Characterization of protein post-translational modifications (PTMs)

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**Abstract**

Protein post-translational modifications (PTMs) are chemical alterations to proteins following their ribosomal synthesis, vastly expanding the diversity and complexity of the proteome. These modifications, involving the covalent addition of chemical groups to amino acid residues, can significantly impact protein structure, function, and interactions. Acting as molecular switches, PTMs modulate protein activity, conformation, stability, localisation, and half-life, enabling dynamic regulation of cellular processes.

PTMs play critical roles in numerous cellular processes, including metabolism, cell signalling, gene expression, immune responses, and apoptosis, and their dysregulation is implicated in various diseases such as cancer and neurodegenerative disorders.

The characterisation of PTMs is especially vital for biopharmaceuticals like monoclonal antibodies (mAbs), as these modifications can affect their safety and efficacy. Identifying and analysing PTMs using sophisticated analytical techniques such as mass spectrometry (MS) and chromatography are crucial for defining critical quality attributes. These methods allow for the identification, localisation, and quantification of diverse PTM types, ultimately contributing to a deeper understanding of protein function in both normal and disease states and ensuring the quality of therapeutic proteins.

**1. Introduction**

Protein post-translational modifications (PTMs) are chemical changes to proteins after their synthesis, significantly increasing proteome diversity. These modifications involve the addition of chemical groups to amino acids, altering protein structure, function, and interactions. PTMs act as molecular switches, modulating protein activity and affecting conformation, stability, and localisation. They are crucial in many cellular processes like metabolism, signalling, and gene expression, and their dysregulation is linked to diseases. PTMs can also interact with each other (PTM crosstalk), and disruptions can lead to pathological events. Characterising PTMs is especially important for biopharmaceuticals like monoclonal antibodies (mAbs), as these modifications can affect their safety and efficacy. Identifying and analysing PTMs helps define critical quality attributes (CQAs) during drug development and manufacturing.

**2. Literature Review**

**2.1. Post Translational Modifications**

Post-translational modifications (PTMs) are chemical modifications to proteins that occur after their synthesis on the ribosome. These modifications involve the covalent addition of various chemical groups to amino acid. PTMs greatly expand the diversity and complexity of the proteome. Over 400 different types of PTMs have been identified, each with the potential to modify a protein in unique ways, which can significantly alter protein structure, function, and interactions (Wang et al., 2025). Figure1, represents the most common types of PTMs.

PTMs can act as molecular switches, modulating protein activity by either activating or suppressing it. They can affect protein conformation, stability, localisation, and half-life (Pandey and Gayen, 2024). Many PTMs are reversible, allowing for dynamic regulation of cellular processes (Zhou et al., 2024). This reversibility is often controlled by specific enzymes, such as kinases and phosphatases for phosphorylation (Pandey and Gayen, 2024).

**A diagram of different types of molecules

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Figure 1. Categorisation of common types of PTMs (Wang et al., 2013).

PTMs play crucial roles in many cellular processes, including metabolism, cell signalling, gene expression, immune responses, and apoptosis. They also regulate stress responses and can help maintain cellular homeostasis (Zhou et al., 2024). Aberrant PTMs and mis regulations are implicated in various diseases, such as cancer, metabolic disorders, and neurodegenerative diseases. Understanding PTMs is essential for designing more effective diagnostic tools and targeted treatments (Lai et al., 2024). PTMs do not act in isolation, and can co-regulate each other, a phenomenon called PTM crosstalk. Disruptions to PTM crosstalk can lead to pathological events (Lai et al., 2024).

**2.1.a. Glycosylation**

Glycosylation is a prevalent and complex PTM that involves the transfer of sugars (glycans) to specific amino acid residues on proteins, creating glycosidic linkages (Lai et al., 2024). Types of Glycosylation includes N-, O-, C-, S-, and P-glycosylation, where glycan is attached to the nitrogen (N), oxygen (O), carbon (C), sulphur (S) and phosphate (P) of amino acids. The conversion of proteins into glycoproteins starts in the endoplasmic reticulum (ER) and continues in the Golgi apparatus (Figure 2), (Pandey and Gayen, 2024).

A diagram of a cell line

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Figure 2. Visual representation N-linked and O-linked protein glycosylation in the endoplasmic reticulum (ER) and Golgi apparatus (Wang et al., 2013).

Glycosylation impacts protein stabilization, folding, and solubility. It serves as a marker linking proteins for cell-to-cell or cell-to-matrix communication (Pandey and Gayen, 2024).

Glycosylation influences the functions of trophoblasts, decidual stromal cells, and decidual immune cells, contributing significantly to the regulation of immune tolerance during pregnancy (Lai et al., 2024). Improper or aberrant glycosylation processes can directly contribute to the development or progression of metabolic dysfunction-associated steatosis liver disease (MASLD), cancer, Alzheimer’s disease, and cardiovascular disease (CVD) (Raju and Sankaranarayanan, 2025).

