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Comprehending Dynamic Protein Methylation with Mass Spectrometry

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Abstract

Protein methylation is a post-translational modification (PTM) which modulates cellular and biological processes including transcription, RNA processing, protein interactions and protein dynamics. Methylation, catalyzed by highly specific methyltransferase enzymes, occurs on several amino acids including arginine, lysine, histidine and dicarboxylic amino acids like glutamate. Mass spectrometry (MS) based techniques continue to be the methods of choice for the study of protein PTMs. These approaches are powerful and sensitive tools that have been used to identify, quantify and characterize protein methylation. In addition, metabolic labeling strategies can be coupled to MS detection in order to measure dynamic and differential *in vivo* protein methylation rates. In this review, different applications of mass spectrometry technologies and methods to study protein methylation are discussed.

Introduction

Post-translational modifications (PTM)s play crucial roles in modulating protein activity, turnover, and protein-protein interactions. Protein methylation is a fairly common type of protein PTM and has been implicated in several biological processes such as transcriptional regulation, RNA processing, metabolism and signal transduction [1]. Although methylation has been most commonly observed on lysine and arginine residues, methylation of other amino acids including histidine (H), cysteine (C), aspartic acid (D), glutamic acid (E), serine (S) and threonine (T) has been reported [2, 3]. Lysine-methylation occurs by transferring one to three methyl groups from S-Adenosyl-Methionine (SAM) to the lysine ε -amine side chain, which leads to monomethylated (me1), dimethylated (me2) or trimethylated (me3) lysines (Figure 1). In the case of arginine, one or two methyl groups are added to its

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guanidine group which leads to mono- or di-methylation [2] (Figure 1). Methylation specific enzymes (methyltransferases) can read specific protein sequence/motifs and further propagate existing methylation marks [2]. For example, arginine methyltransferase enzymes often target proteins sequence including an RGG- RNA binding motif [4]. Furthermore, methylation has been shown to depend on a protein's existing methylation state and to be a dynamic modification. For instance, methylated lysines within histones have been shown to have measurable differential turnover rates [5].

PTMs, including protein methylation, have been traditionally identified by Edman degradation, amino acid analysis, radio isotope labeling or antibody based methods including peptide and protein arrays. These methods suffer from being unspecific, low throughput, and having a low dynamic range for quantitative measurements. In addition, they fail to identify specific modification sites, cannot distinguish methylation state, and they often rely on prior knowledge of the modification. For instance, protein methylation has been detected by radioactive methods (review in [6]) including using tritiated methyltransferase cofactor S-Adenosyl methionine (SAM) as a methyl-donor. The weakness of this radioactive method is that radioisotopes of carbon and hydrogen are weak radio emitters and it is difficult to detect modified peptides efficiently. Another issue with current technologies is the small size of the methyl group which makes it challenging to develop high quality methylation specific antibodies. Protein methylation substrates can be identified by protein and peptide arrays, however, any hit needs to be validated with purified endogenous proteins by mass spectrometry. In recent years, MS based methodology has proven to be superior for the analysis of PTMs including methylation due to improvements in the accuracy and sensitivity of MS instrumentation. MS methods have been developed to identify proteins carrying PTMs, to map (novel) PTM sites, to quantify the changes in PTM abundance at individual sites, and to characterize the cooperativity between interrelated PTMs at several sites on proteins [6–9] (Figure 2).

Mass Spectrometry based technology for PTM analysis: application to protein methylation

Post-translational modifications are functional groups including chemical species (phosphate, carbohydrate or methyl-group) and functional polypeptides (ubiquitin and SUMO). PTMs can be added or removed from an amino acid side chain or protein termini or created by the cleavage of signal peptides from proteins or by covalent cross linking between separate protein domains [10]. These chemical changes on modified amino acids form a mass shift that can be measured by mass spectrometry (MS). Tandem mass spectrometry (MS/MS) provides valuable information about modified peptides. However, during MS/MS sequencing, it can be challenging to assign the mass shifts because the identified mass shifts may represent isobaric modifications or the sum of a few modifications. For example, the mass difference between tri-methylation (+42.05 Da) and acetylation (+42.01 Da) is very small (0.0364 Da) and can only be discriminated within <30 ppm mass accuracy on sensitive instruments such as the Fourier-Transform Ion Cyclotron MS (FT-ICRMS) or Orbitrap systems. An additional way to discriminate between tri-methylation and acetylation is by the presence of diagnostic marker ions and neutral loss in MS/MS spectra [11].

