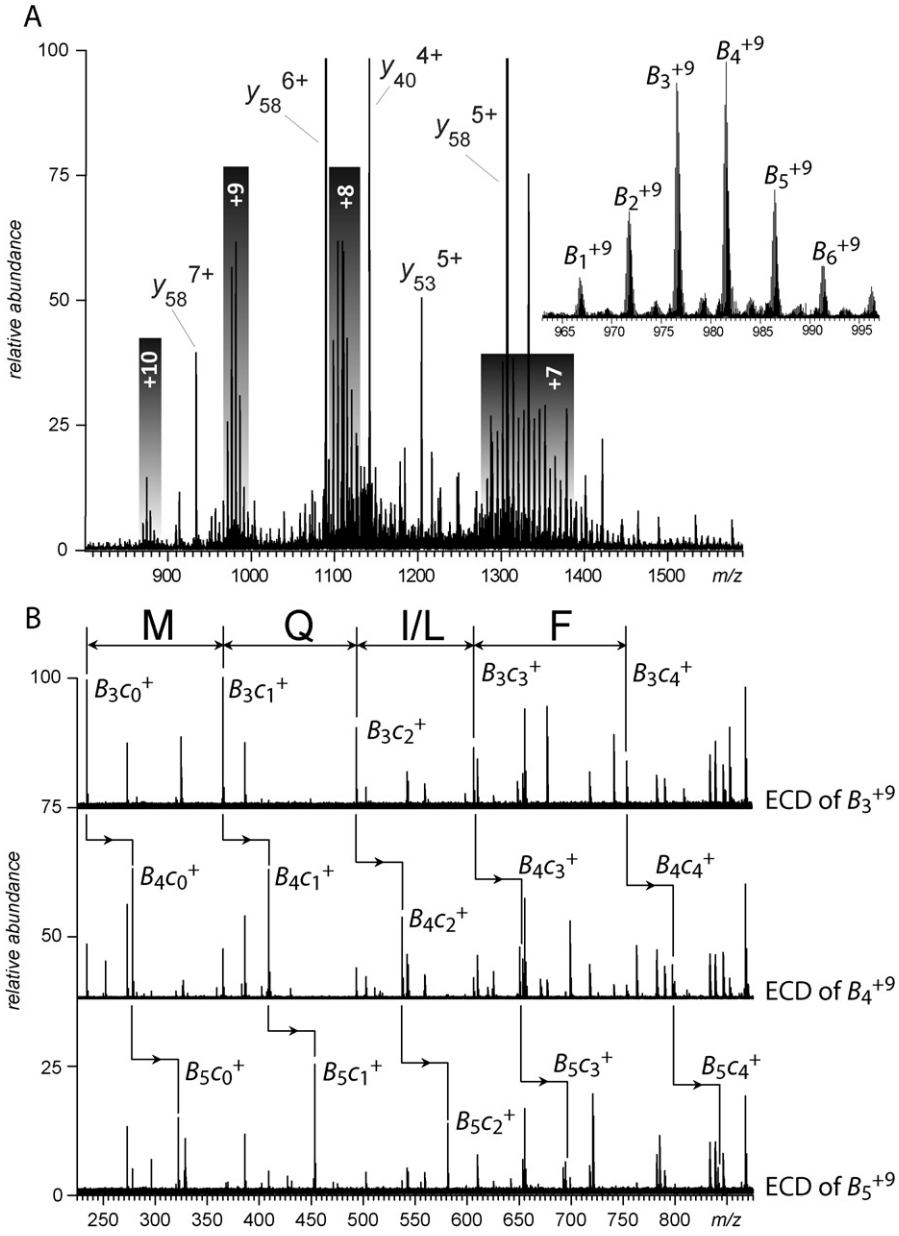



**Fig. 4.** Mass spectrum of fragment ions produced by collisional activation of PEGylated ubiquitin ions in the ESI interface region of a hybrid quadrupole/FT ICR MS (A). Clusters of fragment ions with truncated PEG chains are shaded and their charge states are indicated. The inset shows a zoomed view of one such cluster (charge state +9), where $B_3^{9+}$, $B_4^{9+}$ and $B_5^{9+}$ fragment ion were used to produce second-generation fragment ions using ECD in the ICR cell (B). Refer to Scheme 3 for the explanation of the fragmentation nomenclature.

a range of charges (from +18 to +21), yielding a mass distribution centered around 18.7 kDa, which is in close agreement with the calculated mass of di-PEGylated ubiquitin.

