

# Post-translational modifications and their applications in eye research (Review)

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**Abstract.** Gene expression is the process by which genetic information is used for the synthesis of a functional gene product, and ultimately regulates cell function. The increase of biological complexity from genome to proteome is vast, and the post-translational modification (PTM) of proteins contribute to this complexity. The study of protein expression and PTMs has attracted attention in the post-genomic era. Due to the limited capability of conventional biochemical techniques in the past, large-scale PTM studies were technically challenging. The introduction of effective protein separation methods, specific PTM purification strategies and advanced mass spectrometers has enabled the global profiling of PTMs and the identification of a targeted PTM within the proteome. The present review provides an overview of current proteomic technologies being applied in eye research, with a particular focus on studies of PTMs in ocular tissues and ocular diseases.

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## 1. Introduction

Over the last two decades, genomics has been regarded as the most popular and productive research field in biological science. However, the intermediate mRNA transcript may rapidly degrade (1) or undergo alternative splicing (2), which leads to a number of variable outcomes that renders the study of biological systems more challenging. Unlike ribosomal proteins or enzymes, which are relatively stable, the majority of proteins involved in the cell cycle demonstrate a rapid turnover rate and may function alongside a degradation process (3). As functional products of the biological system, proteins are direct and crucial participants in all downstream biochemical pathways.

Post-translational modification (PTM) is a key step in protein biosynthesis, whereby the addition, folding or removal of functional groups leads to drastic alterations in protein function (4). For instance, an Alzheimer's disease associated protein, Tau, is phosphorylated at 40 different sites, which produces site-specific phosphorylation that are responsible for different stages of the disease (5,6). Large-scale studies of proteins and PTMs are often included in proteomic research at present. Robust peptide separation methods that employ strong cation exchange, high performance liquid chromatography (HPLC), novel mass spectrometry (MS) designs with high resolution and sensitivity, such as Orbitrap and TripleTOF, as well as PTM-specific bioinformatics tools and databases, have rendered the profiling of unique or multiple PTMs in biological proteomes possible. Proteomic analysis of PTMs has facilitated the identification of novel biomarkers for a number of diseases, including prostate cancer (7), pancreatic cancer (8) and rheumatoid arthritis (9). However, few proteomic-based studies have been performed to explore PTMs associated with eye diseases. It is thought that gaining an increased understanding of the characteristics of PTMs through the use of emerging MS techniques, is important for the development of effective diagnostic and therapeutic strategies for various disorders, including eye

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diseases (10). The present review will discuss the technological challenges in protein research, provide an overview of the types and mechanisms of PTMs, as well as their application in the research of eyes and associated diseases.

## 2. Proteomics analysis technology

**Protein separation methods.** Large-scale proteomic analysis typically involves gel-based and liquid chromatography-based separation strategies prior to MS analysis. It is possible to categorize these into electrophoretic and chromatographic approaches. For electrophoretic separation strategies, two-dimensional gel electrophoresis (2DE) has been widely used for separating soluble proteins based on their isoelectric points and molecular weights (11). However, membrane proteins, which serve vital functions in signal transduction pathways, are frequently difficult to separate using 2DE due to their high hydrophobicity (11). In addition, these approaches are technically insufficient due to a low dynamic range and throughput, which are important for analyzing complex biological samples. Therefore, the use of chromatography, including ion-exchange chromatography (12), hydrophobic interaction chromatography (13), size-exclusion chromatography (14), affinity chromatography (15), and the most popular, reverse-phase high-performance liquid chromatography (16,17), have become the more common separation strategies used in high-throughput liquid chromatography-based proteomic research.

**Protein identification strategies.** MS has advanced qualitative and quantitative analysis of unknown organic and inorganic compounds in numerous fields, including environmental contaminant monitoring (18), forensic toxicology, doping analysis (19) and analysis of clinical samples (20), and is currently an indispensable tool in proteomics research. A typical mass spectrometer consists of an ionization source, a mass analyzer and a detector. Analytes are ionized to a gas phase and are subsequently processed by the mass analyzer and detector. The resultant mass spectra, expressed as mass/charge ratios, are compared with protein databases for identification (21).

Currently, there are two major ionization strategies employed for MS. The first is a matrix-assisted laser desorption/ionization (MALDI) approach, by which analyte desorption is induced by a laser beam and then absorbed by the matrix material (22). The molecules are then ionized to gases (23). The remaining approach is electrospray ionization (ESI), which was invented by Dole *et al* in 1968 (24). This technique was developed further by John Fenn, who was awarded a Nobel Prize in 2002 for this invention (25). Acidic liquid analytes acquire a positive electro-charge as they are sprayed into small droplets. They subsequently produce ions and enter the mass spectrometer. ESI allows a continuous flow of analytes with a variable flow rate, and generates multi-charged positive ions depending on the acidity of the solvent. This technique permits the identification of large molecular weight proteins by MS (26).