1-Top-down, Bottom-up and Middle-down mass spectrometry approach: application to histone-methylation

MS-based analysis of PTMs, can globally be classified into three groups based on whether the fragmentation is carried out on: intact protein ions (Top-down) [12–14], short peptide ions post *in vitro* protein digest (Bottom-up) [12, 15], or more recently on large polypeptides (3–20 kDa) (Middle-down [12, 16–18]). These technologies differ in their capacity for shotgun or large-scale discovery and differ in MS instrumentation. The Top-down approach normally requires high mass accuracy instruments and has been used mostly with FT-ICR-MS, but this method has now been extended to other MS instruments [19]. Top-down is suitable for combinatorial PTM analysis, but less suitable for a shotgun sequencing based identification of low stoichiometric modification sites. For Bottom-up experiments, trap-based instruments with high speed MS/MS scanning, which have the capacity for MS/MS sequencing and for shotgun proteomics are mostly in use. The Middle-down method was introduced after the development of recent fragmentation methods including Electron Capture Dissociation (ECD) [20] and Electron Transfer Dissociation (ETD) [21] which allows efficient ionization of large peptides. The Middle-down technique has been developed to analyze larger peptides, in addition to labile and backbone modifications. While Middle-down has a comparable quantitative capacity to Bottom-up, it also has the capacity to characterize combinatorial PTMs like Top-down.

These methodologies have been extensively applied to study histone modifications, revealing new methylation, phosphorylation, ubiquitination and acetylation sites (review in [22]). With a Bottom-up approach, mono-, di- and tri-methylation at lysine 4, lysine 27 and lysine 36 in H3 were identified. Lysine 27 has been reported to be predominantly mono-methylated while lysine 36 was mostly tri-methylated [23]. A Top-down approach using ECD fragmentation revealed several acetylation, phosphorylation and methylation sites on different H3 forms. Lysine 4 mono-methylation was observed in 5% and lysine 9 dimethylation in about 50% of histone H3.1 in Hela cells [13]. Garcia et al., developed a Middle-down approach using ECD fragmentation to identify and characterize H3 variants and the site occupancy of the most abundantly modified H3 residues: lysine K4, K9, K23, K27 and K36 in ten different rat tissues [24]. Young et al., reported methods to detect histone modification combinations in a single experiment by a Middle-down strategy using a novel saltless pH gradient for weak-cation exchange-hydrophilic interaction chromatography (WCX-HILIC). Using ETD fragmentation, they were able to characterize over 200 modified histone H3.2 forms and 70 histone H4 forms [17].

2- Methylation-specific mass spectrometry

Since protein modifications are often transient and labile with a low stoichiometry (< femtomole), a number of complementary methods such as PTM-targeted MS/MS and selective enrichment of modified peptides prior to MS/MS analysis are needed to improve the sensitivity and quality of mass spectrometric analysis. Although different enrichment strategies are available for the efficient enrichment of various modified peptides, only relatively weak affinity based enrichment methods are available for methylation (review in [7]). Most of these methylation enrichment strategies utilize either methyl-lysine [24, 25] or

methyl-arginine [26, 27, 28] specific antibodies, for purification of methylated peptides by immunoprecipitation (IP) (Table 1). Ong et al. [25], used anti-methyl lysine and -arginine antibodies to enrich for methylated peptides following methyl-specific labeling of proteins using heavy methionine SILAC labeling. The authors were able to use SILAC in combination with enrichment to identify 59 methylation sites, corresponding to 58 methylated peptides, in HeLa cells. These methylation sites were identified in 33 different proteins (Table 1). More recently, Uhlmann et al., improved the enrichment by combining different separation methods including Strong-Cation-Exchange (SCX), Isoelectric-Focusing (IEF) and HILIC prior to immunoprecipitation to increase the enrichment efficiency and reduce the sample complexity. Individual comparisons of different separation methods with antibody enrichment demonstrated that HILIC identifies 3–5 times more methylation sites compared to other methods. In total, Uhlmann et al., identified 249 arginine methylation sites in 131 proteins. They identified 190 new methylation sites and 93 proteins which were not previously described to be methylated as well [28] (Table 1).