**2.1.b. Methylation**

Methylation is a prevalent and reversible post-translational modification involving the transfer of a methyl group to a protein or DNA residue. In proteins, methylation commonly occurs on lysine, arginine, histidine, cysteine, asparagine, glutamine, aspartate and glutamate residues (Figure 3). In DNA, it is considered a potential mechanism of recurrent spontaneous abortion (RSA) (Lai et al., 2024). Methylation influences various cellular processes, including gene expression, protein stability, protein-protein interactions (PPIs), and signal transduction (Raju and Sankaranarayanan, 2025).

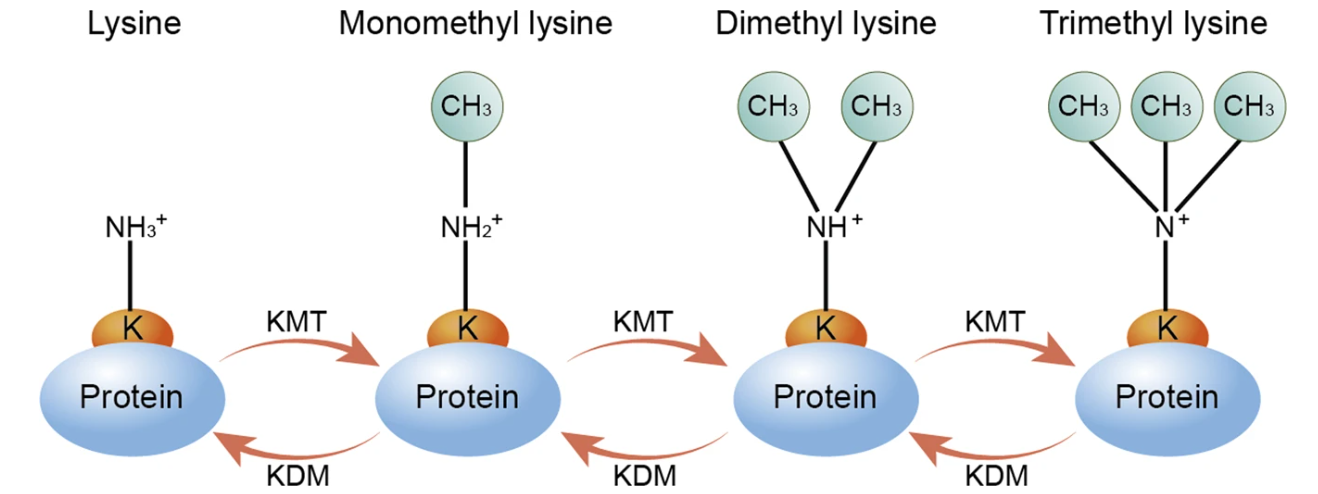


Figure 3. Visual representation of lysine methylation and demethylation. The lysine ε-amino groups can accept up to three methyl groups, resulting in mono-, di-, or trimethyllysine. Lysine methyltransferases (KMTs) catalyse the addition of methyl groups to substrates, while lysine demethylases (KDMs) remove methyl groups (Han et al., 2019).

Based on the proteins modified, protein methylation can be categorised into histone and non-histone protein methylation. Histone Methylation occurs at lysine and arginine residues on histone tails it can either activate or suppress gene expression depending on the specific site of methylation. For example, methylation at H3K9 is linked to gene silencing, whereas methylation at H3K4 results in gene activation. H3K27me3 (histone H3K27 methylation) is involved in gene silencing, cellular differentiation, developmental processes, and disease progression (Lai et al., 2024). Non-Histone Methylation regulates cellular signalling pathways, protein stability, mRNA translation, and other biological processes. It affects the function of non-histone proteins, either positively or negatively, depending on the methylation location and quantity (Wang et al., 2013). Aberrant methylation patterns are observed in cancer and Recurrent Spontaneous Abortion (RSA) (Lai et al., 2024).

**2.1.c. Phosphorylation**

Phosphorylation is a prevalent and well-characterised type of post-translational modification that plays an important role in modulating cellular signalling. It involves the transfer of a phosphate group, typically from ATP or GTP, to the side chain of hydroxylated amino acid residues in a protein. While it primarily occurs on serine, threonine, and tyrosine residues, phosphorylation of histidine, cysteine, and aspartate has also been reported in various organisms including plants (Pandey and Gayen, 2024).

Phosphorylation is a reversible modification that can be undone through dephosphorylation, a process facilitated by phosphatase enzymes, while kinases are responsible for catalysing phosphorylation (Figure 4) (Raju and Sankaranarayanan, 2025). Notably, approximately 1–2% of eukaryotic genes encode protein kinases, emphasizing the crucial role of phosphorylation in cellular regulation. This process influences nearly all essential biological activities, including the cell cycle, growth, gene expression, differentiation, apoptosis, defence signalling, and cellular communication. It is estimated that about 30% of the entire eukaryotic proteome undergoes phosphorylation at least once in its lifetime. The continuous interplay between phosphorylation and dephosphorylation serves to activate or inhibit protein activity, receptors, enzyme functions, and cellular processes across different compartments (Pandey and Gayen, 2024).

