^{18}O Stable Isotope Labeling in MS-based Proteomics

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Abstract

A variety of stable isotope labeling techniques have been developed and used in mass spectrometry (MS)-based proteomics, primarily for relative quantitation of changes in protein abundances between two compared samples, but also for qualitative characterization of differentially labeled proteomes. Differential $^{16}O/^{18}O$ coding relies on the ^{18}O exchange that takes place at the C-terminal carboxyl group of proteolytic fragments, where two ^{16}O atoms are typically replaced by two ^{18}O atoms by enzyme-catalyzed oxygen-exchange in the presence of $H_2{}^{18}O$. The resulting mass shift between differentially labeled peptide ions permits identification, characterization and quantitation of proteins from which the peptides are proteolytically generated. This review focuses on the utility of $^{16}O/^{18}O$ labeling within the context of mass spectrometry-based proteome research. Different strategies employing $^{16}O/^{18}O$ are examined in the context of global comparative proteome profiling, targeted subcellular proteomics, analysis of post-translational modifications and biomarker discovery. Also discussed are analytical issues related to this technique, including variable ^{18}O exchange along with advantages and disadvantages of $^{16}O/^{18}O$ labeling in comparison with other isotope-coding techniques.

Keywords: *^{18}O labeling; enzyme-mediated isotope incorporation; stable isotope labeling; MS-based proteomics; relative protein quantitation; LC/MS/MS*

INTRODUCTION

A major goal of proteomics is to develop methods enabling the systematic quantitation of protein abundances within the cell/tissue or the comparative measurement of changes in protein abundances between two different states (e.g. healthy versus disease). Therefore, mass spectrometry (MS)-based approaches that quantify changes in protein abundances play an important role in systems biology, improving our understanding of fundamental biological processes or facilitating the identification of specific protein biomarkers [1]. The absolute quantitation of proteins using isotopically labeled synthetic peptides is typically employed in an experimental setting in which proteins of interest are known and physical changes in their abundances are expected to be regulated by particular stimuli or pathological processes. To identify and quantify unknown proteins presumably implicated in certain physiological or pathological responses, global quantitative profiling techniques that measure changes in protein abundances between two samples are required.

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Differential stable isotope labeling that relies on isotope incorporation at the protein or peptide level is primarily employed in the realm of liquid chromatography-mass spectrometry (LC-MS)-based, shotgun proteomics. Recent developments in stable isotope labeling and LC-MS offer significant advantages over 2D-PAGE-based comparative proteomics, including better coverage/quantitation of membrane proteins, proteins with extreme molecular weight and/or pI. Currently, two distinct techniques are used for the incorporation of stable isotopes into the proteome of interest: (i) *in vivo* labeling, which is accomplished metabolically by supplying the cell/organism of interest with nutrients highly enriched in stable isotopes [2], using simultaneous anabolic isotope incorporation into all cellular proteins; (ii) *in vitro* stable isotope labeling, which relies on chemical [3, 4] or enzymatic incorporation of isotopes into the proteome of interest at the protein and/or peptide level [5] after cell lysis or tissue homogenization.

Although the $^{16}O/^{18}O$ labeling is not the most commonly used isotope-tagging technique, its simplicity and instantaneous applicability to clinically relevant and amount-limited samples make this technique easily applicable for protein biomarker discovery that relies on MS-based profiling of human specimens. These specimens typically include tissues obtained by laser-capture microdissection or biofluids obtained by a variety of biopsy procedures. This review focuses on recent developments in the realm of enzyme-mediated $^{16}O/^{18}O$ stable isotope labeling and its overall utility in MS-based proteomics.