ESI MS analysis of another large IXC fraction (comprising the second cluster of peaks in IXC chromatogram) also reveals a convoluted signal distribution (Fig. 2C). Charge state assignment carried out using the approach described in the previous paragraph yields a distribution of charges ranging from +11 to +15 (this assignment was later confirmed using isotopically resolved FT ICR mass spectra, data not shown). Calculation of masses of the ionic species based on this charge assignment generates a distribution centered around 13.7 kDa, which is in close agreement with the expected mass of mono-PEGylated ubiquitin.

### *3.2. Top-down fragmentation of PEGylated ubiquitin: localizing the conjugation site in the high primary amine-density region of the polypeptide chain using a combination of CAD and ECD*

While most of the peaks in IXC chromatogram of PEGylated ubiquitin are not well-resolved, one of the mono-PEGylated products (peak 1 in Fig. 2A) can be easily separated from the rest. In-source collisional activation of ions produced by ESI of this IXC fraction generates a very convoluted spectrum (Fig. 3A), although careful examination of the spectrum reveals the presence of two abundant fragments of ubiquitin lacking conjugation ($y_{58}^{5+}$ and $y_{58}^{6+}$). These ions (as well as complementary $b_{18}$ fragment ions) are among the most abundant CAD-generated fragments of intact ubiquitin [21], although the spectrum in Fig. 3A contains no unmodified

$b_{18}$ fragments. This observation indicates that the conjugation site in this product of ubiquitin PEGylation is localized within the first eighteen residues of the protein, three of which possess primary amine groups (Met-1, Lys- 6, and Lys-11, see Scheme 2).

Another feature of the CAD spectrum of PEGylated ubiquitin is the presence of a group of ionic species whose mass distributions indicate the presence of few PEG repeat units (shaded in Fig. 3A). Masses of these ionic species slightly exceed that of unmodified ubiquitin, suggesting that these fragment ions are formed by removal of most (but not all) of the PEG chain from the protein–polymer conjugate in the gas phase. The remaining linker and a few ethylene glycol repeat units can be used as convenient marker of the conjugation site. Masses of these truncated ions are in agreement with the notion of the PEG chain cleavage following the route that produces B and C ions using the nomenclature proposed by Lattimer and co-workers [22]. The proposed structures of these first-generation fragment ions are presented in Scheme 3. High-resolution mass measurements of these ions produced by in-source CAD in the ESI interface region of FT ICR MS (Fig. 4A) further supported this assignment by providing mass values that are within 5 ppm of the calculated masses for $B_k{}^{9+}$ ions.

Unfortunately, CAD of $B_k{}^{m+}$ ions carried out in the collision cell of a hybrid quadrupole/time-of-flight mass spectrometer is not very informative, as the fragmentation of the polypeptide backbone gives rise to abundant *b*-ions carrying the placeholder tags (*e.g.*, $B_3b_{12}{}^{2+}$ through $B_3b_{18}{}^{2+}$ derived from $B_3{}^{9+}$ in Fig. 3B, refer to Scheme 3 for the explanation of the fragmentation nomenclature) that correspond to cleavages of amide bonds in the $Ile^{13}$-$Pro^{19}$ segment of the polypeptide chain (Scheme 2). No fragmentation is observed in the segment comprising first eleven amino acid residues, which does not allow the conjugation site to be localized with precision higher than that offered by the first stage of fragmentation (*vide supra*).