A diagram of a phosphatase

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Figure 4. Visual representation of reversible phosphorylation catalysed by kinase and phosphatase (Chauhan, 2021).

Phosphorylation exhibits significant crosstalk with other PTMs. For example, there is a large amount of overlap between phosphorylation, acetylation and ubiquitination (Wang et al., 2025).

**2.1.d. Acetylation**

Acetylation refers to reversible process of transferring acetyl groups, often from acetyl-coenzyme A (Ac-CoA) to protein lysine residues. This covalent addition is orchestrated by specific enzymes called lysine acetyltransferases (KATs), (Figure 5). Lysine deacetylases (KDACs) catalyse the inverse reaction by removing acetyl groups from proteins (Di Martile et al., 2016). It is a widespread PTM with critical roles in numerous cellular processes (Pandey and Gayen, 2024).

A diagram of a catheter

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KDACs

Figure 5. Visual representation of reversible acetylation catalysed by lysine acetyltransferases and lysine deacetylases (Di Martile et al., 2016).

Acetylation is involved in processes like genome maintenance, epigenetic regulation, protein synthesis/degradation, signalling pathways, apoptosis, cell cycle, autophagy DNA repair, transcriptional regulation, and stress tolerance (Wang et al., 2025). Aberrations in acetylation are frequently associated with various diseases, highlighting its importance in maintaining cellular homeostasis and its potential as a therapeutic target.

**2.1.e. Ubiquitination**

Itis a post-translational modification that involves the covalent binding of ubiquitin, a 76-amino acid protein, to target substrate proteins. This process is catalysed by a series of enzymes: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s) (Figure 6), (Song and Luo, 2019). The E1s activate ubiquitin in an ATP-dependent manner, E2s determine the specific linkage of the ubiquitin chain, and E3 ligases link the target protein to a specific E2, selecting the protein to be ubiquitinated (Lai et al., 2024). The ubiquitin chain can consist of four or more ubiquitin molecules to mark a protein for degradation (Pandey and Gayen, 2024). Ubiquitination regulates a wide variety of cellular processes, including DNA repair, cell cycle, autophagy, and transcriptional regulation. It is also involved in cell signalling, apoptosis, protein processing, and immune responses (Pandey and Gayen, 2024). Due to the ubiquity of signalling molecules, protein phosphorylation and ubiquitination exert a profound influence on most life processes. Abnormalities in this PTM are implicated in various physiological and pathological conditions, including reproduction, cancer, stress responses, inflammation, and metabolic diseases (Lai et al., 2024).

A diagram of a molecule

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Figure 6. The visual representation of ubiquitination cascade. (Song and Luo, 2019).

**2.1.f. SUMOylation**

SUMOylationis a reversible PTM that involves the covalent and reversible addition of small ubiquitin-like modifiers (SUMO) to specific target proteins. SUMO enzyme is linked to the specific substrate via SUMO-activating enzyme(SAE or E). For example, SUMO E3 ligase transfers SUMO-GG to substrates in a similar way to ubiquitination, forming the target proteins. SUMOylation is regulated by the family of sentrin-specific proteases (SENPs), which primarily play a decoupling role in SUMOylation (Figure 7).

A diagram of a structure

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Figure 7.  Illustration of the protein SUMOylation cascade (Wild et al., 2024).

In mammalian cells, there are six SENP isoforms that cater to deSUMOylation (Raju and Sankaranarayanan, 2025). SUMOylation influences protein function by either concealing or exposing interaction sites, which affects protein binding. This modification also plays a role in subcellular localization, alters enzymatic activity, and enhances the transcription of specific genes by attracting essential transcription factors (Pandey and Gayen, 2024).

**2.2. Characterization Methods**

The increasing significance of biopharmaceuticals, such as monoclonal antibodies (mAbs) and recombinant proteins, necessitates thorough analytical characterization to ensure their safety and efficacy. A critical aspect of this characterization involves the identification and analysis of PTMs, which can significantly impact a biopharmaceutical's structure, function, stability, and immunogenicity. Monoclonal Antibodies (mAbs), being complex glycoproteins, are particularly susceptible to various PTMs, including glycosylation, oxidation, deamidation, and glycation. Several sophisticated methods have been developed and applied to this task, playing a crucial role in drug development by identifying critical quality attributes (CQAs) and guiding process optimisation (Prinston et al., 2025). The characterization of protein post-translational modifications relies on a range of sophisticated analytical techniques to identify, locate, and quantify these modifications, and to understand their functional implications (Wang et al., 2013).