PRINCIPLE AND PRACTICE OF $^{16}O/^{18}O$ LABELING

Enzyme-facilitated ^{18}O labeling is a simple technique for tagging peptides in the presence of $H_2{}^{18}O$. It typically relies on class-2 proteases (e.g. trypsin) to catalyze the exchange of two $^{16}O_2$ atoms for two $^{18}O_2$ atoms at the C-terminal carboxyl group of proteolytic peptides, resulting in a mass shift of 4 Da between singly charged, differentially labeled peptide ions observed in MS^1 mode (Figure 1). The first study describing an enzyme-catalyzed oxygen exchange in the presence of $H_2{}^{18}O$ was reported in 1951 by Sprinson and Rittenberg [6], while MS spectra obtained by Antonov *et al.* using electron-beam MS explicitly showed a mass shift resulting

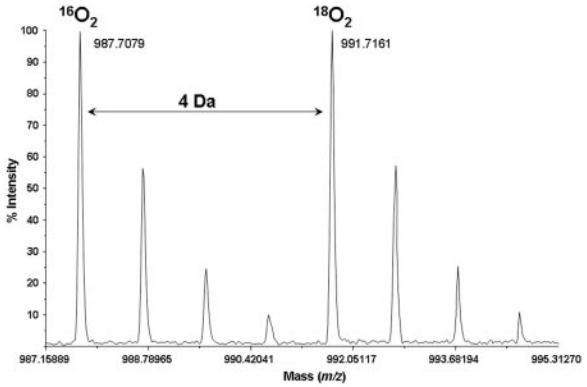

Figure 1: MALDI-MS depicting natural isotopic pattern of selected pair of differentially $^{16}O/^{18}O$-labeled peptides, exhibiting complete incorporation of both ^{18}O atoms.

from enzyme-catalyzed ^{18}O incorporation at the carboxylic group of proteolytic peptides [7]. Desiderio and Kai employed enzyme-catalyzed ^{18}O exchange for the preparation of internal standards for MS-based quantitation of peptides in biological extracts [8]. Mirgorodskaya *et al.* and Stewart *et al.* [9, 10] proposed the use of $^{16}O/^{18}O$ labeling for MS-based quantitation of proteins; the application of this technique as an effective quantitative solution-based, shotgun proteomic tool was first reported by Yao *et al.* [5]. Coupling the SDS–PAGE-based quantitative approach with post-digestion ^{18}O exchange for differential proteomics of protein complexes was first proposed by Bantscheff *et al.* [11].

$^{16}O/^{18}O$ labeling has also been used for non-quantitative proteomic investigations. Shevchenko *et al.* [12] described a method for *de novo* peptide sequencing that employs protein tryptic digestion in the presence of equal ratios of $^{16}O/^{18}O$ water for derivatization of tryptic peptides; this method greatly facilitates *de novo* sequencing due to simplicity of MS/MS spectra interpretation assisted by the presence of long Y ion series showing characteristic $^{16}O/^{18}O$ ratio throughout the spectrum. Kosaka *et al.* [13] employed tryptic digestion in the presence of 50% $H_2{}^{18}O$ for C-terminal characterization of proteins resolved by 2D-PAGE, while Park *et al.* [14] applied this approach to characterize plasma gelsolin as a substrate for matrix metalloproteinase and its potential role in the context of severe trauma. Back *et al.* [15] proposed the use of ^{18}O labeling for detecting cross-linked peptides within protein complexes. El-Shafey *et al.* [16] further developed this technique and applied it to protein–protein interaction analysis and characterization of the 3D structure

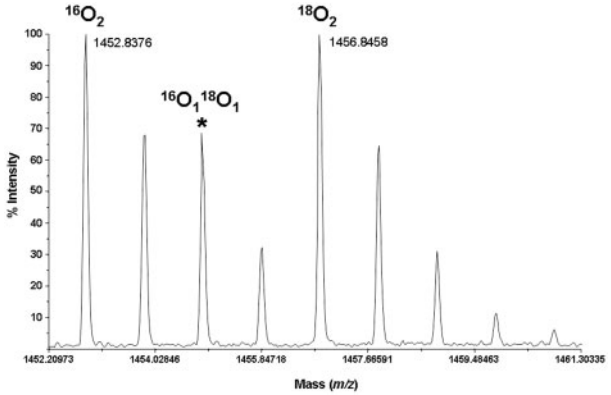

Figure 2: MALDI-MS depicting altered isotopic pattern of selected pair of differentially $^{16}O/^{18}O$-labeled peptides, indicting the presence of peptides with single ^{18}O atom incorporation [$^{16}O_1{}^{18}O_1$] characteristic for variable oxygen incorporation (marked by asterisk).

