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The use of fast photochemical oxidation of proteins coupled with mass spectrometry in protein therapeutics discovery and development

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Structural analysis of protein therapeutics is a challenging task owing to complexities associated with large molecular size and 3D structures. Recent advances in fast photochemical oxidation of proteins (FPOP) coupled with mass spectrometry (MS) have provided a means to characterize higher order structure (HOS) of protein therapeutics in solution. In this review, the utility of FPOP–MS in protein therapeutics discovery and development is presented, focusing on the principles and applications of epitope mapping for target-protein–protein-therapeutics interactions and biopharmaceutical HOS comparison studies. Future trends in the application of FPOP–MS in protein therapeutics characterization are also described.

Introduction

Protein therapeutics represent an important class of medicines in drug discovery pipelines and have been developed to treat a wide variety of clinical indications. This class of medicines includes monoclonal antibodies, fusion proteins and many other proteins with much larger sizes compared with small-molecule drugs, with molecular weights often exceeding 100 kDa. Because of the large size and structural complexity, protein therapeutics offer high specificity and complex sets of functions that cannot be mimicked by synthetic small molecules, and are less likely to interfere with normal biological processes and cause adverse effects [1]. To better design protein therapeutics, it is imperative to understand their structures and interactions with drug targets.

The higher order structure (HOS) of protein therapeutics is a key component in understanding the molecular structure, biological function and pharmacological function of a protein. Changes in HOS can have significant impact on quality, stability, safety and efficacy of protein therapeutics with increased risk of immunogenicity and loss of biological function. Biophysical characterization of HOS of protein therapeutics provides crucial data in selection of drug candidates, formulation development, quality control, stability and biocomparability studies [2]. Several biophysical approaches have been developed for assessment of HOS of protein therapeutics [3,4], including circular dichroism (CD) [5], Fourier transform infrared spectroscopy (FTIR) [6], electron microscopy [7,8] and differential scanning calorimetry (DSC) [9]. These methods offer the advantages of higher throughput and low sample consumption; however, they are limited by resolution issues. X-ray crystallography [10] and NMR spectroscopy [11] provide atomic resolution structure for understanding of protein–protein and protein–ligand interaction. However, these methods are labor intensive and usually require large amounts of protein. NMR is typically limited by size of protein (<25 kDa). It is challenging to monitor protein conformational dynamics by X-ray crystallography. It is often difficult to obtain suitable crystals of large protein therapeutics for X-ray studies. In the past decade, mass spectrometry (MS)-based protein HOS analytics have emerged as complementary approaches to provide a set of orthogonal methods for HOS assessment of protein therapeutics, including hydrogen/deuterium exchange (HDX) and fast photochemical oxidation of proteins (FPOP) coupled with MS.

HDX–MS relies on the exchange of the protein backbone amide hydrogens with deuterium in solution [12–15]. The rate of deuterium exchange is dependent on H-bonding and solvent accessibility. By measuring differences in HDX rates, one can obtain information on protein dynamics and conformation perturbation induced by a change of solvent environment or interaction with ligands [16]. HDX–MS applications include epitope mapping

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https://doi.org/10.1016/j.drudis.2018.12.008

[17–20], aggregation assessment [21,22], conformational analysis and comparability studies [23,24]. The reversible labeling nature in HDX–MS requires the HDX reaction to be quenched at low temperatures and pH 2.5; and digested by acidic proteases (pepsin) immediately after labeling. These stringent conditions often limit its spatial resolution of binding regions to the peptide level, although amino acid residual level resolution could be achieved under favorable activation conditions for certain peptides [25,26].

FPOP utilizes photolysis of hydrogen peroxide (H_2O_2) to generate hydroxyl radicals, which have a comparable size to water and high reactivity with a majority of protein amino acid side-chains to produce detectable oxidized products [27,28]. There are other methods to generate hydroxyl radicals including the use of synchrotron and plasma-induced modification of biomolecules [29,30]. One important feature of FPOP is that the time-scale can be controlled by using a radical scavenger to limit the primary radical life to a very short lifetime (around a microsecond), enabling studies of rapid kinetics [31]. The FPOP platform can be readily applied to protein analysis. In addition, the production of stable modifications by hydroxyl-radical exposure enables a wide range of sample sizes, proteases and solution conditions to be examined. In this review, we will focus on the general experimental workflow and current applications of FPOP–MS in epitope mapping and conformational analysis in protein therapeutics.

