A Peptidoform Matching Strategy in Bottom-up Proteomics for Studying Functions of Post-translational Modifications

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Abstract: Protein translational modifications (PTMs) generate an enormous, but as yet undetermined, expansion of the expressed *proteoform*s. In this Viewpoint, we firstly differentiate the concepts of *proteoform* and *peptidoform* by reviewing and discussing previous literature. We show that the current PTM biological investigation and annotation largely follow a *PTM site-specific* rather than *proteoform-specific* approach. We further illustrate a potentially useful matching strategy in which a particular "modified *peptidoform*" is matched to the corresponding "unmodified *peptidoform*" as a reference for the quantitative analysis between samples and conditions. We suggest this strategy could provide directly relevant information for learning the *PTM site-specific* biological functions. Accordingly, we advocate for the wider use of the nomenclature "*peptidoform*" in the future bottom-up proteomic studies.

Proteins and PTMs.

Protein is the preeminent molecule carrying many cellular functions and processes. Currently, it is widely accepted that one gene frequently generates multiple forms of proteins, rather than only one distinctive protein form. These forms include a variety of e.g., sequence variation, RNA alternative splicing isoforms, and protein post-translational modifications (PTMs). Together, they render the organism extensive biological variability and phenotypic diversity at the protein level, even with the lower-than-anticipated gene numbers [1, 2].

Protein PTMs, include phosphorylation, glycosylation, acetylation, polyubiquitination, and methylation, among a few hundred others [3]. PTMs can decorate a protein during its synthesis or after it is produced, altering its physicochemical and biological properties – frequently in a modification-site-specific manner. For example, protein phosphorylation can mediate protein-protein interaction [4, 5], alter the three-dimensional structure, stability, turnover [6, 7], and subcellular localization of proteins [8]. In the proteomics field, the application of mass spectrometry (MS) to detect and quantify PTMs in various biological and clinical samples for deciphering their functions has been a popular topic in the last twenty years [9].

Top-down and Bottom-up Proteomics: Proteoform and Peptidoform.

There are two principal proteomic methodologies for analyzing proteins and their PTM forms – the *bottom-up* approach and the *top-down* approach. In a general bottom-up workflow, proteins are digested into peptides using trypsin and then identified by liquid chromatography coupled with tandem MS analysis. Consequently, in bottom-up approach the connection between the peptides and their proteins of origin is lost during the enzymatic digestion step. This information loss makes resolving of PTM forms at the whole-protein level challenging. In contrast, in top-down workflows, intact proteins and protein variants are measured for their masses and then fragmented and identified by tandem MS, allowing a comprehensive characterization of the protein level molecular composition.

The concept of *proteoform* is highly relevant for understanding top-down perspectives. The Consortium for Top-down Proteomics has defined the nomenclature "*proteoform*" to *designate all the different molecular forms in which the protein product of a single gene can be found*, which *include changes due to genetic variations, alternatively spliced RNA transcripts, and posttranslational modifications* [10]. In essence, each individual molecular form of expressed proteins is a *proteoform* [10].