of freeze-dried protein complexes. Mirgorodskaya *et al.* [17] proposed an interesting approach for analysis of protein–protein interactions, which employs differential $^{16}O/^{18}O$ labeling to distinguish between endogenous protein-complex components and those that were non-specifically co-purified. These non-quantitative studies depict the variety of applications of trypsin-catalyzed ^{18}O tagging for functional profiling of peptides/proteins mixtures.

With the advent of this technique, it instantly became evident that the enzyme-catalyzed ^{18}O exchange is not always homogeneous (complete) and results in a mixture of peptides having one [$^{16}O_1{}^{18}O_1$] or both [$^{18}O_2$] oxygen atoms exchanged at their C-termini. The variable ^{18}O incorporation alters the natural isotopic distribution and forms a complex isotope pattern, depicted in Figure 2, complicating the calculation of the $^{18}O/^{16}O$ ratios. Many factors are responsible for the variable degree of ^{18}O incorporation, including variable enzyme substrate specificity, oxygen back-exchange, pH dependency and peptide physical–chemical properties.

Diverse upstream labeling approaches were developed to optimize oxygen exchange and achieve homogenous (complete oxygen) incorporation. Significant advancement was reported by Yao *et al.* [18], who proposed decoupling of ^{18}O tagging from the digestion step. This modification allowed targeted optimization of conditions for incorporating ^{18}O and minimized $H_2{}^{18}O$ consumption. This study also confirmed that trypsin-facilitated ^{18}O exchange of both C-terminal ^{16}O atoms is a two-step catalytic reaction; the first hydrolytic reaction, $RC^{16}ONHR' + H_2{}^{18}O \rightarrow RC^{16}O^{18}O^- + {}^+H_3NR'$, is followed by the second hydrolytic reaction, $RC^{16}O^{18}O^- + H_2{}^{18}O \rightarrow RC^{18}O^{18}O^- + H_2{}^{16}O$. Both trypsin-catalyzed oxygen exchanges were confirmed to be strictly substrate (Lys and Arg)-specific. This investigation showed weaker substrate binding for Lys-ending peptides than for Arg-ending ones. Subsequently, Hajkova *et al.* [19] showed that the incorporation of the second ^{18}O atom can be substantially accelerated if the post-digestion ^{18}O labeling is carried out at a pH in the range of 5–6, depending on the enzyme used in this step. Storms *et al.* [20] observed that prohibition of ^{18}O back-exchange can be efficiently accomplished by heating differentially labeled samples at 80°C for 10 min before combining them for subsequent MS analysis. Sevinsky *et al.* [21] proposed the use of immobilized trypsin for both the proteolysis and the labeling step to provide protection for the isotopic tags throughout the IPG–IEF process and prevent the ^{18}O back-exchange. A significant increase of the ^{18}O labeling rate was reported by Mirza *et al.* [22], describing accelerated oxygen-exchange if the trypsin was immobilized in the micro-spin column. Wang *et al.* [23] proposed inverse ^{18}O labeling for improved peptide/protein quantitation accuracy, particularly for peptides/proteins exhibiting extreme abundance changes.

For the past several years, our laboratory has been investigating the utility of $^{16}O/^{18}O$ for proteomic profiling of a complex membrane protein mixture that relies on buffered methanol to facilitate solubilization and proteolysis of membrane proteins. We have shown, using an α-N-benzoyl-L-arginine ethyl ester (BAEE) assay, that trypsin exhibits higher activity in 20% MeOH than in pure aqueous buffer, resulting in improved labeling efficiency when used for post-digestion labeling of membrane proteins [24]. The workflow depicting this modification is shown in Figure 3.