Electron capture dissociation (ECD) often provides structural information complementary to that deduced from CAD experiments, and its incorporation into the top-down MS routine typically enhances the level of sequence coverage [23]. In order to enhance spatial resolution in localizing the conjugation site of PEGylated ubiquitin species represented by IXC peak 1, ECD fragmentation of ubiquitin ions containing placeholder tags was carried out in the ICR cell of a hybrid quadrupole/FT ICR MS. The precursor ions for ECD fragmentation were produced by truncating the PEG chain by carrying out CAD in the ESI interface region (Fig. 4A), followed by mass-selection of $B_3{}^{9+}$, $B_4{}^{9+}$ and $B_5{}^{9+}$ in the front-end quadrupole as precursor ions for further dissociation. ECD of these ions produces a series of *c*-ions carrying the placeholder tags ($B_kc_m{}^{+}$, refer to Scheme 3 for the explanation of the nomenclature) that cover the first few amino acid residues in the N-terminal segment of ubiquitin (Fig. 4B). The only primary amino group in this segment of the protein is its N-terminus, which indicates that the conjugation site of the PEGylation product represented by IXC peak 1 is Met-1.

### 3.3. Assignment of PEGylation sites in poorly resolved IXC fractions

Identification of the conjugation site within mono-PEGylated ubiquitin discussed in the preceding section was possible due to the ability to separate this species from other PEGylation products. While the separation of the products differing by the extent of conjugation can usually be accomplished using various chromatographic techniques (SEC or IXC), complete separation of isomeric forms (differing by the location of the conjugation site within the polypeptide sequence) is usually more difficult to achieve. An example of this is presented in Fig. 2A, where IXC peaks 2 and 3 representing mono-PEGylated ubiquitin are only partially resolved, and the convoluted structure of peak 2 (a shoulder between 31′15″ and 31′40″) suggests the presence of at least two different species whose elution times are very close to each other. Assuming that ideal chromatographic peaks should have Gaussian profiles, the crowded region of the IXC chromatogram between 30 and 33 min can be deconvoluted to yield contributions of four individual species (inset in Fig. 2A).

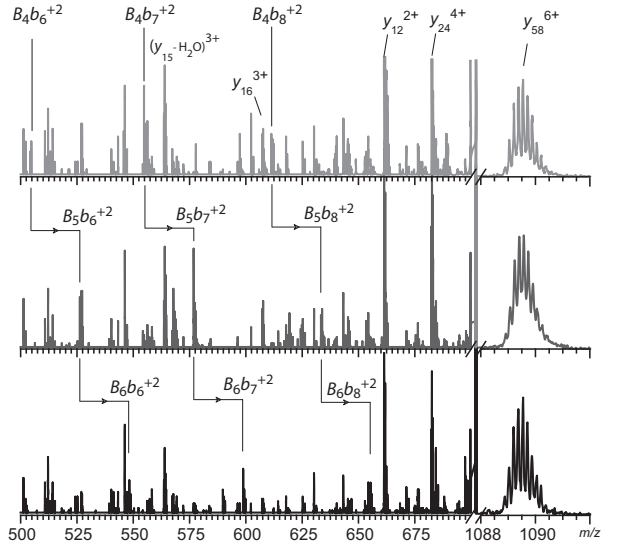
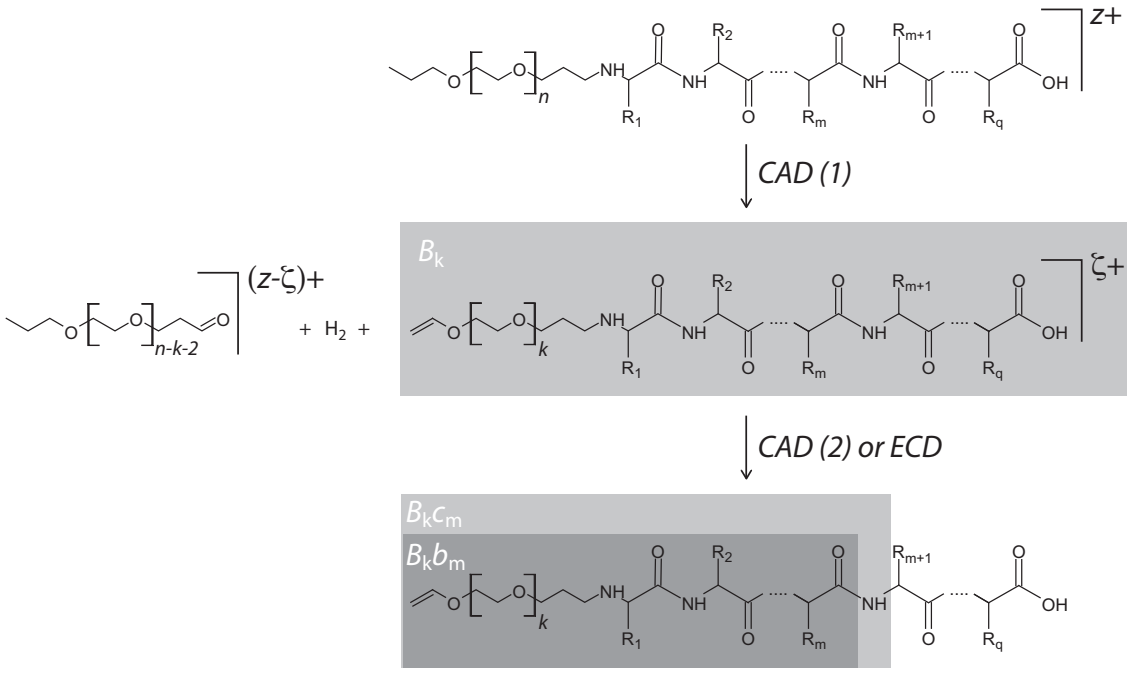