Collision-induced dissociation (CID) is the most widely used fragmentation method. It allows ions to collide with neutral gas molecules, which leads to internal energy conversion and fragmentation of the precursor ion (27). CID is effective in

detecting small, low-charged peptides and single-charged ions (28); however, is not as effective at detecting long peptides. Due to the presence of PTMs, the digestion of long peptides by CID fragmentation becomes less effective. Therefore, labile PTMs, such as phosphorylation and S-nitrosylation, may be lost during CID collision (29). Furthermore, the presence of several basic amino acid residues in PTM proteins has been demonstrated to inhibit random protonation along peptide backbones by CID (28). These factors hinder the generation of efficient fragment ions by CID. To date, electron-transfer dissociation (ETD) is considered to be more favorable for PTM studies (30). This method transfers electrons to multi-protonated proteins or peptides, which leads to N-C $\alpha$  backbone bond cleavage. ETD is useful for the identification of labile PTMs. It provides the protein sequence information and modification sites, and it is frequently used for PTM analysis (31). Previous studies have reported that ETD is particularly suitable for detecting peptides with >2 charges (32,33). However, it has been suggested that ETD yields a reduced number of identified proteins due to its lower scanning rate when compared with CID (29,31). Several studies have compared the efficiency of CID and ETD in identifying PTMs (34-36). In general, CID and ETD are able to detect stable PTMs successfully, including acetylation and methylation, while ETD is the optimal strategy for identifying labile PTMs, including phosphorylation, ubiquitination and glycosylation. Multiple studies have suggested combining CID and ETD in order to gain higher competency and accuracy in PTM studies (34-40).

**Protein quantification strategies.** Advantages of applying large-scale proteomics strategies for biological research include profiling of the proteome for protein identification and quantification of protein expression. It is possible to achieve such quantification using chemical labeling strategies, in which the ion intensity between labeled and unlabeled peptides or differentially labeled peptides is compared. Isobaric tagging for relative and absolute quantification uses isobaric amine-specific tandem mass tags to label the N-terminus and lysine residues of digested peptides. It is then possible to compare or analyze up to eight different sets of samples in a single experiment (41). Additional popular labeling strategies include isotope protein coded labeling (42), which uses isotope-coded tags instead of isobaric tags to label the N-terminus and lysine residues. Dimethyl multiplexed labeling is an economical option, whereby the N-terminus of peptides and  $\alpha$ -amino groups of lysine residues are labeled with water-soluble formaldehyde via reductive methylation (28). Stable isotope labeling by amino acids in cell culture (SILAC) is an *in vivo* strategy, which involves feeding cell cultures with amino acids containing stable isotopes. During SILAC, lysates of labeled and normal cell cultures are mixed, digested and analyzed by MS, and the relative peak intensities in the MS spectrum are subsequently compared and analyzed (43). Despite the effectiveness of using labeling strategies in the quantification of the proteome, these are time-consuming, expensive and complicated in terms of sample preparation procedures and data analysis. Novel and accurate label-free quantification methods are gradually emerging.

Label-free quantification is a fast and low-cost strategy for measuring large-scale differential proteomic expression.

Table I. List of typical and important PTMs and their biological significance.

PTMs	Modification subtypes	Biological functions
Phosphorylation	pSer, pThr, pTyr (most common), pHis, pCys and pAsp (least common)	Reversible; regulating signaling pathways by activating and inhibiting enzymes
Glycosylation	N-linked, O-linked, C-mannosylation and GPI anchor.	Molecular interaction; signal transduction; cell recognition
Acetylation	N-terminal (most common), C-terminal and histone (less common)	Reversible; protein localization, stability and synthesis; cell-to-cell interaction; apoptosis
Ubiquitination	-	Protein degradation
Sumoylation	-	Reversible; protein-protein interaction, protein stability and localization
Methylation	N-/O-terminal	Gene transcription and signal transduction
Lipidation	GPI anchors, N-myristoylation, S-palmitoylation and S-prenylation	Protein activities and targeting

PTM, post-translational modification; GPI anchor, glycosylphosphatidylinositol anchor.