In addition to efforts focused on optimizing the labeling conditions, several advanced computational tools were developed with the aim of accounting for the variable oxygen incorporation. Halligan *et al.* [25] developed an algorithm that employs a calculation method previously described by Yao *et al.* [18]. The algorithm relies on differences between experimentally obtained isotope abundances and those obtained theoretically, while the method developed by Johnson and Muddiman [26] relies

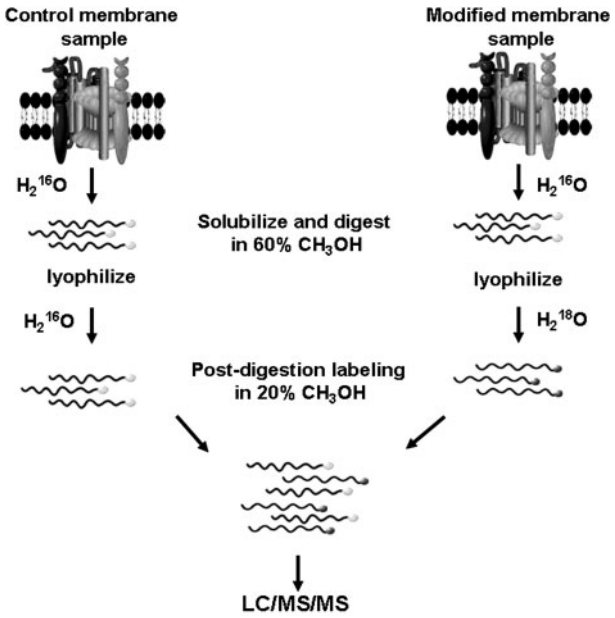

Figure 3: A workflow depicting differential $^{16}O/^{18}O$ labeling of membrane proteins. Isolated membrane samples (control and modified one) are first solubilized and digested in the buffer containing 60% MeOH/$H_2{}^{16}O$. After lyophilization, compared sample is digested in 20% MeOH/$H_2{}^{18}O$ while control sample is digested in 20% MeOH/$H_2{}^{16}O$ buffer. Samples are then combined and analyzed by LC-MS.

on average-based calculations to account for variable oxygen incorporation. Eckel-Passow *et al.* [27] described a method for estimating the ^{18}O incorporation directly, relying on a multivariable regression model in the context of post-digestion ^{18}O exchange. Ramos-Fernandez *et al.* [28] describe a kinetic exchange model that is incorporated within the quantification algorithm and is able to eliminate artifacts caused by variable oxygen incorporation; this model is readily amenable to quantitative profiling of complex protein mixtures. The algorithm developed by Mason *et al.* [29] utilizes a linear regression model to automatically interpret the spectra of ^{18}O-labeled isotope clusters, correcting for artifacts caused by variable ^{18}O incorporation. This approach uses centroid peak data obtained by MS with high-resolution power. We are in the process of testing software developed in-house that accounts for variable ^{18}O incorporation. The assumption that the integrated area of each peak within the isotopic manifold represents overlapping Poisson distributions is used as a basis for accurate $^{18}O/^{16}O$ peptide ratio calculation.