Indeed, the concept of *proteoform* has greatly sharpened our views on protein diversity. Following the definition of *proteoform*, protein subpopulations carry a phosphate moiety at two sites could generate four possible *proteoform*s – three modified at each or both sites and another unmodified *proteoform*. Likewise, the same or different types of PTMs (e.g., PTM crosstalk) at different amino acid residues might decorate a given protein in any combinatorial patterns. Therefore, an exceptional increase in the theoretical number of *proteoform*s due to PTMs could be expected (**Figure 1**, Upper view). Moreover, certain PTM types such as polyubiquitination and glycosylation can generate additional constitutive variation at the same site. For example, if we consider the variable structural feature of oligosaccharides, the number of *proteoform*s carrying distinct glycan structure will be fairly large. Very interestingly, despite such a theoretical "*proteoform* explosion", the real number of human *proteform*s seems to be much lower, according to a community-level estimation [3]. A total of ~one million *proteoform*s were roughly estimated based on e.g., the current practice of histone modification analysis, the technical detection threshold, cell type uniqueness, and the practical cellular constraints in controlling the enzymatic writing and maintenance of PTMs. The authors nevertheless acknowledged that a precise estimate of the number of human *proteoform*s is difficult to provide [2, 3]. As such, for a specific protein the pool of all possible *proteoform*s can be immense [11]. Although top-down studies have proven to be an exceptionally powerful resource for hypothesis-driven research on defined protein targets, there is a fairly long way to go for the top-down technique to routinely detect most (e.g., one million) *proteoform*s in a sample, due to its current limited sensitivity which is still perceived as worse than in the bottom-up approach [12, 13].
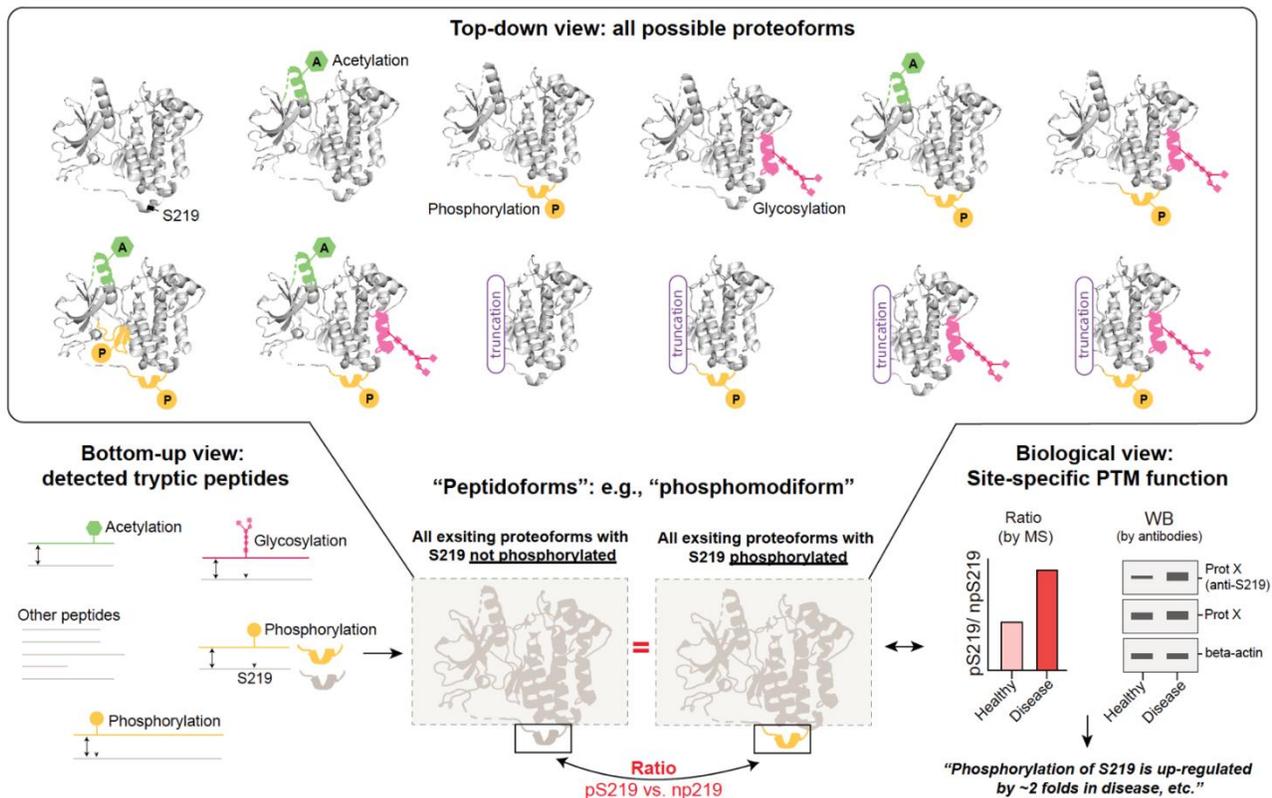
Figure 1. The top-down and bottom-up views for studying PTM biology. A virtual example of protein X (representing any protein) is shown. This protein X can be modified by different enzymes at different amino acid residues, forming a variety of possible *proteoform*s carrying acetylated, phosphorylated, or glycosylated sites and even the combinatorial PTMs in the cells. Additionally, the mRNA alternative splicing could create some truncated *proteoform*s of X. In bottom-up proteomics, the modified *peptidoforms* can be measured in PTM enriched samples. If we take the non-modified *peptidoform* counterpart (measured by total proteomics) as a comparative reference, we can extract a pair of PTM/non-PTM *peptidoform*s, irrespective of the total *proteoform* pool, for interrogating the impact of a site-specific PTM among samples. Such information can be verified by e.g., western-blot, illustrating site-specific PTM functions. Protein structural regions of different colors denote the location of respective *peptidoform*s. The phosphorylated S219 represents *any* PTM site of any type. The red equals sign highlights the *proteoform* background which is *the same* in PTM-enriched and non-PTM measurements.