Identification and site localization of methylation by Tandem Mass spectrometry

Tandem mass spectra generated by different fragmentation techniques can provide valuable information for peptide sequencing, PTM identification, the PTM subtype and PTM site localization. Collision Induced Dissociation (CID) [25, 29–31, 35] as well as ETD [29–33] spectra of methylated peptides contain methylation specific ion signals in the low mass range. These low mass ions resulting from neutral losses and other peptide backbone fragments can be used to identify, validate and localize the methylation sites (Table 2). The methylation related losses in CID spectra are mostly water losses [33], but other abundant low-mass losses including methylamine, methylguanidine, or methylcarbodiimide have been identified [25, 29, 30]. ETD fragmentation has been applied to study lysine [32] and arginine [31–33] methylation. Methylarginine-associated neutral losses from charge reduced precursor ions during ETD fragmentation [32] result in highly-abundant low-mass product ions which allow for the reliable discrimination of symmetric and asymmetric dimethylarginine [29, 30] (Table 2). In contrast, lysine methylated peptides do not produce significant losses during ETD fragmentation. In addition to neutral loss and immonium ions identification, there are other PTM-specific tandem MS based strategies including precursor ion scanning [34, 36] and multistage MS/MS [37]. Couttas et al. [36], applied immonium ion scanning to discover new histone methylation sites and this method improved the discovery rate of modified peptides 4 fold in comparison to control experiments.

3- Quantitative Mass spectrometry to study PTM: Application to methylation

Quantitative MS-based proteomics techniques [38] are classified into two main groups: shotgun (discovery, large scale) and targeted MS (review in [39]). Shotgun MS [38] is based on Data-Dependent Acquisition (DDA) and intensity-based product ion scanning. Here, there is no need for information on predefined peptides. In targeted methods, the mass spectrometer- often a triple quadrupole instrument - identifies specific and predefined peptide/fragment ion pairs called transitions over time in a Data-Independent Acquisition (DIA) mode. The most common targeted method is “selected reaction monitoring” (SRM)

[39], which has the advantages of a better detection limit and extended dynamic range compared to shotgun approaches. Most recently, the Coon group, proposed a new targeted approach “Parallel Reaction Monitoring”(PRM) on a Q-Exactive instrument (Hybrid Quadrupole-Orbitrap) [40]. The advantages of PRM over traditional SRM are a wider dynamic range, high specificity, parallel detection of all target product ion in one concerted high resolution mass analysis rather than 3–5 transitions to validate the peptide identity, minimum upfront method development, and higher tolerance for co-isolated background peptides/species [40].

Label-free and labeling based quantitative MS methods have been applied to study and quantify histone modifications (Review in [42]). In a Middle-down label free ETD based approach, 74 discrete combinatorial modification codes on the tail of histone H4 in differentiating human embryonic stem cells (ES) were identified and quantified. Significant changes in the methylation and acetylation patterns of histone H4 isoforms during differentiation were observed, thus describing a context-specific PTM pattern. For example, H4R3 methylation was found only in the presence of H4K20 dimethylation [43]. Chemical derivatization in combination with stable isotope labeling is another quantitative method which has been widely used to study histone modification. Smith et al [44], used stable isotope labeling (deuterated acetic anhydride) to quantify Histone H4 lysine acetylation. In 2005, Garcia et al., introduced a double derivatization technique to quantify histone PTMs [45]. The first derivatization was applied to the free amino group in the N-terminus and unmodified or mono-methylated internal lysine so that similar sized fragments were formed from reproducible cleavage of histone protein by trypsin C-terminal to Arginine mimicking an ArgC-digest. They modified carboxylic acid groups with a normal (d0-methanol) or stableisotope labeled reagent d4-methanol (esterification reaction on peptide level) for relative quantitation. Further optimization of this method was done using d0- or d10-propionic anhydride to label the newly formed free N-terminal amino group during the second round of derivatization after digestion to overcome some limitations of labeled methanol [46]. With this method histone methylation can be directly quantified by comparing peak pairs separated by a +5 Da mass shift.