$^{16}O/^{18}O$ LABELING APPLICATIONS

Global proteomic investigations

Currently $^{16}O/^{18}O$ labeling is primarily used for proteome-wide quantitative profiling of biological samples. The first attempt on using ^{18}O labeling for global quantitative profiling of two different adenovirus serotypes was proposed by Yao *et al.* [5]. Hathout *et al.* carried out analysis of doxorubicin-resistant MCF-7 breast cancer cells using shotgun proteomics [30]. In this study, the cytosolic proteome of MCF-7 cells was fractionated using C-4 reversed-phase column. It was found that superoxide dismutase showed no significant expression changes in either of these two distinct cell lines. The authors hypothesize that up-regulation of FK-506 (4.1 ± 0.4) and the telomerase-binding protein (2.7 ± 0.1) might contribute to doxorubicin resistance since these proteins can inhibit cell sensitization to doxorubicin. Rao *et al.* [31] employed Lys-N to differentially label cytokine/lipopolysaccharide (LPS)-treated human retinal pigment cells and compared them with non-treated human retinal pigment cells. Lys-N tags the carboxyl terminus with only a single ^{18}O atom, allowing for homogenous ^{18}O incorporation and no ^{18}O back-exchange when complete digestion is accomplished. However, variable cleavage has been observed for the amino acids –Lys–X_{0-3}–Lys– (two lysine residues separated by no more than three other amino acids), –Glu–Lys–, and –Pro–Lys–, indicating slow hydrolysis and incomplete cleavage for given sequences between the two samples. This 2D-LC-MS shotgun analysis resulted in relative abundance measurements for 562 proteins identified from eight SCX fractions. A total of 11 proteins were found to be up-regulated and 49 to be down-regulated in cytokine/LPS-treated cells. Patwardhan *et al.* [32] compared proteomes of various breast cancer cell lines with the human mammary epithelial cell (HMEC) line proteome, using trypsin-catalyzed ^{18}O labeling coupled with an accurate mass and time (AMT) tag strategy. This analysis resulted in the identification of 33 631 peptides, allowing the identification of 2299 non-redundant proteins with at least two unique peptides, which were used as potential AMT tags. Measured changes in protein abundances between HMEC and cancer cell lines resulted in 86 proteins exhibiting at least a 3-fold change in their abundances.

Results obtained by global solution-based, multidimensional shotgun investigations suggest that $^{16}O/^{18}O$ labeling is a reliable and powerful tool

for comparative proteomics and offers significant advantages over the 2D-PAGE-based comparative proteomics by allowing unbiased proteome coverage [33] and high analytical throughput [34]. It is important to stress that relative changes in protein concentrations obtained by shotgun proteomics depict changes in protein abundances only at a given point in time. These calculations are based on MS-acquired data displaying identities and intensities of differentially labeled proteolytic fragments. The relative change in concentration observed for a particular protein may be instigated by a variety of cellular processes, including increased or decreased protein synthesis, increased or decreased protein degradation/trafficking, post-translational modifications (e.g. phosphorylation/dephosphorylation), or simply by artifacts induced by upstream sample preparation. Hence, the interpretation of these changes should be carried out cautiously. A variety of independent/orthogonal validations should be employed before inferring that measured changes in protein abundances represent genuine changes in the biological system.