FPOP–MS workflow

The main components of the FPOP apparatus include: (i) a KrF pulsed excimer laser at 248 nm; (ii) a set of focusing lenses; (iii) a syringe pump connected to a fused silica capillary tubing; and (iv) a laser optical table for alignment and mounting of all devices (Fig. 1). The pulsed KrF laser beam generates a sufficient amount of hydroxyl radicals (~1 mM) for oxidative labeling of protein side-chains without disturbing its native conformation because proteins generally have very low absorbance at 248 nm. A simple capillary flow system with syringe pump injection is designed to deliver protein solution for FPOP labeling. To afford rapid labeling with a pulsed laser in a flow system, the fused silica capillary tubing is positioned vertical to the laser beam, with a segment (3–4 mm) of the polyimide coating removed as a UV transparent window to enable laser transmission. The frequency of the laser can be controlled by an external pulse generator. The reaction duration and protein exposure to hydroxyl radicals are tuned by adding histidine or other radical scavengers for rapid labeling in the sub-millisecond scale [32]. When the laser is pulsed 'on', a small 'plug' of protein solution in the window passes through, and H_2O_2 is dissociated into hydroxyl radicals for FPOP labeling. When the laser is pulsed 'off', the remaining hydroxyl radicals are removed by reaction with radical scavengers and self-quenching reactions, and the nearby 'plug' of solution is not labeled. As a result, FPOP can capture a 'snapshot' of protein conformation – an added advantage of FPOP over HDX for investigating protein conformational changes with rapid kinetics.

In a typical FPOP experiment, the protein of interest is mixed with H_2O_2 and radical scavenger (e.g., histidine, glutamine, etc.) and continuously injected into a 150 μm fused silica tubing by a syringe pump. Post FPOP labeling, the flow-through containing labeled and unlabeled proteins is collected in a microcentrifuge tube, and any remaining H_2O_2 is removed by adding a quenching solution (i.e., catalase and methionine) to avoid post-labeling oxidation. Labeled solutions can be snap-frozen in liquid nitrogen or immediately used for bottom-up proteolysis MS analysis. Oxidized samples are usually digested by enzymes (e.g., trypsin, etc.) and analyzed by reverse-phase ultra-high-pressure liquid chromatography for separation and high-resolution tandem MS for identification. FPOP labeling sites with oxidative modifications can be identified by database search software, and modification site assignments are validated by examining product-ion mass spectra. FPOP oxidation products usually elute at different times than the unmodified species, and the extent of modification can be calculated using accurate mass-based extracted chromatograms. The fraction of modified peptide is derived from the ratio of the peak area of modified species to total peak areas of all the species (unmodified and modified). Significance of the difference between conformational states can be assessed by statistical analysis, and level of differences in oxidations can be ranked and reported. In the cases where modification occurs on multiple sites of the sample peptide evidenced by multiple chromatography peaks eluting at a defined retention time, level of modification at a specific site is calculated to localize the residues that contribute to the conformational changes in different states. It is worth mentioning that reactivity of different amino acid residues with hydroxyl radicals can impact the detectability of oxidation products.

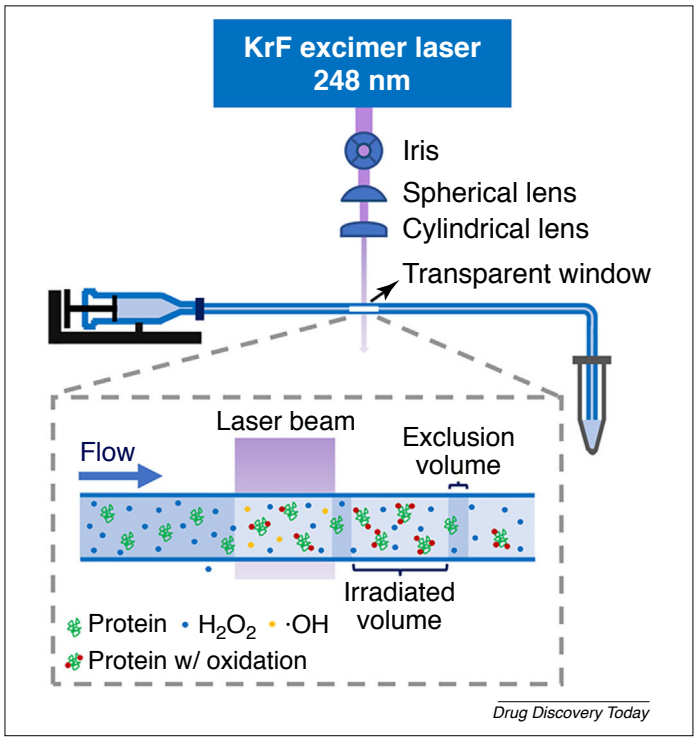

FIGURE 1

Schematic representation of the fast photochemical oxidation of proteins (FPOP) apparatus. FPOP solution mixture is injected into a fused silica capillary tubing by a syringe pump. The laser beam passes through a transparent window on the silica tubing for FPOP labeling. Post FPOP labeling, the flow-through containing labeled and unlabeled species is collected in a microcentrifuge tube to quench remaining hydrogen peroxide (H_2O_2). Adapted, with permission, from [28].