Another quantitative method to study methylation, is MS quantitation using isotopic reductive methylation (MassSQUIRM) [47]. This method differentiates between lysine methylation states by reductive methylation with heavy formaldehyde, causing the addition of up to two methyl-groups to lysine residues. All the peptides (mono-, di- and unmethylated) are converted to the same chemical species to have the same ionization efficiency. The dimethylated peptide has 28 Da (m/z), with one heavy methyl (30 Da) and with two-methyl groups (32 Da) in the unmodified form. MassSQUIRM can measure demethylase dynamics and their capacity to remove mono- and dimethyl marks from lysine residues. As briefly mentioned earlier, in a variation of SILAC (Stable Isotope Labeling by Amino acids in Cell culture), cells can be cultured in media with $^{13}CD_3$-methionine instead of heavy leucine, lysine, or arginine as in regular SILAC approaches [25]. The heavy methionine can be converted to $^{13}CD_3$-adenosyl methionine, a biological methyl-donor (Figure 1), which, in turn can be used by methyl transferases to label with heavy amino acids methyl groups. The relative peak intensity of methyl-modified peptide pairs are used to identify and quantify methylated species (Figure 2). Zee et al, reported the steady state

kinetics of global methylation of histones on a residue-specific basis using such heavy methyl-SILAC labeling [5]. Their work showed a progressively slower rate of formation of mono-, di-, and trimethylated residues and different methylation rates associated with either active or silent genes.

4- Identification of methylation cross talk with other modifications by quantitative MS

Identification of multiple PTMs in peptide sequences may be a hint at the synergistic or antagonistic interaction of these modifications. Methylation cross talk with other modifications such as acetylation, phosphorylation and ubiquitination has been described [6, 48]. Lysine methylation increases protein half-life by blocking ubiquitination (review in [6]). Darwanto et al., quantified (absolute and relative) 20 histone modification sites on H2A, H2B, H3 and H4 including acetylation, propionylation, methylation and ubiquitination by a MRM approach. The authors discovered an inverse correlation between Histone H2B ubiquitination and H3-Lysine 79 methylation [48].

5- Conclusion

Taken together, the advancements in the mass spectrometry field including new developments in instrumentation, technologies and methodology have been applied to improve research into protein methylation. Besides lysine and arginine methylation, there are scarce publications on other methylated amino acids [49] and there are insufficient validation strategies able to discriminate between real modification sites and artifacts [50], technical methylation and functional methylation. Therefore, there is room for the improvement of methylation detection strategies that will undoubtedly advance these fields.

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modified on lysine, arginine, histidine, and glutamic acid residues with a mass increase of 14 or 18. Using Methyl-SILAC method, they described that technical methylation (gel-based method) can cause complications in correctly of endogenous methylation.

Highlights

- Mass spectrometry based technologies and methodologies are powerful tools to confidently identify and characterize methylated amino-acids.
- They also enable to distinguish methylation subtypes, and to localize modification sites.
- Mass spectrometry can quantitatively measure protein methylation states and their dynamics.