These approaches are divided into two major strategies; the first requires cross-checking of the numbers of MS/MS spectra acquired for peptides of proteins between different standard samples (44), and the second strategy is based on the measurement and comparison of chromatographic peak areas of peptide precursor ions between different samples (45). There are currently multiple open source and commercial software packages available for the processing of label-free quantification data, including MZmine, MsInspect, MapQuant, SIEVE, Elucidator and OpenSWATH for SWATH analysis (46-49). These software programs facilitate peak detection and matching, data alignment, normalization and statistical analysis. However, limitations, such as the variation between different samples, remain a major concern.

### 3. Introduction of PTMs

To date, >400 types of PTMs have been reported, and >90,000 individual PTMs have been identified (50). Glycosylation, ubiquitination, phosphorylation, methylation and acetylation are commonly reported (4), while additional PTMs, such as adenosine monophosphate adenylation, are less frequently reported (51). As PTMs influence almost every aspect of cell biology, they are an important molecular factor for understanding biological and pathological mechanisms, and are summarized in Table I.

**Phosphorylation.** Phosphorylation is a chemical reaction that involves the transfer of a phosphate group from the  $\gamma$ -locus of adenosine 5'-triphosphate (ATP) or guanosine 5'-triphosphate to the side chain of an amino acid residue of a substrate protein molecule. This reaction is mediated by protein kinase catalysis, whereas its counterpart enzyme, phosphatase, catalyzes the de-phosphorylation reaction (52). Phosphorylation is the most widely studied PTM, due to its involvement in a wide range of cellular functions. For instance, it activates or inhibits various enzymes or receptors, thus regulating different signaling pathways that govern cell metabolism, growth and differentiation, the immune response, oncogenesis

and apoptosis (53-55). Phosphorylation of eukaryotic proteins has been demonstrated to occur most commonly on serine, threonine and tyrosine residues, which mediates the activity of numerous signaling networks involved in cell differentiation and proliferation (56). A higher frequency of phosphorylation on serine and threonine residues occurs when compared with tyrosine residues at a ratio of ~1,800:200:1 (pSer: pThr: pTyr) (57). By contrast, phosphorylation of histidine, cysteine and aspartate residues has been reported in bacteria, fungi and plants, as part of bi- and multi-component phospho-signaling transduction pathways (58,59). Furthermore, phosphorylation of histidine has been demonstrated to be involved in regulating metabolic signaling pathways in eukaryotic cells (60,61). In the last decade, various human genome projects have confirmed that genes coding for kinases and phosphatases comprise >2% of the human genome (62). In total, ~30% of the whole human proteome has been postulated to undergo phosphorylation during its life cycle (63).

**Glycosylation.** Glycation (non-enzymatic glycosylation) is the covalent bonding of a carbohydrate molecule to another molecule, which may occur under *in vivo* or *in vitro* conditions (64). In biological systems, the process of attaching glycans to lipids, proteins or additional organic molecules by catalysis is termed glycosylation (65). It is possible to divide glycosylation into four main subcategories, including N-linked and O-linked glycosylation, C-mannosylation and the glycosylphosphatidylinositol (GPI) anchor. N-linked glycosylation involves the attachment of an oligosaccharide to aspartic acid residues of secreted or membrane-bound proteins, and occurs primarily in the endoplasmic reticulum of eukaryotic cells (66). A similar binding strategy occurs between various sugars and serine and threonine residues primarily in the Golgi, nucleus and cytoplasm (67), termed O-linked glycosylation. C-mannosylation refers to the addition of a mannose oligosaccharide to the first tryptophan residue in the amino acid sequence via a carbon-carbon bond (68). Formation of the GPI anchor involves the covalent linkage of the C-terminus of a protein with the glycolipid portion of the membrane phospholipid located

on the extracellular side of the plasma membrane (69). This occurs in >50% of proteins (70) and affects multiple molecular activities, including the regulation of cellular interactions, signal transduction and molecular interactions. In addition, GPI anchors affect processes including the pathogenesis of several diseases, such as paroxysmal nocturnal hemoglobinuria (71), immunological protection, intercellular adhesion, cellular proliferation, inflammation, oncogenesis and viral replication (72,73).