**2.2.a. Mass Spectrometry (MS)-based methods**

Mass Spectrometry is analytical technique used across various scientific disciplines for the identification and quantification of analytes by measuring their mass-to-charge ratio (m/z)

For an analyte to be analysed by MS, it must be in an ionic form. This is achieved using an ion source, which is typically the first component of a mass spectrometer (Hariharan and Johnson, 2024). Various ionisation techniques exist, including:

Electron Ionization (EI), which involves bombarding the vaporised sample with high-energy electrons, leading to the formation of radical cations and often causing fragmentation.

Chemical Ionization (CI), utilising ion-molecule reactions between an ionised reagent gas and the analyte to produce molecular ions with less fragmentation.

Electrospray Ionization (ESI), based on generating ions from a liquid solution by forming charged droplets that undergo solvent evaporation and fission. ESI is known for producing ions with little to no fragmentation.

Photoionization (PI), Involves the absorption of a photon by an atom or molecule, causing the ejection of an electron and the formation of a cation (Hariharan and Johnson, 2024).

After separation, the ions are detected by a detector, which measures the abundance of ions at each m/z value. This data is then used to generate a mass spectrum, a plot of ion abundance versus m/z (Figure 8).

A graph of a graph showing different types of ion

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Figure 8. An example of mass spectrum. Axis Y represents ion abundance, axis X represents mass-to-charge ratio (m/z) (Kitson et al., 1996).

Different types of mass analysers offer varying resolution and mass accuracy. Mass spectrometers are broadly classified into low-resolution (LR-MS) and high-resolution (HR-MS) instruments. HR-MS can distinguish between ions with very small differences in mass, allowing for the identification of different chemical species, including isomers and isotopologues, which is particularly important for complex mixtures like atmospheric particles (Hariharan and Johnson, 2024).

Quadrupole Mass Analyser (QMA/QMS) is a low-resolution mass analyser suitable for quick identification and determining the abundance of different chemical components.

Time-of-Flight (TOF) is a high-resolution technique where ions are accelerated through an electric field and their mass is determined by the time it takes them to reach a detector.

Orbitrap, another high-resolution mass analyser known for its high mass accuracy.

Finally, Fourier Transform Ion Cyclotron Resonance (FT-ICR) is a high-resolution technique that uses a magnetic field to trap ions and determine their m/z based on their cyclotron frequency.

**2.2.b. Chromatography**

In essence, chromatography is a separation technique that relies on the differing interactions of mixture components with stationary and mobile phases, enabling their isolation and subsequent analysis. There are number of different types of chromatography techniques and specific of employed depends on the nature of the analyte and the desired separation (Khan Academy, 2018).

In the pharmaceutical analysis context, Hydrophilic Interaction Liquid Chromatography (HILIC) separates polar, hydrophilic, and charged analytes which often include modified biomolecules. It is a crucial technique for glycosylation analysis at the peptide and protein levels, maintaining site-specific information and allowing direct UV detection, avoiding the need for fluorescence labelling (Wei et al., 2025).

Reversed Phase Liquid Chromatography (RPLC) is the most used liquid chromatography technique in pharmaceutical analysis and is widely applied in LC-MS/MS for peptide analysis, which is essential for characterizing post-translational modifications. In RPLC, the stationary phase is non-polar, and the mobile phase is polar. This means that more hydrophobic molecules will have a greater affinity for the stationary phase and will be retained longer. It enables the separation of modified protein variants based on subtle changes in their hydrophobic properties, and its compatibility with MS makes it a powerful tool for the identification and characterization of these modifications. It plays a significant role in the analysis of therapeutic proteins and recombinant monoclonal antibodies (Fekete and Guillarme 2012).

Ion-exchange chromatography (IEX) is a separation method where molecules are separated based on their net surface charge. It utilises a solid stationary phase (resin) that carries ionic charges and a liquid mobile phase (buffer). The separation occurs due to the electrostatic interactions between the charged molecules (analytes) in the mobile phase and the oppositely charged groups on the stationary phase (Khan Academy, 2018).

Mixed-Mode Chromatography (MMC) combines multiple separation mechanisms, including HILIC, ion-exchange (IEX), size exclusion, and reversed-phase, offering unique selectivity for complex analytes, including those with PTMs (Figure 9). MMC's diverse interaction capabilities are valuable for challenging analyses of modern pharmaceuticals and biotechnological products, and it is also compatible with MS detection (Wei et al., 2025).

A graph of a chemical reaction

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Figure 9. Chromatogram of the separation of 25 commonly used pharmaceutical counterions using a mixed-mode chromatography technique (Wei et al., 2025).

Affinity chromatography is a separation technique that relies on the specific binding interaction between an analyte and a ligand that is immobilised on a stationary phase. This technique is employed in various applications, including the enrichment of antibody variants with differential affinity to a target, the purification of biopharmaceuticals, and the purification of vaccines (Ma et al., 2025).