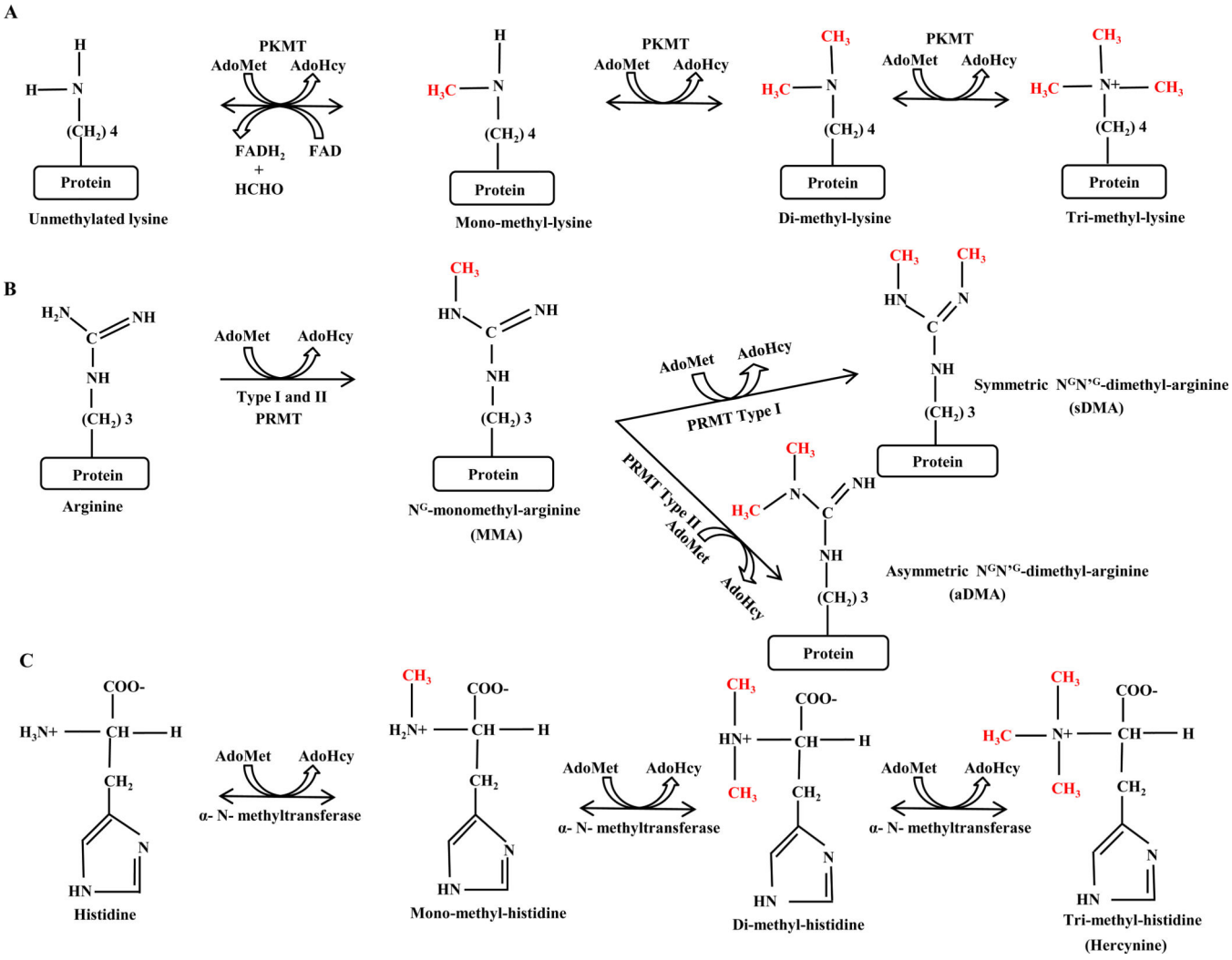

Figure 1.

Biochemical mechanism of lysine, arginine and histidine methylation. (A) Lysine methylation. Formation of mono-, di- and tri-methylated lysine by adding methyl-group to ε amine of lysine residue. Conversion of S-adenosyl-L-methionine (AdoMet) to S-adenosyl-Lhomocysteine (AdoHcy) leads to methyl-group transfer to a protein. The methylation reaction is catalyzed by Protein Lysine-Methyl-Transferase (PKMT). The reversibility of the methylation reaction in presence of Fe (II) and α-ketoglutarate has been proved by the discovery of a demethylase. (B) Arginine methylation. Addition of methyl groups to guanidine nitrogens of arginine forms NG -monomethyl-, NGN'G -dimethyl- symmetric (sDMA) and asymmetric (aDMA) - arginines. Type-I and –II protein Arginine methyltransferase (PRMT) are the catalytic enzyme for Arginine methylation. (C) Histidine methylation. A methyl-group will be added to the α-amino nitrogen atom of Histidine and result in mono-, di- and trimethyl- histidine (Hercynine). Histidine methylation is catalyzed by a single enzyme, Histidine-α-N-methyltransferase [3].