Several aspects need to be taken into consideration when designing a successful FPOP workflow. First, the flow rate of the syringe pump and the frequency of the laser pulse need to be carefully calculated to ensure an exclusion window (usually ~20%) to avoid repeated exposure of the same protein 'plug' to laser irradiation. Consistency in protein buffer compositions is important for comparison experiments. Second, FPOP and 'no-laser control' experiments need to be performed to correct for any oxidation induced by oxygen and H_2O_2 during the labeling process. Finally, the accuracy and resolution of FPOP–MS results usually rely on optimization of proteolytic digestion conditions. Incorporation of multiple enzymes for digestion for complete sequence coverage is often required, permitting the identification of site-specific oxidations with high accuracy.

FPOP–MS applications

Epitope mapping

The key functional feature of a therapeutic monoclonal antibody is the ability to selectively bind to its epitope or antigen. A detailed characterization of the epitope provides crucial structural information for understanding the mechanism of action, selection of antibody with better properties for drug development, protection of intellectual property rights and regulatory filings. Typical epitopes are classified as linear and conformational epitopes (also termed as discontinuous epitopes). There are a number of approaches in epitope mapping experiments, including peptide microassay, limited proteolysis, alanine scanning mutagenesis, yeast display, site-directed mutagenesis, X-ray crystallography and HDX–MS [33,34]. FPOP–MS has been used as an effective approach for epitope mapping [35–40]. To probe binding epitopes, FPOP experiments are usually performed on the free antigen as a reference experiment and on the antigen–antibody complex. The data generated from the two experiments are compared and peptide regions and/or amino acid residues in the protein that possess different oxidation levels are highlighted as potential epitopes.

In a recent study, a combination of HDX–MS, FPOP–MS, alanine shave mutagenesis and binding analytics was employed to map the energetic epitope of interleukin (IL)-23 by determining the interfacial hotspot that dominates the binding for an anti-IL-23 antibody [35]. IL-23 is an important therapeutic target of several autoimmune diseases, and IL-23-specific antibodies have shown remarkable effectiveness for the treatment of autoimmune diseases [41,42]. Compared with wild-type IL-23, site-directed mutagenesis with 3–5 residues mutated to alanine showed differences in binding and signaling blockade by the anti-IL-23 antibody. The mutant with five alanine substitutions represents the dominant energetic epitope and shows a dramatic decrease in binding to anti-IL-23 antibody while maintaining functional activity and biophysical properties similar to wild-type IL-23. HDX–MS was utilized to identify epitope of IL-23 interacting with antibody and found five peptide regions on IL-23 with reduced backbone amide solvent accessibility upon binding to anti-IL-23 antibody. These potential epitope regions were further characterized by FPOP–MS, consistent with findings from HDX–MS results (Fig. 2a). In addition, FPOP–MS analysis at the residue level reveals key interacting amino acid residues, in agreement with mutagenesis results (Fig. 2b,c). It was noted from this study that mapping antibody epitopes efficiently and accurately using this integrated orthogonal approach and pinpointing the crucial binding residues constituted a major analytical advance for the discovery and development of antibody therapeutics. The combination of MS-based methods along with computational modeling and targeted mutagenesis allows this crucial information to be readily obtained for important therapeutic targets in the absence of an X-ray structure.

FPOP–MS has also been used for structural characterization of antibody–antigen interaction of crucial reagents used in a quality-control lot-release assay for a sandwich ELISA [43]. FPOP–MS results indicated suppression of labeling across the antigen upon binding either of the two antibodies employed in the ELISA and identified the putative epitopes that appeared to span regions containing N-linked glycans. Further deglycosylation of the antigen resulted in loss of potency in the ELISA, supporting the FPOP–MS labeling data. In addition, negative stain transmission electron microscopy data provided visualization for linear and diamond antibody–antigen complexes with a similar binding orientation to that predicted from FPOP–MS analysis. Interestingly, mapping of the epitopes onto the antigen crystal structure revealed an orientation optimal for a noncompetitive binding ELISA format.