Targeted proteomic investigations

To alleviate the analytical issue related to the wide dynamic range [35] of protein concentrations and facilitate the identification of low-abundance proteins, targeted proteomic strategies have been developed to isolate cellular organelles, or protein complexes [36]. Comparative subcellular proteomics represents an essential tool for the investigation of protein sorting and protein trafficking between different cellular compartments in response to various stimuli. When coupled with traditional cell-biology techniques, these investigations provide the link between proteomic data and organelle function, including information on protein location and the mechanisms regulating their functions [37]. Wang *et al.* [38] isolated soluble mitochondrial fractions to study relative changes in abundance of mitochondrial proteins implicated in the drug resistance of MCF-7 human cancer cells. Using forward and reverse ^{18}O labeling coupled with solution-based isoelectric focusing, Wang *et al.* identified 278 proteins, of which 12 exhibited at least a 2-fold change in their abundances. Based on data obtained by reverse labeling, Galectin-3-binding protein was detected only in the drug-resistant MCF-7 cells. Chen *et al.* [39] isolated the secretome of rat adipose cells to investigate differences in the secretory subproteome in response to insulin treatment. Reversed-phase liquid chromatography was used to fractionate secreted proteins prior to labeling and LC-MS/MS. The analysis resulted in the identification of 183 proteins, of which adiponectin and GM2 were up-regulated, while complement factor B and osteonectin were found to be down-regulated among proteins affected by insulin treatment. This investigation showed that ^{18}O labeling is the technique of choice for comparative proteomic profiling of amount-limited tissue specimens obtained from animals or those procured in clinical settings. Bantscheff *et al.* [11] used SDS–PAGE coupled with ^{18}O labeling to investigate the differential TNF-α-dependent protein complex assembly around the NF_kB transcription factor p65. The analysis indicated up-regulation of tubulin beta and complete removal of FK506-binding protein upon stimulation with TNF-α. Lane *et al.* [40] employed a similar approach, using SDS–PAGE to resolve the liver microsomal proteome followed by ^{18}O labeling and to examine the effect of dichloropyridylbenzene on the expression of P450 proteins in immuno-deficient mice previously receiving human colon carcinoma xenograft. A total of 16 P450 protein isoforms were quantified, of which 13 exhibited significant dysregulation in response to dichloropyridylbenzene treatment. Western blot analysis confirmed up-regulation of CYP1A2 and down-regulation of CYP2E1. Lopez-Ferrer *et al.* [41] demonstrated the applicability of linear ion-trap MS for accurate large-scale $^{16}O/^{18}O$ quantitation of proteins isolated from nuclear fractions of mesenchymal stem cells using high-resolution ZoomScans. The logarithmic chart of all calculated ratios showed a Gaussian distribution, enabling measurements of relative ratios for identified peptide ion pairs lower than 0.52 and higher than 1.95 at the 95% confidence level. Our laboratory employed post-digestion ^{18}O labeling to profile a subproteome of plasma membrane detergent-insoluble microdomains. Using ^{18}O labeling in 20% methanol buffer, we examined differences in protein abundances between control and Iota b-treated Vero cells [24]. We identified at least 10 lipid-raft marker proteins including caveolin, flotillin and CD44. Only CD44 showed a significantly higher expression level in Iota b-treated cells. The overall CV was in the range of 17–38% for quantified lipid-raft marker proteins. Also, we observed a 3-fold up-regulation of guanine

nucleotide-binding regulatory protein subunit 1 and G-protein-regulated inducer of neurite outgrowth, which might be involved in Iota b uptake. In the study focused on the effects of Triton X-100 and Brij-96 on enrichment of detergent-insoluble membrane proteins isolated from detergent-resistant membrane microdomains (DRMMs), we described the use of simultaneous $^{16}O/^{18}O$ and cICAT labeling to increase the proteome coverage [42]. The analysis revealed that a much greater fraction (i.e. 63.4%) of detergent insoluble proteins was more readily isolated using Triton X-100 compared to Brij-96 (10.4%). Notably, Triton X-100 also extracted larger quantities of non-DRMM-associated proteins. Stockwin *et al.* [43] carried out a comparative proteomic analysis of plasma membrane isolated from hypoxia-adapted mouse B16 F10 melanoma cells. The authors employed differential post-digestion ^{18}O labeling coupled with multidimensional liquid chromatography tandem mass spectrometry to discover novel hypoxia-induced membrane proteins. Consistent increases at the proteomic and transcriptomic levels were observed for aminopeptidase N; carbonic anhydrase IX; potassium-transporting ATPase; matrix metalloproteinase 9; and stromal cell-derived factor 1. Western blot analysis of a panel of human melanoma cell lines confirmed that aminopeptidase and stromal cell-derived factor 1 were consistently up-regulated during hypoxia. All these investigations indicate that ^{18}O labeling is particularly suitable for tagging size-limited samples in which every proteolytic fragment is accessible for identification/quantitation, allowing for better profiling of low abundant proteins as well.