**Acetylation.** Acetylation is the process of adding an acetyl group to a molecule. Its counterpart reaction, de-acetylation, is the removal of the acetyl group from a chemical compound. Acetylation of various proteins, including histone (74), p53 (75) and tubulin (76) have been reported. According to previous studies, three main categories of acetylation have been identified thus far; N-terminal acetylation, histone acetylation and acetylation of C-terminal residues (75,77). N-terminal acetylation is one of the most common PTMs and is catalyzed by N- $\alpha$ -acetyltransferases, which have been demonstrated to be expressed in >50% of cytosolic yeast proteins (78) and ~80-90% of mammalian proteins (78). N-acetylation is known to affect protein stability and prevent or generate specific degradation signals (79-81). It is involved in regulating and determining protein localization (82,83), as well as regulating the cellular life cycle and apoptosis (84,85). Previous studies have demonstrated its importance in protein synthesis (86) and protein-protein interactions (85,86). The  $\alpha$ -amino group of histone lysine residues was revealed to be consistently acetylated or de-acetylated by enzymes (87,88), which was subsequently demonstrated to be pivotal in regulating gene expression (79,89). Further studies have confirmed a close association between histone acetylation and the regulation of gene transcription in inflammation and cancers of the immune system (90-92). In addition, acetylation of C-terminal serine and threonine residues has been observed (93). Acetylation has been suggested to compete with phosphorylation of these C-terminal amino acid residues (94), and thus may be involved in regulating phosphorylation-associated signaling pathways. However, a more complete understanding of the involvement of C-terminal acetylation on PTMs remains to be elucidated.

**Ubiquitination and sumoylation.** Ubiquitin is a highly conserved protein (76 amino acids in length) that is abundant in eukaryotic cells. The process by which the last amino acid residue of ubiquitin is attached to a lysine residue of a protein by enzymatic catalysis is known as ubiquitination (95). The ubiquitin-bound protein then undergoes sequential reactions catalyzed by three different enzymes, namely ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3). Ubiquitination is commonly involved in the intercellular degradation of proteins, such as short-life and abnormal-life proteins (95). Abnormalities in the ubiquitin system have been associated with a number of pathogenesises, including neurodegenerative disorders (96,97), malignancies (98,99) and immunological disorders (100,101).

In a previous study, a reversible PTM modifier known as the small ubiquitin-like protein modifier (SUMO), was identified (76). The SUMO protein is covalently bound to a variety of proteins at their lysine residues. This process

is known as sumoylation, which is mediated by the SUMO-specific E1-activating enzyme heterodimer termed activator of Sentrin/SUMO-ubiquitin-like modifier activating enzyme 2, the E2 conjugating enzyme UBC9 and SUMO E3 ligase (102-105). SUMO proteins are ubiquitously expressed in numerous biological systems, and previous studies have demonstrated that a high frequency of sumoylation occurs in cell nuclei (106,107). Additional studies have revealed that sumoylation occurs in additional cellular structures, including the plasma membrane (108), endoplasmic reticulum (109) and mitochondria (110). Four SUMO protein isoforms, SUMO1, SUMO2, SUMO3 and SUMO4, have been identified in humans thus far (111). Although SUMO is functionally similar to ubiquitin, it has not been demonstrated to promote protein degradation, and is instead involved in a number of additional molecular reactions. For instance, it affects protein-protein interactions (108,112,113), promotes intracellular protein trafficking and localization (114-116) and prevents protein degradation (117).

**Additional types of PTMs.** In addition to the aforementioned PTMs, previous studies have demonstrated that a number of relatively simple, yet significant additional PTMs have been identified. For instance, oxidation of specific amino acids as a result of exposure to oxidative stress, activates *in vivo* protease activities, which leads to apoptosis induction (118,119). The transfer of a carbo-methyl group by methyltransferases to the N- or O-terminal of histidine, proline, arginine, lysine or carboxyl groups (known as methylation), is associated with a number of vital biological functions, such as gene transcription and signal transduction (120,121). By contrast, the lipidation PTM describes the covalent binding of various lipids to peptide chains. This PTM is subdivided into GPI anchors, N-myristoylation, S-palmitoylation and S-prenylation (122). In addition, lipidation has been demonstrated to affect the function of proteins and membrane proteins by further increasing their hydrophobicity (123).

**Sample purification and enrichment methods prior to MS analysis.** Despite advancements in improving the sensitivity of HPLC/MS systems, together with progress in the generation of powerful algorithms for database searching, the efficiency of PTM identification by proteomic approaches remains unsatisfactory. The identification of low-abundant PTM proteins remains a major challenge, as their MS signals are easily disguised by more abundant non-PTM proteins in a complex mixture. Therefore, extensive purification and enrichment of PTM proteins is necessary prior to the performance of mass analysis. Antibody-based affinity purification has been widely used to purify target proteins with specific PTMs. This approach has been adopted successfully for the analysis of tyrosine phosphorylation (124), arginine methylation (125) and lysine acetylation (126), in order to enhance the sensitivity and accuracy of PTM identification. However, the running costs of this enrichment procedure are relatively high. Alternatively, chemical tagging is a common approach for labeling PTMs for *in vivo* and *in vitro* studies. Chemical tags are sequentially conjugated to affinity linkers, such as biotin (127) or lectin (128). Biotin-containing tags have been successfully used to isolate proteins with PTMs including S-nitrosylation (129), O-linked



Table II. Overview of purification strategies for PTMs.