**2.2c. Electrophoresis**

Electrophoresis is a method that utilizes an electric field to separate charged molecules such as proteins, nucleic acids, and carbohydrates. This technique helps determine the size, charge, structural features, and relative concentration of biological molecules (Hagness et al., 2023). Gel electrophoresis (e.g., SDS-PAGE, PAGE) separates biomolecules based on their size and charge through a porous gel medium like polyacrylamide. It can be used to estimate the Molecular Mass (MM) or Molecular Weight (MW) of unknown molecules and assess the shape or conformation of a protein. However, it has limitations such as limited resolution for complex mixtures, potential interference from the sample matrix, and less accurate quantification based on band absorbance. High concentrations of molecules are also often needed, making it potentially expensive and time-consuming. Figure10, illustrates an electrophoresis gel layout. Non-reducing SDS-PAGE can be used to detect the unfolding of IgG domains. Zone Gel Electrophoresis or PAGE uses a polyacrylamide gel as the medium for separating proteins based on their Molecular Weight (Santamaría et al., 2024).

A diagram of a gel

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Figure 10. Visual representation of (a) Electrophoresis gel layout where all components involved in the process, (b) antibodies fragments with their corresponding Molecular Mass (Santamaría et al., 2024).

Phosphoprotein Differential Gel Electrophoresis (Phospho-DIGE) technique has been used to identify phosphoproteins involved in specific biological pathways, such as those related to tumour growth in endometrial cancer (Figure11). It uses fluorescent labels and gel electrophoresis to visually compare the amounts of phosphorylated proteins in different samples, allowing researchers to pinpoint which proteins have altered phosphorylation levels in various conditions (Capaci et al., 2023). Phospho-DIGE allows for the comparison of phosphorylation levels between different protein samples (Raju and Sankaranarayanan, 2025).

A close-up of a microscope

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Figure 11. A 2D-DIGE map comparing phosphoproteomics of normal endometrium (C) and endometrial cancer (EC) enriched using IMAC columns. The first dimension utilized immobilized pH gradient (IPG) strips with a pH range of 4–7, while the second dimension was performed on a 12% polyacrylamide gel. Numbered circles highlight differentially phosphorylated spots (Capaci et al., 2023).

Capillary electrophoresis (CE) is a technique based on the principle of electrophoresis, which involves the directional movement of charged species in the presence of an electric field. As a variant of electrophoresis, CE utilizes a capillary tube as the separation channel. When an electric field is applied, charged molecules migrate towards the electrode with the opposite charge, and their separation is influenced by their size and charge (Hagness et al., 2023).

Different designs exist within CE, such as microchip capillary electrophoresis (MCE), which can be used for techniques like MCE with sodium dodecyl sulphate (SDS) for protein analysis (Prinston et al., 2025). Furthermore, CE can be adapted for specific applications like affinity capillary electrophoresis to determine binding strengths and immunoaffinity capillary electrophoresis for quantifying low-abundance substances. Importantly, CE can be coupled with other analytical techniques, such as mass spectrometry, to provide more detailed information about the separated molecules (Prinston et al., 2025). This makes CE a versatile tool in various scientific disciplines, including the analysis of proteins and nucleic acids. In the context of biosensors, CE can be used to separate sample components before they are detected by a sensing element (Hagness et al., 2023).

**2.3. Limitations of individual techniques combination of analytical methods**

High-resolution mass spectrometry (HRMS) can struggle to detect and quantify low-abundance analytes, including proteins with specific PTMs, especially amidst a background of highly abundant endogenous molecules. Targeted low-resolution mass spectrometry often exhibits better sensitivity for known compounds.  (Flasch et al., 2023). In mass spectrometry imaging (MSI) and other MS-based techniques, the presence of certain ions can suppress the ionisation of others, limiting the detectability of low-abundant analytes and those with low ionisation efficiency. This can hinder the identification of specific PTM-containing molecules in complex mixtures (Mavroudakis et al., 2025) While HRMS offers high resolution, some MS techniques like MALDI-TOF MS can have limited resolution and mass accuracy, particularly in linear mode. This makes it challenging to definitively identify proteins or specific PTMs based solely on mass, as observed in attempts to identify serotype-specific proteins in *Streptococcus pneumoniae* (Zintgraff et al., 2023). Mass spectrometry alone may struggle to distinguish between isomers (molecules with the same mass and formula but different structures) and isobars (molecules with the same nominal mass but slightly different exact masses). This limitation makes it difficult to definitively annotate specific metabolites or modified proteins without prior separation, as illustrated in the spatial metabolomics study where valine and betaine (isomers) could not be distinguished by MSI alone (Mavroudakis et al., 2025). In native mass spectrometry (nMS), there are limitations in studying solely hydrophobically mediated interactions, as these can be more labile in the gas phase environment of the mass spectrometer (Britt and Robinson 2025)