Post-translational modifications

Many vital cellular processes are regulated by post-translational modifications of proteins. Thus, quantitative profiling of post-translationally modified proteins using MS-based proteomics is critical for understanding regulation of important cellular processes/pathways. Gonzalez *et al.* [44] were first to propose the use of enzyme-catalyzed digestion in the presence of $H_2{}^{18}O$ to facilitate MS-based identification of N-glycosylation sites in a glycoprotein. They used digestion by peptide-*N*-glycosidase (PNGase) F in the presence of 40% $H_2{}^{18}O$, which generated a complex isotopic MS^1 pattern of N-glycosylated peptides because of the partial incorporation of ^{18}O at the carboxyl group of corresponding Asp-residues. This technique was further extended proteome-wide by Kaji *et al.* [45] to identify *N*-glycosylated proteins using lectin-facilitated enrichment of glycopeptides coupled with PNGase-mediated deglycosylation in the presence of $H_2{}^{18}O$.

Phosphorylation is the most common post-translational modification of protein and is implicated in the regulation of a variety of cellular processes. Bonenfant *et al.* [46] employed $^{16}O/^{18}O$ labeling coupled with IMAC to enrich for phosphopeptides and alkaline phosphatase to quantitate the phosphorylation changes in nitrogen permease reactivator protein kinase isolated from wild-type and rapamycin-treated yeast, respectively. They were able to measure changes in the phosphorylation of proteins enriched from two different cellular states utilizing trypsin-catalyzed ^{18}O exchange. Smith *et al.* [47] proposed an interesting concept for relative quantitation of protein phosphorylation without phosphopeptide enrichment: following labeling, the ^{16}O-tagged sample is dephosphorylated using a cocktail of phosphatases, and differentially labeled samples are then combined and analyzed by LC-MS. The intensity of dephosphorylated peptide peaks is used to calculate the extent of phosphorylation present before the phosphatase treatment. The proof of the principle was shown by employing this technique on synthetic peptides followed by its application to a complex protein mixture extracted from yeast lysate.

In our laboratory significant effort has been put into detecting *in vitro* kinase-generated protein phosphorylation sites. Zhou *et al.* [48] employed a 1:1 mixture of adenosine triphosphate for *in vitro* kinase reaction, in which four ^{16}O atoms at the terminal phosphate group were replaced by four ^{18}O atoms. After tryptic digestion, the phosphorylated peptides were easily recognized by the presence of peptide ion pairs separated by 6.01 Da. This stable isotope labeling method positively detects the phosphorylation sites generated by *in vitro* enzymatic phosphorylation. Although few exist, previous investigations make a strong case for further investigation of post-translational modifications using differential $^{16}O/^{18}O$ labeling [46, 47, 49].

Biomarker discovery

Recent advances in MS-based proteomics have resulted in increased interest in the discovery of protein biomarkers for early disease diagnosis,

therapy, follow-up and prognosis. Heller *et al.* [50] showed that ^{18}O exchange can be successfully employed for quantitative profiling of low-molecular-weight (LMW) human plasma, indicating its utility for biomarker discovery from clinically relevant samples. A similar approach was employed by Hood *et al.* [51] for quantitative profiling of LMW serum isolated from xenografted tumor-bearing mice (^{18}O-labeled) and control mice (^{16}O-labeled), resulting in 1650 quantified proteins. The analysis resulted in 211 proteins exhibiting a significant increase and 246 proteins showing a significant decrease in abundance within the LMW serum obtained from mice bearing Lewis lung cancer. Vascular endothelial growth factor receptor 1 (VEGFR-1) was found to be significantly increased in the lung carcinoma xenografted mice. VEGF is a key angiogenic factor known to be expressed in advanced malignancies. Qian *et al.* [52] employed AMT tag strategy coupled with 2D-LC-FTICR-MS to analyze $^{16}O/^{18}O$ differentially labeled human plasma obtained from an individual before and after lipopolysaccharide administration. The analysis resulted in quantitation of 429 plasma proteins, of which 25 exhibited significant changes in abundance. In the quest for breast carcinoma biomarkers, Zang *et al.* [53] examined differences in protein abundances between metastatic ductal carcinoma and normal ductal epithelium obtained by laser capture micro-dissection (LCM). Tissue specimens were digested, differentially $^{16}O/^{18}O$ labeled and subjected to LC-MS analysis. Samples contained ~1–4 μg of proteins and yielded identification and quantitation of 76 proteins. Of these, mitochondrial isocitrate dehydrogenase and actin were found significantly increased in the breast tumor specimen. These investigations strongly suggest that $^{16}O/^{18}O$ labeling has a great potential for biomarker discovery in the realm of clinical proteomics that relies on amount-limited human proteome specimens, including LCM-procured specimens and needle biopsy–acquired samples.