On the other hand, the concept of *peptidoform* has been used in several studies [14-24], but not widely. *Peptidoform* stands for *specifically modified or mutated peptides with the same backbone amino acid sequence* [20]. In the early days, *peptidoform* was mentioned always together with *proteoform*, to deliver the analogous concept at the peptide level. More recently, *peptidoform* was independently used in bottom-up studies, such as in a few data-independent acquisition (DIA) MS-based [25, 26] quantitative PTM investigations [14, 16, 20]. For example, we and colleagues have developed Inference of Peptidoforms (IPF), a computational algorithm for confident, systematic identification and quantification of *peptidoforms* in DIA-MS datasets [20] and applied it in analyzing plasma proteomes of human twin individuals [20, 27].

PTM function: Proteoform-specific or PTM Site-specific?

With the concepts of *proteoform* and *peptidoform* clarified above, it becomes inspiring to revisit how we study PTM biology currently. To learn the function of a protein PTM, the researchers normally perform experiments to measure the abundance and other properties of it (e.g., localization, stability, etc.) in a biological process such as drug-induced perturbation or disease development. The researchers would then refer to published literature or PTM databases and sometimes perform new validation experiments to fully establish a functional link between the PTM site and the biological question. Although both bottom-up and top-down approaches both support well the broad, relative proteomic quantification between samples and conditions, they measure the *de facto* individual peptides (digested) and *proteoform*s, respectively. In an ideal scenario, if distinctive *proteoform*s are precisely identified and quantified between biological/clinical samples (see a nice example in Ref. [28]), and with such knowledge being accrued over time, the top-down measurement will pinpoint the different functions of every detectable *proteoform*. This type of *proteoform-specific* knowledge would also nicely fit the structural view of protein complexity, because actions taken by the each intact *proteoform* species in the cellular processes should be anyway depending on their unique molecular structure.

However, it is crucial to stress that, ***different proteoforms of the same gene can share the same site-specific PTM***, whereas lots of PTMs are currently studied and annotated in a *site-specific*, rather than a *proteoform-specific*, manner. For a virtual example, please see **Figure 1**. Herein, the S219 of protein X (can be any protein) is able to be phosphorylated by a particular kinase Y, resulting in phosphorylated S219 (i.e., pS219, highlighted in *orange color*). All the *proteoform* species carrying pS219 (n=7 in this case) might co-exist in the cell after the enzymatic kinase-substrate reaction. Currently, the most concrete functional studies are performed for pS219 itself, but not for one of the seven *proteoform*s carrying pS219 (which would otherwise need the protein-level separation or purification [29]). Also, the resultant knowledge and annotations are built on pS219 (e.g., it is a substrate of kinase Y) [30], but not on each of the seven *proteoform*s.