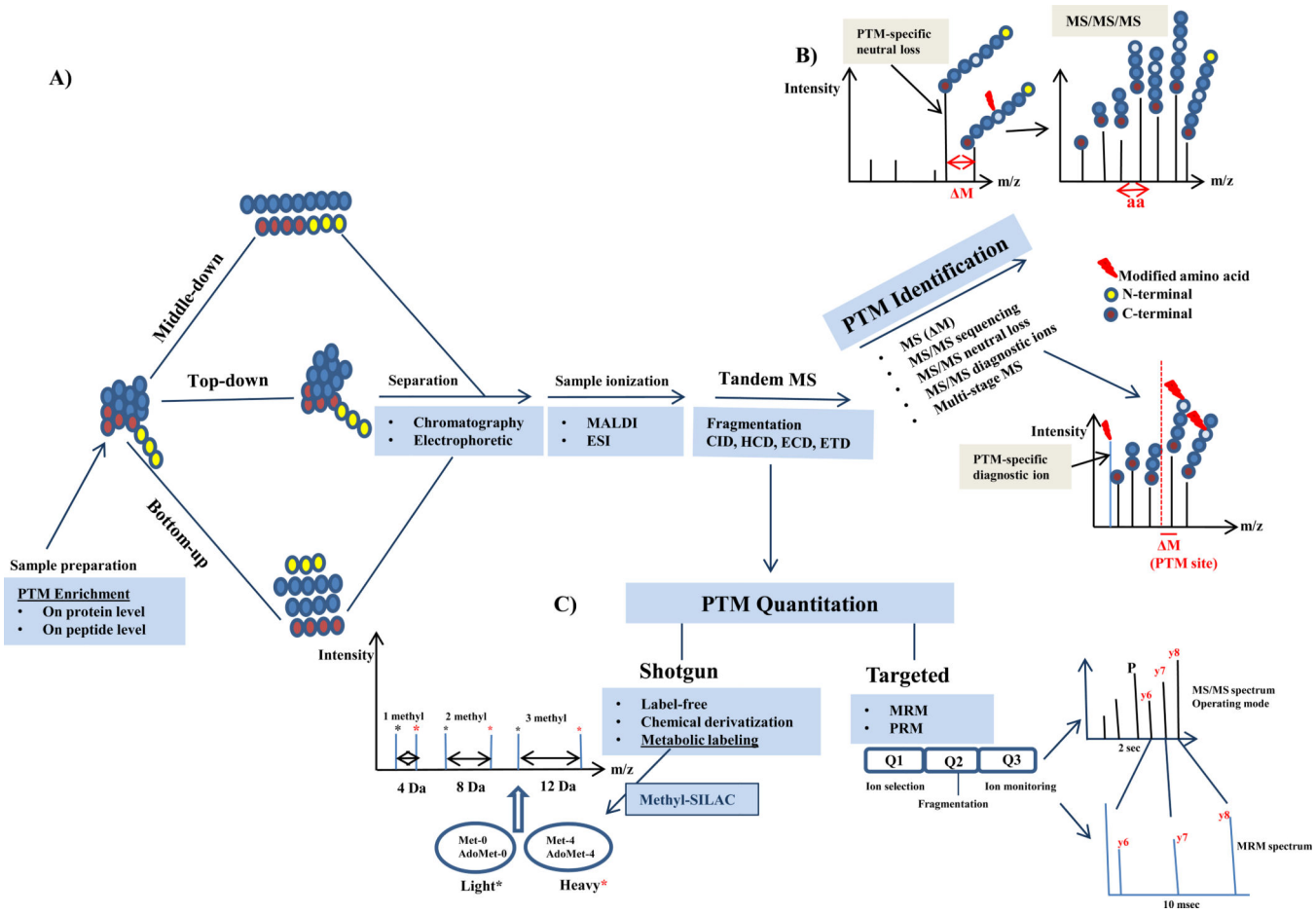

Figure 2.