**Fig. 5.** CAD fragment ion spectra of tag-carrying ubiquitin ions $B_4{}^{9+}$ (top), $B_5{}^{9+}$ (middle), and $B_6{}^{9+}$ (bottom) produced by collisional activation of intact PEG-ubiquitin from fraction 3 in the ESI interface of a hybrid quadrupole/time-of-flight MS showing tag-carrying *b*-ions and tag-free *y*-ions.

Although the chromatographic peaks 2 and 3 have a significant overlap, it is possible to isolate a species giving rise to peak 3 in a pure form by collecting an IXC fraction between 29′50″ and 30′15″, as indicated by a box labeled "fraction 3" in Fig. 2A inset. Localization of the PEGylation site within this species is relatively straightforward. MS/MS analysis of protein ions with truncated PEG chain (generated by collisional activation of intact ions in the ESI interface region) yields abundant $y_{58}$ fragment ions, whose masses are consistent with the absence of a placeholder tag in this protein segment spanning residues 19–76 (Fig. 5). Presence of a series of small *b*-fragments carrying the mass tags ($B_kb_6{}^{2+}$, $B_kb_7{}^{2+}$, and $B_kb_9{}^{2+}$) further reduces the number of possible PEGylation sites to Met-1 (N-terminus) and Lys-6. Since the former site has been already identified as the conjugation site of the species giving rise to peak 1 (*vide supra*), the only remaining possibility for peak 3 is conjugation at Lys-6.

Unfortunately, it is nearly impossible to isolate the two species giving rise to peak 2 in IXC chromatogram. Fraction collection carried out within a time window between 30′40″ and 31′35″ (to eliminate contribution of peaks 1 and 3) yielded a mono-PEGylated product, which clearly is a mixture of different protein–polymer conjugates. Indeed, for this fraction the MS/MS analysis of protein ions with truncated PEG chain (generated by collisional activation of intact ions in the ESI interface region) yields abundant $y_{24}$, $y_{40}$, and $y_{58}$ fragment ions both with and without the mass tag (Fig. 6). Since the protein segment corresponding to $y_{24}$ fragment contains only one potential conjugation site (Lys-63), one of the isomers in this fraction must be conjugated at this residue. Another isomer must be conjugated at Lys-11, since this is the only remaining potential conjugation out of three sites lying outside of the segment represented by $y_{58}$ fragment. In order to understand which site (Lys-11 or Lys-63) corresponds to the major species in peak 2, mass analysis of fragment ions produced by in-source CAD was carried out on two fractions that were collected such that (i) neither