In another study, FPOP–MS was used in combination with molecular modeling for epitope mapping of a highly glycosylated HIV-1 gp120 envelope glycoprotein and a broadly neutralizing antibody b12; and detected direct interactions and conformational changes upon b12 binding without the need to introduce mutations or site-specific chemical labels that might alter the native conformation [44]. These studies are excellent examples of FPOP–MS for epitope mapping, demonstrating the utility of FPOP–MS as a stand-alone method or in conjunction with orthogonal methods for detailed structure characterization of antibody-antigen interactions.

Conformational analysis in protein therapeutics

The conformation of protein therapeutics is often sensitive to small changes during production, formulation and manufacturing. Protein therapeutics with improper conformation can lose stability, efficacy or even become highly toxic [45]. A recent study illustrated the utility of FPOP–MS in conformational analysis of protein therapeutics, including Neupogen®, an FDA-approved granulocyte colony-stimulating factor (GCSF), and several expired samples of recombinant GCSF and heat-treated Neupogen® [46]. Overall, the expired recombinant GCSF samples are all different from Neupogen®, although the extent of the differences varies. FPOP–MS experiments were carried out to identify all differences in the recombinant GCSF samples and provide localized information on changes in conformation dynamics. In addition, FPOP–MS results confirmed the identical conformations shared between the Neupogen® and the Neupogen22 sample held at room temperature for 22 h before analysis. The robustness and sensitivity of FPOP–MS methodology was further evaluated with different preparations of other protein therapeutics (i.e., interferon-α-2A, erythropoietin) generated at different times during the past 12 years. Overall, FPOP–MS could identify differences in the conformation between 12 different pairings of samples across three different protein therapeutics. This comparison study highlighted the potential of FPOP–MS for conformational assessment of