Reversed-phase liquid chromatography (RPLC), a predominant LC mode in pharmaceutical analysis, often struggles with retaining highly polar analytes, including some PTMs or modified peptides. This necessitates the use of alternative techniques like HILIC and MMC for such analyses (Wei et al., 2025). Some HPLC methods developed for specific applications, such as petroleum analysis, may suffer from a lack of validation using real-world samples, lack of calibration data, and incomplete analytical figures of merit, which restricts their wider practical use for PTM analysis in other fields (Bhanu et al., 2024). While HILIC is useful for polar compounds and PTM analysis is not universally adaptable to all proteins or stationary phases, and its complex retention mechanism makes it difficult to predict analyte behaviour. This can complicate method development for PTM analysis (Tengattini et al., 2024). Compared to RPLC, hydrophilic interaction chromatography (HILIC) still faces challenges in terms of method consistency, robustness, and validation success, which can limit its application in routine quality control for proteins with PTMs. Issues like unpredictable chromatographic behaviours and poor reproducibility remain. (Tengattini et al., 2024). Certain chromatographic methods, like some HPLC applications in petroleum analysis using perfluorocarbon solvents, can be environmentally unfriendly and operationally costly (Bhanu et al., 2024). Size exclusion chromatography (SEC), while useful for separating molecules based on size, may have limited resolution for complexes of similar size, which could be relevant when analysing proteins with different PTMs affecting their oligomeric state (Wu et al., 2024). Peptide mapping, often employing LC-MS for PTM analysis, can involve tedious sample preparation steps. (Bhanu et al., 2024).

Electrophoresis can have limited resolution when dealing with complex mixtures or closely related analytes, such as protein isoforms with subtle differences due to various PTMs. The sample matrix itself can interfere with analyte migration, leading to distorted results or reduced separation efficiency in electrophoresis. Quantification of PTM-modified proteins based on the absorbance intensity of bands or spots on a gel may not be sufficiently accurate for detailed quantitative PTM analysis. Achieving good results with electrophoresis often requires high concentrations of molecules, which can be limiting when dealing with low-abundance PTM variants. Electrophoresis can be a complex and time-consuming procedure that requires qualified personnel. Some electrophoresis techniques require protein denaturation, which can alter the structure and properties of the protein, potentially losing information about native PTM-dependent interactions or conformations (Santamaría et al., 2024).

In essence the complexity of biological systems and the diverse nature of PTMs necessitate a multi-faceted analytical approach. By combining techniques that offer different separation mechanisms, enrichment capabilities, and detection principles, researchers can gain a completer and more accurate picture of protein post-translational modifications and their functional roles.

**2.4. Combination of analytical methods**

As previously mentioned, mass spectrometry is a powerful tool for studying PTMs primarily because PTMs often result in a change in the mass of a protein or peptide. This mass difference can be detected with high accuracy by a mass spectrometer, allowing for the identification and characterisation of modifications (Du and Cooper, 2025). LC-MS/MS (Liquid Chromatography-Tandem Mass Spectrometry) is frequently employed to analyse peptides and identify the presence and location of PTMs. Its high sensitivity and ability to provide detailed structural information make it invaluable for identifying and quantifying a wide range of PTMs (Guapo et al., 2022). This typically involves separating peptides using liquid chromatography and then analysing their mass-to-charge ratio in the mass spectrometer. Tandem mass spectrometry (MS/MS) further fragments these peptides to provide sequence information and pinpoint the modified amino acid residue. High-resolution mass spectrometry is crucial for the specific and sensitive detection of protein alterations. Software tools are used to process the raw MS data, obtain the intensity of PTM peptides, and identify differentially modified peptides (Hariharan and Johnson, 2024).

Peptide Mapping: LC-MS-based peptide mapping is routinely used for comprehensive characterization of recombinant proteins, including mAbs. Digestion of the protein with proteases like pepsin or trypsin generates peptides that are then separated by LC and analysed by MS. This approach provides sequence coverage and PTM information, aiding in product understanding and manufacturing process development. Studies have reported the identification and quantification of various PTMs on AAV capsid proteins, a delivery mechanism for gene therapy, using fast and efficient digestion methods followed by LC-MS. These identified PTMs represent quality attributes that can be monitored during product development (Guapo et al., 2022).

As highlighted affinity chromatography, in the context of target affinity enrichment, plays a significant role in isolating modified protein variants based on their binding properties. By immobilizing a target ligand, proteins with varying affinities due to PTMs can be separated. Subsequent analysis of the enriched fractions, often by MS, allows for the identification of PTMs that impact binding. This approach is particularly valuable for understanding the functional consequences of PTMs on therapeutic antibodies (Prinston et al., 2025).

For example, MS analysis of weaker binding fractions obtained through affinity chromatography demonstrated the enrichment of common PTMs like deamidation, glycation, and sialylation. The presence and abundance of less common modifications, such as atypical glycosylation of Fab fragments, can also be detected and should be closely monitored (Prinston et al., 2025).