Workflow for PTM analysis including methylation-specific mass spectrometry (MS). A) Protein samples are prefractionated, purified and eventually enriched for methylation either on the protein or peptide level. The samples will be subjected to mass spectrometric analysis based on one of these following strategies: Bottom-up, Middle-down and Top-down methods. B) The methylated peptides are identified by tandem mass spectrometry using different fragmentation techniques. Different PTM-specific MS/MS strategies are used to identify the methylation, localize the methylation sites and characterize the subtype of methylation including di-methylation types (aDMA, sDMA). C) Methylation is quantified by mass spectrometry based on two main quantitative approaches: shotgun (discovery) and targeted MS. Shotgun approaches which have been used for methylation are mainly labelfree, SILAC (methyl-SILAC) as well as chemical derivatization. In the methyl-SILAC approach, the cells metabolically convert [$^{13}CD_3$] methionine to [$^{13}CD_3$] S-adenosyl methionine. Heavy methyl-groups are fully incorporated into methylation sites in vivo. This method allows confident identification and relative quantitation for proteins with methylation sites. The specific mass shifts allow differentiation between mono-, di- and tri-methylated species. Targeted quantitative (absolute and relative) strategies including SRM and most recently PRM can be used to study methylation. Label-free and labeling strategies can be applied to targeted approach as well.

Table 1

Examples of large-scale mass spectrometry analysis of protein methylation

Enrichment	Type of Mass spectrometer	Separation method	Number of identified methylated peptides/proteins	Labeling-method	Reference
Methyl-arginine (R)-specific antibody	Q-exactive	HILIC[1]	Methyl-R sites: 215 Novel methyl-R sites:171	Methyl-SILAC	[28]
		SCX[2]	Methyl-R sites: 39 Novel Meth-R sites: 25		
		IEF[3]	Methyl-R sites: 66 Novel methyl-R sites: 34		
			Total number of methyl-R: 249 sites (190 novel sites) Methylated proteins: 131 (93 not previously described as methylated proteins)		
Methyl-arginine (R)- specific antibody	Q-TOF	Gel-based	Methylated sites: 59 Methylated proteins: 33	Methyl-SILAC	[25]
	LTQ-FT				
Methyl-lysine (K)- specific antibody					
Methyl-arginine (R)-specific antibody	Q-Star	Gel-based	Methylated proteins: 200	Label-free	[27]

[1] HILIC Hydrophilic Interaction Chromatography,

[2] SCX Strong Cation Exchange,

[3] IEF Isoelectric Focusing

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Table 2

Examples for immonium ions, diagnostic ions and neutral losses of methylated peptides using CID and ETD fragmentation by MS

Modification	Abbreviation	Mass shift (Δm) (Da)	Instrument	Neutral loss (Da)	Immonium ions (m/z)	References
Methyl-arginine						
Arginine mono-methylation	MMA	14.0156	LTQ-FT QSTAR Triple-quadrupole LTQ-Orbitrap-velos Quadrupole-ion trap HCT-ultra ion trap	31.0422[1,*] 73.064 [2,*]	143 (not unique for arginine)	[25, 30–33, 36]
Arginine asymmetric di-methylation	aDMA	28.0312	Quadrupole- ion trap LTQ-FT LTQ/Orbitrap LTQ-Orbitrap velos HCT-ultra ion trap	45.0578[3,*] 87.087[4,*]	71.06	[25, 30–34]
Arginine symmetric di-methylation	sDMA	28.0312	LTQ-FT LTQ/Orbitrap	31.0422[1] 87.087	**71.06**	[25, 30]
Methyl-lysine						
Lysine mono-methylation		4.0156	Triple-quadrupole MALDI-TOF		98.096 **84.081** (C-terminal lysine) 101 (N-terminal lysine)	[35, 36]
Lysine di-methylation		28.0312	Triple-quadrupole		98.096 112.1 84.081	[35, 36]
Lysine tri-methylation		42.0470	LTQ-FT QSTAR MALDI-TOF	59.0735		[25, 35]

[1]MMA (mono-methylamine),

[2]MMG (mono-methylguanidine),

[3]DMA (Di-methylamine),

[4]DMG (Di-methylguanidine),

[*]ETD (Electron-transfer-dissociation), The numbers in bold represent the higher ion intensity in mass spectra comparing to unbold