Furthermore, it is intriguing to ask what is really being measured in PTM analyses performed using classic non-MS methods, such as western blotting (WB), immunohistochemistry (IHC), and enzyme-linked immunosorbent assay (ELISA). For these assays, antibodies have to be developed and optimized targeting proteins carrying particular PTMs. During antibody production, the immunogen—the part of the protein that the antibody recognizes (e.g., a continuous stretch of amino acids) or the full-length protein, is the key. In the first production of phosphorylation-dependent antibodies twenty years ago, benzyl phosphonate was injected into rabbits to generate antibodies detecting phosphotyrosine-containing proteins [31][WEB1]. Nowadays, phosphosite-specific antibodies are typically generated by the immunization using synthetic phosphopeptide surrounding the phosphosite of interest. Further, a positive selection is normally performed to remove antibodies detecting the non-phosphorylated version. Therefore, whether WB, IHC, or ELISA measure a specific *proteoform* or multiple *proteoform*s carrying the same PTM site largely depends on the epitope and selectivity of the particular antibody used. Although in WB the molecular weight (MW) of the protein target is obtained by referring to MW markers, the MW information can be lacking in IHC and ELISA assays. Even in WB, due to the limited MW resolution of electrophoresis, a detected protein band might represent multiple *proteoform*s with varied molecular compositions (but sharing a PTM site) that are just too close in MW. In this regard, using full-length *proteoform*s as antigens for production of high-quality affinity reagents using methods like phage display might be helpful to increase the *proteoform*-level specificity in WB, IHC, and ELISA analyses [3].

Although many current research tools largely follow the PTM site-centric assumption, we want to point out that there is no doubt that the link between *proteoform* species and their functional significance would be a major

advance in the future. This will catalyze the fundamental knowledge drift from PTM site-centric to *proteoform*-centric investigation, because eventually, biology research has to be both precise and mechanistic. Ultimately, the complete primary structures of proteins on a proteome scale will be useful [13, 32]. Before that, however, corresponding experimental and informatic paradigms have to be established and widely applied. Recently, interesting workflows have been applied to infer *proteoform*-dependent functions from e.g., peptide co-varying analysis across multiple samples and comparisons [33-35]. Emerging bioinformatic annotation tools, such as PTMsigDB, just started to drift towards *PTM site-specific* annotation following PTM proteomic profiling [36, 37] (which already presented a major conceptual advance compared to the conventional, widely used, *gene-specific* annotating frameworks [38, 39]). Yet, the *proteoform-specific* annotation databases have not been configured proteome-wide, mainly due to the lack of data. In this regard, the recent initiative of The Human Proteoform Project is very timely and extremely important for assembling an atlas with more detailed knowledge by creating a comprehensive *proteoform* index [32].

Peptidoform: the concept revisited.

Due to the above challenges, we reason that the concept of *peptidoform* may facilitate studying PTMs using bottom-up proteomics (**Figure 1,** and below). In particular, herein, we propose that, the usage of *peptidoform* should clearly embrace both unmodified and differentially modified peptides that share the same backbone amino acid sequence. The *peptidoform*s can be generated by trypsin digestion or by other proteases. We further propose that the previously used terms such as *phosphomodiform* [6] can be unified under the nomenclature "*peptidoform*", because *phosphomodiform* essentially means *phosphorylated peptidoform*.

The usage of *peptidoform* instead of "modified peptides", will **a)** remove those modified peptides shared by multiple genes, **b)** create a concept in analogy to *proteoform* but describes the peptide-level diversity, and **c)** most importantly, emphasize the site-specific PTM biology in bottom-up proteomics, considering the enormous number of *proteoform*s in cells. In this regard, taking phosphorylation as an example, the phosphosite abundance profiling experiments in most previous phosphoproteomic studies, the phosphomodiform thermal stability analysis in Huang et al. and others [6, 40, 41], and our previous phosphomodiform lifetime study [7] all belong to the *peptidoform* profiling.

A peptidoform matching strategy for relative quantification between samples.

In the present Viewpoint, we would like to suggest that a ***peptidoform matching strategy*** could be a powerful approach to study PTM site-specific functions. To elaborate, in our recent study [7], by using the pulse experiment of stable isotope-labeled amino acids in cells (pSILAC) [42], we performed a pilot phosphoproteome turnover analysis. Particularly, we adopted a *peptidoform matching strategy* that directly interrogates the impact of individual phosphosites on protein turnover. This particular method is referred to as DeltaSILAC (delta determination of turnover rate for modified proteins by SILAC) [7]. In DeltaSILAC, for each site-specific phosphorylation, we determined the *lifetime difference* between the phosphorylated *peptidoform* (measured by pSILAC in enriched phosphoproteomes) and the non-phosphorylated *peptidoform* counterpart (measured by pSILAC in the same peptide samples without phospho-enrichment). This strategy successfully revealed that phosphorylation of the majority of sites increased protein stability in growing HeLa cells, which was not transparent without the matching strategy [7].