The chemical modifications to proteins after their synthesis can dramatically alter their charge, size, and isoelectric point (pI), properties that form the basis of electrophoretic separation. By exploiting these changes, various electrophoretic techniques provide valuable insights into the presence, heterogeneity, and impact of PTMs on proteins and other biomolecules. Traditional gel electrophoresis, employing porous gels like polyacrylamide, remains a widely used technique for separating biomolecules, including proteins with PTMs, based on their size and charge(Hagness et al., 2023). The power of electrophoresis in PTM analysis is significantly enhanced when coupled with mass spectrometry (MS). Techniques like capillary electrophoresis-mass spectrometry (CE-MS) allow for the direct analysis of electrophoretially separated molecules by MS, providing precise mass information and enabling the identification of the specific PTMs present (Wei et al., 2025).

**2.5. Case Studies**

Glycosylation is a critical quality attribute for many therapeutic proteins. Hydrophilic Interaction Liquid Chromatography coupled with Mass Spectrometry (HILIC-MS) has emerged as a powerful tool for detailed glycosylation profiling at the glycan, glycopeptide, and protein subunit levels (Wei et al., 2025). Advancements in HILIC, such as the development of wide-pore columns around 2015, have facilitated the direct analysis of large glycoproteins like full-length antibodies and their subunits, enabling the resolution of important glycoform (Wei et al., 2025). The use of Immunoglobulin-degrading enzyme from Streptococcus pyogenes (IdeS) to cleave IgG, followed by HILIC-MS analysis of the Fc fragment, has become a standard middle-up/middle-down strategy for glycosylation profiling of mAbs. This detailed characterization of Fc glycans is crucial as they can influence effector functions and pharmacokinetics (Wei et al., 2025).

Glycan profiling (Figure 12) is a process that involves enzymatic cleavage of glycans from proteins using peptide N-glycosidase-F (PNGase F), followed by fluorescent labelling with a tag like 2-aminobenzamide (2-AB). The labelled glycans are then separated using hydrophilic interaction liquid chromatography (HILIC) and analysed through fluorescence detection and mass spectrometry for identification and quantification (Wei et al., 2025).

Another technique used for glycosylation characterization is RapiFluor-MS, a fluorescent labelling method designed for rapid derivatization of N-glycosylamine within five minutes. This approach enables automated, high-throughput analysis of protein glycosylation (Wei et al., 2025).

A graph of a person's body

AI-generated content may be incorrect.Figure 12. HILIC Separation of 2-AB labelled glycans released from human IgG (Wei et al., 2025).

Analytical techniques for studying methylation, particularly protein methylation, are predominantly based on mass spectrometry (MS), often coupled with separation methods such as liquid chromatography (LC) (Khandelwal and Rout, 2023).

PRmePRed and DeepRMethylSite are another helpful tool for methylation site prediction. PRmePRed (Protein Arginine Methylation Prediction), utilizes machine learning-based models trained on experimentally verified methylation sites. It uses sequence-based features such as amino acid composition and physicochemical properties, allowing to predict whether an arginine residue is likely to be methylated (Figure 13). DeepRMethylSite (Deep Learning-Based Arginine Methylation Site Prediction) uses deep learning (e.g., convolutional neural networks, recurrent networks) also to predict methylation sites. Learns sequence patterns directly from raw protein sequences, eliminating the need for manually engineered feature (Raju and Sankaranarayanan, 2025).

A diagram of a data preparation process

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Figure 13. The PRmePRed model designed to predict arginine methylation sites through a multi-step process. (A) Data is collected, redundant sequences are removed, and data balancing is performed using under-sampling. (B) Feature extraction is conducted, incorporating dipeptide composition, physicochemical properties, amino acid composition, and information theory-based features. (C) Various machine learning algorithms are employed to construct the predictive model. (D) The model is evaluated using multiple performance metrics through 10-fold cross-validation. (E) The SHAP algorithm is applied to interpret the model's outputs (Khandelwal and Rout, 2023).

The key analytical technique for studying phosphorylation mentioned in the sources is mass spectrometry, particularly when coupled with liquid chromatography (LC-MS/MS), it is described as a cornerstone of modern phosphoproteomics (Wang et al., 2025). This technique allows for the identification and quantification of phosphorylated peptides. The process typically involves protein extraction and digestion (often using trypsin) to generate peptides. It is followed by peptide fractionation using high-performance liquid chromatography (HPLC) systems. Finally, by analysis of the resulting peptides by mass spectrometry to identify those containing phosphorylation modifications and (Wang et al., 2025). To enhance the detection of low-abundance phosphopeptides, enrichment techniques are commonly used prior to LC-MS/MS analysis. One source specifically mentions using high-select™ phosphopeptide enrichment kits (Wang et al., 2025). Hydrophilic Interaction Liquid Chromatography (HILIC) is highlighted as an effective technique for separating peptides with various post-translational modifications, including phosphorylation (Wei et al., 2025).