Enzymatic ^{18}O labeling versus chemical and metabolic isotope labeling

The focus of this review is the utility of ^{18}O labeling in both qualitative and quantitative MS-based proteomics. Although detailed reviews addressing the utility of stable isotope labeling in quantitative proteomics have been published recently [54, 55], we will briefly address potential drawbacks and advantages of ^{18}O labeling in the context of quantitative proteomics. In general, ^{18}O labeling suffers from two potential drawbacks; inhomogeneous ^{18}O incorporation and inability to compare multiple samples within a single experiment. Unlike ICAT, ^{18}O labeling is simple, free of extensive sample manipulations, free of side reactions, and amenable to all protein species (i.e. proteins that contain no cysteine residues). It is two orders of magnitude less costly than ICAT and SILAC, comparing the price of reagents needed to label 1 mg of protein. In contrast to ICAT there is no lower limit of the protein amount that can be labeled. On the other hand, ICAT should be a method of choice for very complex protein mixtures (i.e. cell or tissue lysates), where the dynamic range of protein concentration is an issue. SILAC should be the method of choice for labeling of cultured cells, while ^{18}O labeling should be preferentially used for size-limited human tissue specimens (i.e. laser capture micro-dissected specimens). For experiments involving multiple-time-point sample collections, iTRAQ is the method of choice when compared to ^{18}O labeling. It is worth mentioning that the iTRAQ labeling is approximately seven orders of magnitude more expensive than reagents for ^{18}O labeling when calculated as cost per 1 mg of labeled protein. In our opinion, no single method or approach warrants elevation above the others for achieving success across the board.

CONCLUSIONS

It is important to note that there is no clear consensus in the literature for a 'best practice' isotope labeling strategy. Our opinion is that the choice of isotope labeling technique is highly dependent upon experimental design, the scope of a particular analysis and the sample or system being analyzed. In contrast to ICAT, ^{18}O labeling does not favor peptides containing certain amino acids (e.g. cysteine), nor does it require an additional affinity step to enrich for these peptides. Unlike iTRAQ, $^{16}O/^{18}O$ labeling does not require a specific MS platform nor does it depend on fragmentation spectra (MS^2) for quantitative peptide measurements. It is amenable to the labeling of human specimens (e.g. plasma, serum, tissues), which represents a limitation of metabolic labeling approaches (e.g. SILAC). Importantly, ^{18}O labeling is far less expensive than all of the stable labeling techniques mentioned earlier, making

it useful in the area of biomarker discovery, where numerous samples are expected to be analyzed concurrently. Taken together, recent advancements in the homogeneity of ^{18}O incorporation, improvements made on algorithms employed for calculating $^{16}O/^{18}O$ ratios and the inherent simplicity of this technique should result in increased use of ^{18}O labeling, particularly for proteomic profiling of human specimens (e.g. plasma, serum, tissues) in the realm of biomarker discovery.

Key Points

- Enzyme-mediated ^{18}O labeling represents a versatile tool for qualitative and quantitative MS-based proteomics due to its inherent simplicity and affordability.
- Differential $^{16}O/^{18}O$ labeling is particularly suitable for comparative proteomics investigations in the area of biomarker discovery, where clinically relevant human specimens, frequently limited in size (e.g. LCM-dissected specimens), are commonly used.
- Recent advancements in software employed for calculation of $^{18}O/^{16}O$ ratios significantly alleviated the issue of variable ^{18}O incorporation, allowing for accurate quantitative measurements of relative peptide/protein ratios.

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