Purification/ enrichment strategies	Ligands	PTMs	Advantages	Drawbacks/ limitations
Antibody-based affinity purification	Antibody	Tyrosine phosphorylation, arginine methylation, lysine acetylation	High specificity	Small PTM alterations are difficult to identify; generation of antibodies against poorly antigenic PTMs is difficult
Chemical derivative tagging	<i>In vivo</i> metabolic and <i>in vitro</i> chemical tagging	Farnesylation ( <i>in vitro</i> ), O-GlcNAc modification ( <i>in vivo</i> and <i>in vitro</i> ), palmitoylation ( <i>in vitro</i> ), myristoylation ( <i>in vitro</i> ), glycosylation ( <i>in vitro</i> ), oxidation ( <i>in vitro</i> )	Wider enrichment range of PTMs	Sample loss; unwanted side products; non-specific binding

PTM, post-translational modification; O-GlcNAc, O-linked  $\beta$ -D-N-acetylglucosamine.

Table III. Overview of chromatography phospho-enrichment strategies.

Strategy	Ligands	Advantages	Drawbacks/limitations
IMAC	Metal ions ( $\text{Al}^{3+}$ , $\text{Fe}^{3+}$ , $\text{Ga}^{3+}$ ) immobilized on a matrix via acidic compounds (IDA, NTA, TED)	High purification capacity of phosphopeptides from complex samples.	Poor affinity of mono-phosphopeptides; non-specific binding of non-phosphorylated peptides with multi-acidic amino acid residues or nucleic acid; susceptible to be influenced by experimental conditions.
MOAC	Metal oxide ( $\text{TiO}_2$ , $\text{ZrO}_2$ , $\text{Al}(\text{OH})_3$ )	High selectivity and sensitivity for complex sample; high tolerance of solvent pH.	Poor affinity of multi-phosphopeptides; non-specific binding of acidic non-phosphorylated peptides.
SIMAC	IMAC+MOAC	Increase the number of discovered phosphoproteins; increase the phosphopeptide spectrum.	Complex operation steps.

IMAC, immobilized metal affinity chromatography; MOAC, metal oxide affinity chromatography; SIMAC, sequential elution from IMAC; IDA, iminodiacetic acid; NTA, nitrilotriacetic acid; TED, ethylenediamine.

$\beta$ -D-N-acetylglucosamine (130) and palmitoylation (131). In addition, multiple glycoproteomics studies have adopted the lectin-based affinity enrichment method (132-134). However, there are a number of limitations associated with these methods, which are summarized in Table II.

Ionic interaction-based enrichment of phosphoproteins or phosphopeptides is currently the most successful and widely used strategy to achieve phosphoproteomic enrichment. Immobilized metal affinity chromatography (IMAC) utilizes metal cations to target the negatively-charged phosphate group and the positively-charged metal ions, which are bound to resins via acidic linkers (Table III). These are subsequently eluted using a buffer with increasing pH (135,136). Previous reports have demonstrated that IMAC enriches the total phosphoprotein

content of a complex sample by up to 90% (137). An alternative method of enrichment is metal oxide affinity chromatography (MOAC) (138). In this strategy, the phosphopeptides are trapped by metal oxide ligands in an acidic solvent, and are consequently desorbed and eluted under alkaline conditions. MOAC exhibits higher selectivity and sensitivity for phosphopeptide enrichment compared with traditional phosphoprotein enrichment methods, such as  $^{32}\text{P}$  labeling (139). In addition, a stronger affinity for phosphopeptides has been observed in MOAC when compared with IMAC (138). The advantages and limitations of these phospho-enrichment strategies are summarized in Table III. In general, IMAC appears to be effective for the enrichment of multi-phosphopeptides, while MOAC favors mono-phosphopeptide enrichment. In 2008,

Thingholm *et al* (140) introduced an enrichment strategy known as sequential elution from IMAC (SIMAC), which combines the two phosphopeptide enrichment strategies with the aim of overcoming the limitations of either technique in isolation. In this combined protocol, IMAC was initially used to enrich multi-phosphopeptides, whilst mono-phosphorylated peptides were eluted by an acidic solvent. The solution was then further enriched by TiO<sub>2</sub>. Using this approach, SIMAC was able to identify a >2-fold higher number of phosphorylated sites when compared with the total number identified by TiO<sub>2</sub> enrichment alone (140). This combined strategy has successfully increased the phosphopeptide spectrum, and a more comprehensive understanding of protein phosphorylation patterns may be gained (140).