**Scheme 3.**

contains contaminations from peaks 1 and 3, and (ii) the relative contributions of the species representing the main peak 2 and its shoulder 2′ are different for these two fractions (shown with boxes in Fig. 2A inset). Mass analysis of fragment ions produced by in-source CAD of PEGylated ubiquitin from these two fractions clearly shows lower abundance of the tag-free $b_{18}$ fragment (as measured against tag-free $y_{58}$ fragment) for the fraction containing a higher proportion of the shoulder peak 2′ (Fig. 7), suggesting that this fraction has a higher proportion of ubiquitin molecules conjugated at Lys-63. This allows peak 2 and its shoulder 2′ to be assigned as the products of conjugation at Lys-11 and Lys-63, respectively.

It is rather surprising that only four (out of possible eight) primary amines are occupied in mono-PEGylated ubiquitin. A detailed analysis of ubiquitin structure (Fig. 8) suggests that only one out of four lysine residues that fail to form mono-PEGylated products has poor solvent accessibility (Lys-26). Furthermore, this lysine residue and two other (Lys-29 and Lys-33) are parts of the same alpha-helix, one of the most structurally stable elements in this protein under both native and denaturing conditions [21,24]. Therefore, one might expect that decreased flexibility of this protein segment may make the reaction with activated PEG less favorable due to higher steric hindrance at these sites. Lys-48 is the only residue that is located outside of this helical region, and yet it fails to participate in the conjugation reaction yielding a mono-PEGylated product, suggesting that its chemical microenvironment might be the reason for its low reactivity.

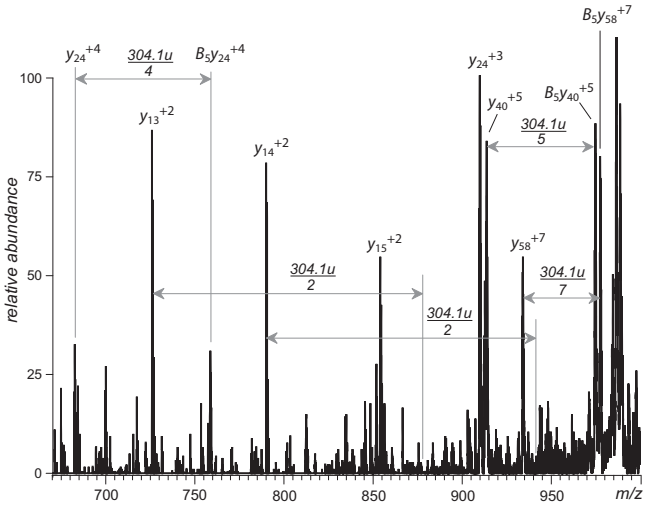



**Fig. 6.** CAD fragment ion spectrum of a tag-carrying ubiquitin ion $B_5^{9+}$ (produced by collisional activation of intact PEG-ubiquitin from peak 2 in the ESI interface of a hybrid quadrupole/time-of-flight MS) showing both tag-free and tag-carrying $y_{24}$, $y_{40}$, and $y_{58}$ fragment ions.

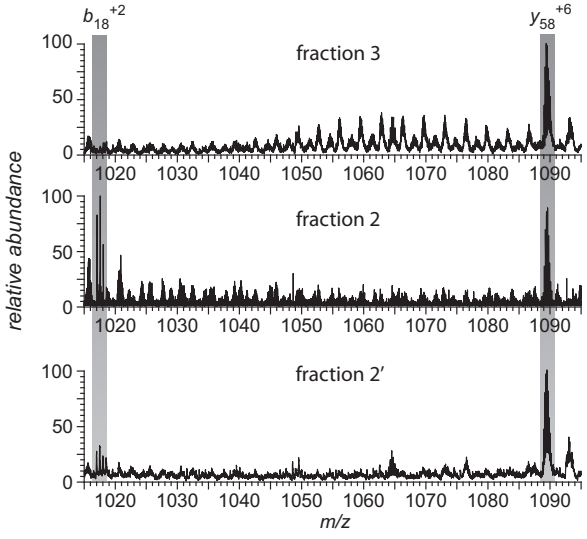



**Fig. 7.** In-source CAD fragment ion spectra of PEGylated ubiquitin from fractions 3 (top trace), 2 (middle) and 2′ (bottom) in the ESI interface of a hybrid quadrupole/time-of-flight MS showing intensity change of the tag-free $b_{18}$ ions relative to tag-free $y_{58}$ fragment ions.