The study of acetylation involves diverse techniques, including biochemical assays, mass spectrometry, and genetic engineering approaches. Genetically encoded PTMs (GCE). By enabling the precise incorporation of modified amino acids or their mimics into proteins, GCE allows researchers to directly investigate the functional consequences of specific PTMs in living cells and to uncover novel regulatory mechanisms. GCE can be used to site-specifically incorporate acetyl-lysine into proteins to study its functional effects. (Gu et al., 2024).

To analyse ubiquitination thoroughly, advanced techniques like Stochastic Orthogonal Recoding of Translation (SORT) are used. This method helps identify ubiquitination sites marked by a di-glycine (diGly) remnant, a key marker of the ubiquitin isopeptide bond that forms after trypsin digestion. When trypsin breaks down proteins, ubiquitinated lysine residues leave behind a distinctive di-glycine (GG) tag on the modified lysine. SORT allows researchers to detect this diGly modification on non-canonical amino acids (ncAAs) incorporated into proteins, using LC-MS/MS analysis. If a specific fragment of the peptide (GGM) is detected, it suggests that ubiquitination has occurred at the site where lysine was replaced by the ncAA (such as the MetK site). In short, SORT is a powerful tool to examine lysine residues across the entire proteome. By substituting these residues with ncAAs and searching for the diGly signature of ubiquitination using mass spectrometry, researchers can map ubiquitination events in cells. This approach is crucial for understanding the role of ubiquitination in various cellular functions and diseases (Gu et al., 2024).

The analytical techniques most relevant to studying SUMOylation that are mass spectrometry and LC-MS-based methods for direct detection and characterisation also genetically encoded non-canonical amino acids (GCE) combined with MS for mechanistic studies, proteomics approaches for global analysis of SUMOylated proteins, functional studies in model organisms to assess the biological impact, and bioinformatic tools for prediction and guiding experimental design (Lai et al., 2024).

**2.6. Impact of PTM and characterization studies**

Successful PTM characterization studies have significantly impacted drug development in several ways:

Firstly, it is an identification of Critical Quality Attributes (CQAs), by thoroughly characterizing the PTM landscape of a biopharmaceutical, critical modifications that affect its safety and efficacy can be identified and defined as CQAs. For instance, the characterization of specific glycosylation patterns on mAbs influencing their effector functions has led to the establishment of these patterns as key quality attributes that need to be controlled during manufacturing (Wei et al., 2025).

Also, the understanding which PTMs occur and under what conditions helps optimise manufacturing processes to minimise undesirable modifications and maximise the formation of beneficial ones. For example, the identification of deamidation as a prevalent PTM can prompt adjustments in cell culture conditions or purification steps to mitigate its occurrence (Guapo et al., 2022).

Detailed characterization of affinity-enriched fractions containing specific PTMs can guide the development of assays that are sufficiently sensitive to detect these modifications during product release, ensuring consistent product quality (Prinston et al., 2025).

When manufacturing processes are changed or biosimilars are developed, comprehensive PTM analysis is crucial for demonstrating product comparability to the originator product. Techniques like HILIC-MS are valuable tools for comparing glycosylation profiles and other PTMs between different batches or products (Wei et al., 2025).

Characterizing PTMs and their impact on binding affinity, stability, and other functional properties provides critical insights into the structure-function relationships of biopharmaceuticals. This knowledge is essential for rational drug design and development (Prinston et al., 2025).

**3. Summary**

In conclusion, protein post-translational modifications are fundamental to the complexity and dynamic regulation of biological systems. These chemical alterations, occurring after protein synthesis, dramatically expand the functional repertoire of the proteome by modifying protein structure, activity, localisation, and stability. As molecular switches, PTMs are integral to a vast array of cellular processes, including metabolism, signalling, gene expression, and immunity, highlighting their central role in maintaining cellular homeostasis. The disruption of PTM mechanisms and the phenomenon of PTM crosstalk are increasingly recognised as key factors in the pathogenesis of various diseases, such as cancer, metabolic disorders, and neurodegenerative conditions.

Given the profound impact of PTMs, their thorough characterisation is of paramount importance, particularly in the development and manufacturing of biopharmaceuticals like monoclonal antibodies. mAbs, being complex glycoproteins, are susceptible to a diverse range of PTMs that can significantly affect their safety, efficacy, and immunogenicity. Therefore, the identification, localisation, and quantification of these modifications are essential for defining critical quality attributes and guiding the optimisation of manufacturing processes. Advanced analytical techniques, including various forms of mass spectrometry and chromatography, coupled with electrophoretic methods, play a crucial role in achieving this detailed characterisation.

Ultimately, a thorough understanding of post-translational modifications (PTMs) and the ability to accurately characterize them are crucial for enhancing our knowledge of basic biological processes, creating better diagnostic tools and targeted treatments for various diseases, and ensuring the quality and effectiveness of important biopharmaceutical products. The continued development and application of sophisticated analytical methodologies will be critical for further elucidating the intricate world of protein post-translational modifications and harnessing their potential for the benefit of human health.

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