#### 4. PTMs in eye research

Proteomic studies of multiple PTMs in ocular tissues have improved our understanding of the physiology or pathology of various ocular conditions. The discovery of co-existing modifications of specific proteins, suggests that mechanisms of disease pathogenesis may involve interplay among these PTMs. The discovery of novel PTM sites on proteins and the study of differential PTM expression patterns have revealed a number of candidates that may be involved in different pathogenic signaling pathways. Using cutting-edge proteomic technology, novel biomarkers for the early diagnosis of ocular diseases are emerging, which may promote the development of novel therapeutic strategies to treat ocular diseases.

**Tears.** The surface of the eyes is overlaid by the tear film, which consists of lipid, aqueous and mucous layers (141). Alterations in any component of the tears may reflect underlying functional disorders of ocular structures, such as the secretory glands or the cornea, and may be an indicator of abnormal systemic conditions. The accessibility of the tear film is a desirable factor when searching for biomarkers in various ocular conditions, including dry eye syndrome, autoimmune thyroid eye disease (142) and diseases of the cornea (143). The proteome of human tears has been well-profiled and thousands of proteins have been identified (144,145). Proteins have been demonstrated to be differentially expressed in the tears of patients with dry eye syndrome (146), keratoconus (147), diabetic retinopathy (148) and in those treated with chronic glaucoma medication (149). Studies investigating the PTM alterations in tears have been performed using proteomic technology. For instance, O-acetylation of sialic acid derivatives on membrane-associated mucins was identified in human tears by the use of LC-MS/MS, and the results suggested that this PTM may be involved in protecting the cellular surface from infection (150). An additional study adopted the hydrazide-resin capture method to enrich N-Linked glycoproteins in the tears of patients undergoing climatic droplet keratopathy, in which 19 unique N-glycosylated proteins were reported for the first time (151). In a study of human tears, three sequential dyes were used to stain phospho-, glycol- and total proteins following 2D-PAGE separation, and a number of novel proteins including dermcidin, glycosylated lipocalin 1, cystatin S, phosphorylated nucleobindin 2 were identified

and their potential ocular functions were discussed (152). In addition, the protein profile of tears from patients with ocular rosacea was revealed using glycan-released, glycan-enriched and solid-phase extraction methods with MS analysis. In total, ~50 N-glycans and 70 O-glycans were profiled, and fucosylated N-glycans were revealed to be significantly underexpressed while sulfated O-glycans was over-expressed. These factors made them potential markers to consider for this particular ocular status (153). Detailed profiling of proline-rich protein 4, a potential biomarker of lacrimal gland acinar cell function (154), in normal human tears was conducted by applying one- and two-dimensional MS analysis. Four co-existing PTMs including methylation, acetylation, oxidation, and the addition of pyroglutamate were identified in human tear samples; however, their functions in physiological and pathological processes have yet to be elucidated (155).

**Cornea.** The cornea is the outermost and key refractive structure of the eye. For this unique structure, >3,000 proteins have been identified by comprehensive proteomic profiling thus far (156). In 2011, phosphorylated sites on mammary serine protease inhibitor (maspin) from human extracellular corneal cells were mapped by nano-HPLC-ESI-MS following immunoprecipitation (157). A total of 8 serine and threonine phosphorylation sites were revealed, while no phosphorylated tyrosine residues were observed (157). These results may enable clarification of the role of selective phosphorylation of maspin in the corneal epithelium during wound healing and anti-angiogenesis. In response to mechanical injury, epidermal growth factor receptor in the corneal epithelium was demonstrated to undergo multiple phosphorylations as revealed by a study using a proteomic workflow (158). In addition, N-glycans and glycosaminoglycans were profiled in a comparative manner in Statens Seruminstitut rabbit corneal (SIRC) cells and rabbit corneal epithelial cells using HPLC-MS. The high mannose-type and a hybrid type of N-glycan were demonstrated to be the most abundant types in SIRC cells, and this observation was considered to have an important pharmaceutical value (159).

**Crystalline lens.** The crystalline lens is a transparent and biconvex structure that functions to refract light rays by altering its curvature. The lens proteome has been thoroughly characterized, with particular interest focused on its association with cataracts. Cataracts, which is elicited by normal aging or various pathologies, is the leading cause of blindness worldwide (160). The association between  $\alpha$ -,  $\beta$ - and  $\gamma$ -members of the crystallin family has been the focus of cataract research for decades. The assemblage of PTMs, including phosphorylation and deamidation in crystallins, was demonstrated to contribute significantly to the formation of cataracts in different animal species (161-171). In addition, the function of phosphorylation in the crystallin family has gained attention, and profiling studies concerning phosphorylated sites in the lens proteome using phospho-enrichment strategies are summarized in Table IV. Using IMAC followed by LC-MS/MS analysis, novel phosphorylation sites have been identified on  $\alpha$ A- and  $\alpha$ B-crystallins, as well as on additional proteins, including  $\beta$ -enolase, heat shock protein 27 and glucose-6-phosphate

Table IV. Summary of identified PTMs on crystallins using the mass spectrometry approach.