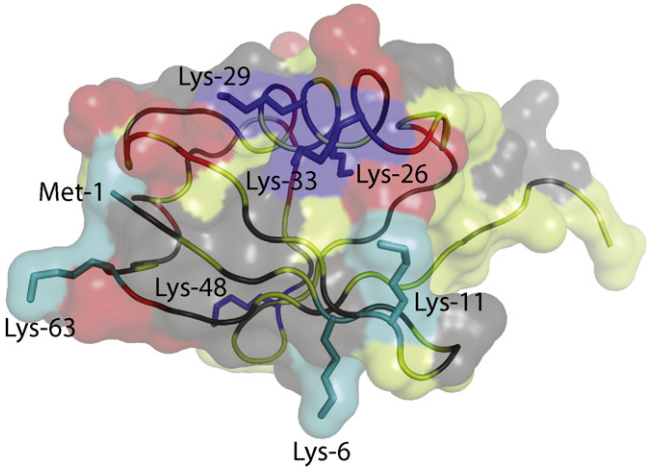



**Fig. 8.** Crystal structure of ubiquitin (PDB ID 1ubq) showing the positions of residues carrying primary amines. Residues participating in conjugation to yield mono-PEGylated products are colored in cyan, and those not participating in formation of mono-PEGylated products are colored in blue. Hydrophobic residues are colored in green, and acidic residues are colored in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

## 4. Conclusions

Large size and high heterogeneity of protein–polymer conjugates present a formidable challenge *vis-à-vis* their structural characterization. A combination of IXC separation and multi-stage top-down MS characterization allows the complexity of these systems to be dramatically decreased, resulting in confident localization of the conjugation sites. IXC easily separates the products of the PEGylation reaction according to the extent of conjugation, and also provides at least some separation for the isomeric forms of PEGylated proteins (which differ from one another by the location of the conjugation site within the polypeptide chain). The remaining heterogeneity due to the dispersity in the length distribution of the PEG chains attached to the protein may complicate the analysis of MS/MS data, but can be dramatically reduced by truncating the polymer chains in the gas phase using methods of ion chemistry (collisional activation). While this process removes the majority of PEG repeat units, it leaves the linker group and a few PEG repeat units still attached to polypeptide chain, which can be used as a placeholder tag for localizing the position of the conjugation site. The latter is accomplished using a second fragmentation step, where both CAD and ECD can be employed to induce dissociation of tag-carrying protein ions. Electron-based ion fragmentation methods have been already shown to be very useful when dealing with highly heterogeneous biopolymers, such as glycoproteins with very high carbohydrate content [25]. Application of ECD to fragmenting PEGylated ions demonstrated in this work further expands the scope of the application of this technique in structural analysis of heterogeneous protein-based systems.

While tandem MS is extremely powerful as far as identifying specific PEGylation products that are completely or only partially resolved by IXC, it is likely that the separation step itself will become a bottleneck in the analysis of larger and more heterogeneous protein–polymer conjugates. However, continuing improvements in IXC [26] and recent progress in applying other separation techniques to analysis of PEGylated proteins [27] provide firm basis for optimism, suggesting that detailed structural characterization of PEGylated biopharmaceuticals will be possible in the very near future. Hyphenated MS-based methods of protein analysis (both LC/MS and MS/MS) already play a vital role in characterization of protein pharmaceuticals [28–30], and continued improvements of these experimental methodologies will undoubtedly expand the scope of complex protein therapies amenable to MS characterization.

## Acknowledgements

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