The highlight of this *peptidoform matching strategy* lies in ***the total "proteoform pool" that is always the same in the both PTM-enriched and non-PTM measurements***. Taking the example of Protein X again (**Figure 1**, bottom panel), no matter what and how many *proteoform* species of X exist in the cells, these *proteoform*s will generate a

common peptide mixture after protease digestion. The bottom-up measurements on the *peptidoform*s of pS219 and npS2I9 (non-phosphorylated S219) will only extract two *peptidoform*s, compare their difference between samples/conditions, and directly infer the impact of S219 phosphorylation. Similarly, this comparison can be applied to all the other PTM sites and types (such as acetylation and glycosylation, see green and pink sites in **Figure 1**) or even to a simultaneous analysis of multiple PTMs. In the case of PTMs on Lysine and Arginine residues, alternative proteases other than trypsin might be used to generate the PTM/non-PTM pair of *peptidoforms*.

Of note, the relative quantitative comparison between samples is crucial because the pS219 and npS219 *peptidoform*s may have different physicochemical properties, ending up with different flyability and responsiveness in the mass spectrometer. The relative fold-change of the ratio of pS219/npS219 between samples, rather than the pS219/npS219 ratio itself, can provide valuable and relevant information to the "PTM site-specific" biological functions.

Conceivably, the extensive detection and quantification of non-PTM *peptidoform* for each PTM site seem to be crucial for this conceptualized strategy, which can be difficult for PTM sites of high stoichiometry. Indeed, in the DeltaSILAC study, despite we estimated lifetimes for ~13,000 phosphorylated *peptidoform*s, we only measured lifetime for 2,100 phos-/non-phos- peptidorm pairs [7]. Fortunately, the technical barrier in identifying non-PTM peptides currently is lower than the PTM analysis. Normally, the amounts of the non-PTM samples are less limited, allowing for e.g., peptide-level fractionation to increase the coverage of unmodified *peptidoform*. As the final relevant note, our studies have suggested that, if the non-PTM protein-level reference is used to match the quantitative results of the PTM *peptidoform*, ~three times more PTM sites could be matched. This solution is of course less ideal, which impaired the turnover measurement [7] but seemed to be acceptable for the abundance profiling of the PTM sites among steady-state cells [43].

The blind man feels an elephant – to take a part for the whole.

Previously, a variety of experimental and bioinformatic strategies have been developed with the purpose of properly normalizing PTM proteomic data using the protein-level results (or analyzing the two jointly) for which we regret that we do not have space to cite and discuss here. The Viewpoint summarizes some of our understanding on the important concepts about peptide, protein, and PTM detection by MS, rather than a comprehensive review on the relevant topics.

To conclude, it seems that, even with the high resolving power provided by modern mass spectrometers, the complete, high-throughput quantification of all or most *proteoform*s might be still formidable in the near future. We deduce that the *peptidoform* measurement and a *peptidoform* matching strategy currently can provide direct and relevant information to study the "PTM site-specific" biological functions, even if one is somehow (unfortunately) "blind" to the "*proteoform* universe" in the sample. This "taking part for the whole" strategy will likely work well for quantitative PTM analysis for a fairly long time. We advocate the bottom-up community should consider using the nomenclature "*peptidoform*" more often.

In essence, we argue that "bottom-up" strategy pioneered by Eng and Yates [44] about 25 years ago does not just measure the peptide surrogates, but the *peptidoform*s collapsed from the huge pool of *proteoform*s in a given sample. In fact, the relative quantification of a "*peptidoform*" between different conditions and samples provides quantitative information for understanding the PTM site-specific function, which many biologists care about and study for many years.

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