PTM subtypes	Species	(Refs.)
$\alpha$ -crystallin		
Phosphorylation of soluble $\alpha$ A- and $\alpha$ B-crystallins	Mouse	(170)
Phosphorylation of $\alpha$ A-crystallin on Ser122 and Ser148	Mouse	(166)
Oxidation of Met, deamidation of Asp and Glu, phosphorylation of Ser and Thr residues	Rat (OXY cataract model)	(164)
Phosphorylation of Tyr4, Ser20, Ser45, Ser59, Ser148, Ser155, Ser172/173, N-acetylation and C-truncation of $\alpha$ A-crystallin	Mouse	(168)
Isomerization of several Asp residues	Human	(165)
$\beta$ -crystallin		
Phosphorylation and acetylation of $\beta$ H-crystallin	Bovine	(167)
$\gamma$ -crystallin		
Oxidation of W136 and additional Tyr residues	Human	(167)
Acetylation of Lys2 and Gly1 of $\gamma$ D-crystallin	Human	(205)
Multiple subtypes of crystallins		
Deamidation, oxidation, ethylation, phosphorylation, methylation, acetylation, and carbamylation	Human	(168)
19 phosphorylation proteins (28 phosphorylated sites)	Human	(173)

PTM, post-translational modification.

isomerase (172). Furthermore, differential expression patterns of phosphoproteins have been observed in human cataract lens extracts, in which 28 novel sites were identified as being differentially phosphorylated (173).

In a previous study investigating PTMs in the lens membrane, the most abundant lens membrane protein, aquaporin 0 (AQP0), was demonstrated to be phosphorylated on serine 235 and serine 229 (174). It was also revealed to be truncated at specific residues and racemized/isomerized on two aspartic acid residues, i.e. Asn 259 and Asn 246, in normal human lens cells. In addition, the spatial distribution of PTMs in the bovine lens was investigated, and serine 235 of AQP0 was demonstrated to be significantly phosphorylated in the nuclear and equatorial cortex regions, while C-terminal truncation of AQP0 was detected in the nuclear region. Furthermore, truncations of connexin 50 and connexin 46 were observed primarily in the nuclear region, and the corresponding expression levels of these proteins was significantly lower in the anterior outer cortex region (175). Novel PTMs of AQP0, including fatty acid acylation of the bovine and human lens protein, and an oleic acid modification to a lysine residue, have been detected by direct tissue proteomic profiling (176). These results suggest that various PTMs exist on AQP0, and that these PTMs may be associated with its biochemical functions, and particularly during the aging process of the crystalline lens.

**Vitreous humor.** Profiling of the human vitreous humor proteome has been completed, with at least 460 non-redundant proteins being catalogued; however, the PTMs of these proteins remain to be elucidated (177). The proteome of the vitreous humor of rats with experimental autoimmune uveitis (EAU) has been previously analyzed using 2D-PAGE,

MALDI-time of flight (TOF)/MS and micro-LC/LC-MS/MS approaches. Truncations of  $\alpha$ A- and  $\alpha$ B-crystallin and phosphorylation of  $\alpha$ B-crystallin were identified in the EAU group, indicating that these PTMs may be crucial in regulating the inflammatory reaction during uveitic conditions (178). In a rat model of ischemia-reperfusion (I/R) injury, the vitreous proteome was studied using 2D-PAGE and MALDI-TOF-MS technology (179). The results revealed an increase in the phosphorylation of three serine residues on  $\alpha$ B-crystallin, and a decrease in extracellular signal related kinase 1/2 phosphorylation at 48 h following I/R injury. It has been suggested that phosphorylation of  $\alpha$ B-crystallin may be involved in suppression of the inflammatory process in I/R.