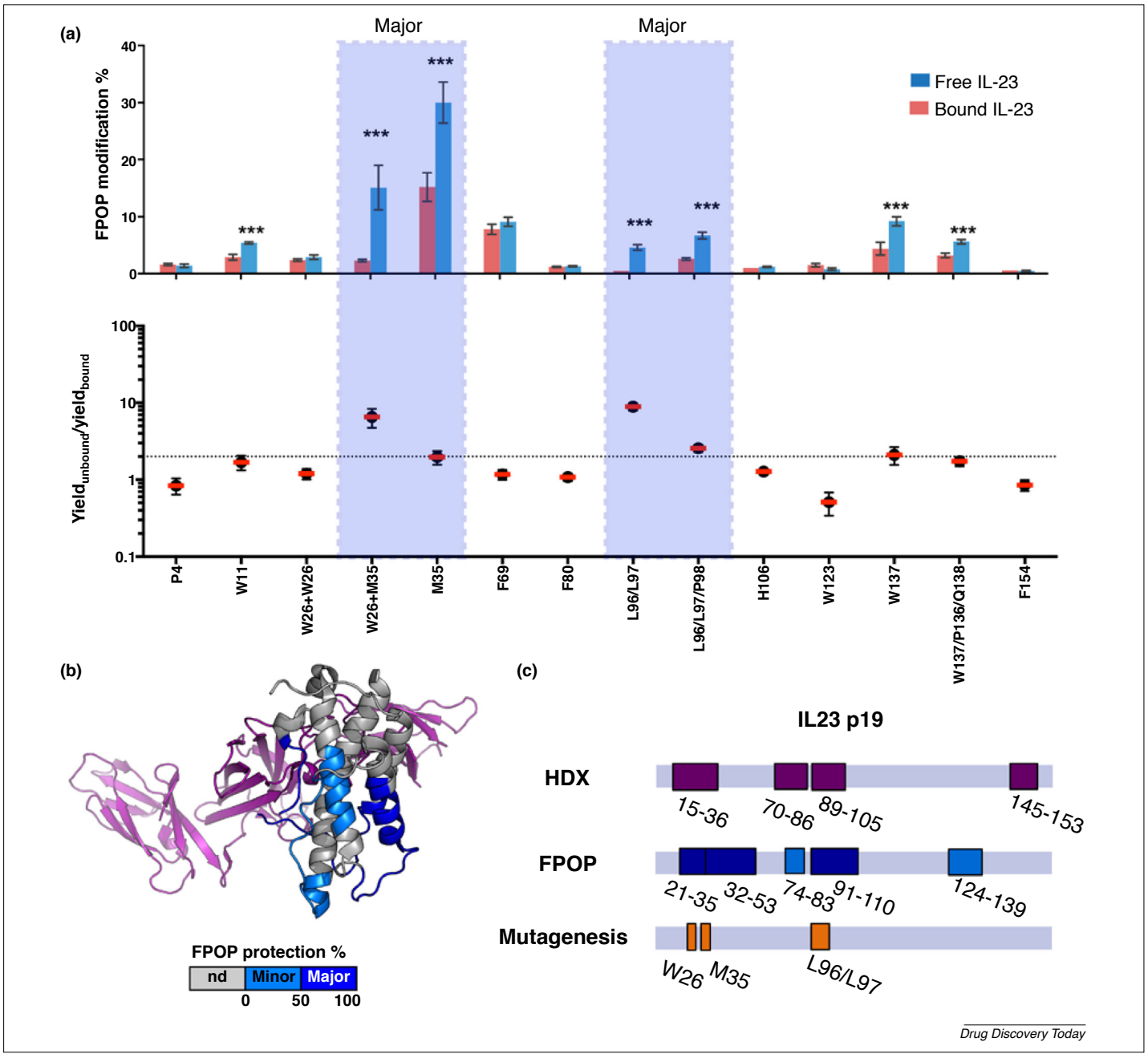

FIGURE 2

(a) Extent of fast photochemical oxidation of proteins (FPOP) labeling in free and bound interleukin (IL)-23 at the residue level. **(b)** Epitope regions determined by FPOP mapped on the crystal structure of IL-23. **(c)** Epitope regions determined by FPOP, hydrogen/deuterium exchange (HDX) and Ala shave energetics as mapped on the linear sequence of the IL-23 p19. Adapted, with permission, from [35].

protein therapeutics during the development and production stage and for quality control.

In another study with wild-type and four mutants of an IgG2 monoclonal antibody, FPOP–MS experiments were designed to probe differences between IgG2 isomers [47]. Molecular dynamics simulations on model human IgG2 antibodies indicated that the absence of disulfide bonds caused the two heavy chains to pack against one another in the hinge via noncovalent interactions. When the disulfide bonds were removed in the hinge, as for mutants C223S, C224S and C223S/C224S, the structures in these regions became more compact. Ion-mobility MS data verify that the wild-type is composed of two major isomers and that each of the mutants is more compact than the dominant wild-type component. Top-down MS and FPOP–MS detected localized conformational changes, and results from both experiments suggest low flexibility in the N and C terminus of the light chain, owing to structural constraints of the disulfide bond. In addition, the middle of the light chain undergoes higher levels of oxidative modification, pointing to higher solvent accessibility of reactive residues. Major differences in the disulfide bonding pattern of IgG2 antibodies occur in the hinge region. The high labeling extent (>60%) of wild-type IgG2 is consistent with the high flexibility of the

hinge. All mutants exhibit decreased oxidation, especially for hinge mutants C223S, C224S and C223S/C224S (oxidation <10%) and, to a lesser extent, C135S mutant (~50%). Although the substitution is not located in the hinge, the altered S–S bonding of Cys214 (light chain) to Cys223 (heavy chain) must induce a structural change in the hinge, inducing a more compact conformation for mutants. Notably, the binding region on IgG2 antibody, which is responsible for antigen specificity and affinity, displays no significant FPOP differences for mutants versus wild-type, suggesting that antigen-binding affinity of the mutants and wild-type is probably similar.

Concluding remarks

As illustrated in this review, FPOP–MS has become one of the attractive protein HOS analytical strategies for epitope mapping and conformational analysis in protein therapeutics discovery and development. Ongoing development in FPOP–MS analytics includes discovery of new labeling chemistry with novel reagents targeting specific amino acid residues [48,49] in cell analysis by FPOP [50,51], dosimetry experiments enabling reliable measurements across applications [52], development of automation for high-throughput FPOP sample preparation [53] and data analytics for rapid and accurate readouts with residue-level resolution including the electron-transfer dissociation approach [54]. Low sample consumption and flexibility in sample handling and digestion are key advantages of this platform technology, creating opportunities to address key structural and conformation questions in challenging target protein systems such as membrane proteins and large multimeric protein complexes [40,55]. There has been an increasing number of studies utilizing orthogonal protein HOS analytics (e.g., HDX, FPOP, computational modeling) as combined approaches for evaluation of protein structure and conformation dynamics [35–37]. It is expected that development and integration of FPOP–MS with protein HOS analytics will further expand the impact of FPOP–MS in protein therapeutics discovery and development.

Acknowledgments

The authors thank Dr Olafur Gudmundsson and Dr Lois Lehman-McKeeman from Bristol-Myers Squibb for their support of this project, and Professor Michael Gross from Washington University (St Louis) for a research collaboration.

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