**Retina.** A number of previous studies have investigated retinal proteomes, and the proteomic alterations that occur in response to various retinopathies (180-183). In terms of PTMs, a previous study profiling porcine rhodopsin documented an extensive phosphorylation pattern on the C-terminus and unusual glycosylation pattern, which is a significant discrepancy when compared with that observed in bovine and rat rhodopsin proteins (184). Among the 13 differentially expressed mitochondrial proteins in normal mice and those with early experimental autoimmune uveitis, oxidation and carbamidomethylation were revealed to be the most common PTMs (185). By contrast, pre-isolation by column chromatography coupled with ESI-triple-quadrupole MS, enabled the characterization of bovine cone transducin (Ty), T $\beta$ 3 $\alpha$ 8, which is similar to T $\beta$ 1 $\gamma$ 1 following isoprenylation. This suggested a weak involvement of the interaction between T $\beta$ 3 $\alpha$ 8 and phosducin during cone specialization (186). A similar top-down proteomic study of the isoprenylation of transducin examined the rod outer segment membrane of mice. In this study, similar modification

sites were observed on murine  $\gamma$  when compared with those in bovine  $\gamma$  (187). Using the proteomic PTM approach, a high heterogeneous pattern of glycosylation on the 5-hydroxytryptamine receptor 4 (5-HT<sub>4</sub>R) in 5-HT<sub>4</sub>R-containing rod cells was discovered in transgenic mice (188). The use of SDS-PAGE, TiO<sub>2</sub> phosphopeptide enrichment and LC-MS/MS has revealed seven N-glycosylation sites and five phosphorylation sites on the ATP-binding cassette, subfamily A, member 4 protein in bovine rod outer segments, which has been suggested to be part of the disease mechanism in Stargardt disease (189). In addition, proteomic research has contributed to understanding the dynamic nature of histones in the I/R injured rat retina. With the application of linear ion trap-orbitrap hybrid MS/MS analysis, 34 histone PTMs were revealed to be differentially expressed in this ocular condition, of which three histone H4 marks were further confirmed by western blotting (190). This indicated that these histone PTMs may predispose towards DNA damage following I/R injury (190).

The increasing global prevalence of myopia in recent decades, especially in eastern Asian countries, such as China, Japan and Singapore, make it a non-negligible health issue (191,192). The morbidity of myopia can reach up to 80-90% in younger age groups in these areas (193,194). Previous studies have demonstrated that the retina is the major site that receives signals and determines the extent of eyeball elongation (195,196). Thus far, proteomic research has revealed a number of candidate proteins that may be associated with myopic eye growth (197-199). However, none of these studies have specifically focused on PTMs. The first global screening of the retinal phosphoproteome in a myopic chick model was conducted using TiO<sub>2</sub> enrichment and nano-LC-TripleTOF MS/MS analysis (200). In this pilot study, 560 phosphoproteins were profiled, in which 45 were upregulated and 30 were downregulated during myopic eye growth. In addition, using the phosphoenrichment approach, acetylated retinal proteins including carbonic anhydrase, ubiquitin carboxyl-terminal hydrolase and fatty acid-binding protein were revealed to be upregulated while nucleophosmin, 40S ribosomal protein S12 and histone H1x were significantly downregulated in myopic eyes. These results may provide an insight into the analysis of retinal phosphoproteome alterations during myopic eye growth.

**Retinal pigment epithelium (RPE).** The single layer of pigmented cells located on the outside of the neurosensory retina, is known as the RPE, which functions primarily to nourish and support the photoreceptors. Alterations in the PTMs of proteins have been studied in RPE cells following exposure to light. The results indicated that phosphorylation of crystallins may be important in protecting RPE against light-induced oxidative damage (201). In addition, profiling of the secretome in bovine RPE demonstrated the presence of three tyrosine-sulfated proteins. This included tyrosine-sulfated complement factor H, which may be involved in age-associated macular degeneration (202). Following H<sub>2</sub>O<sub>2</sub> challenge, a novel 45 kDa truncated modification on the retinoid isomerohydrolase (RPE65) protein was identified by LC-MS/MS in murine RPE, suggesting that the RPE65 cleavage process may be affected by oxidative stress (203). The results of a previous MS-based study put into question

the conventional belief that the palmitoylation of cysteine residues on RPE65 is responsible for membrane affinity (204). However, neither palmitoylation nor additional PTMs were identified on RPE65 in this study.

## 5. Conclusion

Proteomic approaches have evolved tremendously in the past decade. Previous methods for protein separation and fractionation limited the study of PTMs; however, advances in enrichment methods have overcome these limitations and enabled the identification of different disease-specific PTM-associated biomarkers in ocular diseases (151,172,173). This progress will improve our understanding of different ocular disease mechanisms, and will be useful for the development of novel diagnostic strategies to improve treatment efficiency. Although research into proteomic PTMs in ocular diseases is at a preliminary stage, the continuous improvement of proteomics technologies will facilitate a more detailed study of PTMs and their applications in ocular therapy in the near future.

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