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Proteomics and pulse azidohomoalanine labeling of newly synthesized proteins: what are the potential applications?

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Abstract

Introduction: Measuring the immediate changes in cells that arise from changing environmental conditions is crucial to understanding the underlying mechanisms involved. These changes can be measured with metabolic stable isotope fully-labeled proteomes, but requires looking for changes in the midst of a large background. In addition, labeling efficiency can be an issue in primary and fully differentiated cells.

Area covered: Azidohomoalanine (AHA), an analog of methionine, can be accepted by cellular translational machinery and incorporated into newly synthesized proteins (NSPs). AHA-NSPs can be coupled to biotin via CuAAC-mediated click-chemistry and enriched using avidin-based affinity purification. Thus, AHA-containing proteins or peptides can be enriched and efficiently separated from the whole proteome. In this review, we describe the development of mass spectrometry (MS) based AHA strategies and discuss their potential to measure proteins involved in immune response, secretome, gut microbiome, and proteostasis, as well as their potential for clinical uses.

Expert Commentary: AHA strategies have been used to identify synthesis activity and to compare two biological conditions in various biological model organisms. In combination with instrument development, improved sample preparation and fractionation strategies, MS-based AHA strategies have the potential for broad application, and the methods should translate into clinical use.

Keywords

Azidohomoalanine; Proteomics; Newly synthesized protein; Application; Biological studies

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1. Introduction

Cells can adapt to changing environmental conditions by synthesizing new proteins and altering the ensemble of the proteome. Measuring how proteomes respond to perturbations or disease is crucial to understanding the underlying mechanisms involved. In order to understand discrete changes that occur during immune response or disease development, it is critical to be able to specifically label proteins that are synthesized within a narrow time window. One classic way in which MS-based analysis can reveal proteome dynamics is through metabolic incorporation with stable isotope labeled amino acids, but it usually takes days to weeks to generate fully-labeled proteomes, and proteins present in low abundance can be missed. In addition, labeling efficiency can be an issue in primary cells and fully differentiated cells; consequently, monitoring the immediate proteome response to stimuli or early changes remains a challenge[1]. The development of new biorthogonal reactions have provided a new, transformational strategy for labeling proteomes. Dieterich et al. synthesized azidohomoalanine (AHA)[2], an analog of methionine containing an azide moiety, which can be accepted by the endogenous methionine tRNA *in vivo* and inserted into proteins without adverse effects on cellular functions [3, 4]. The AHA azide group reacts with an alkyne-bearing biotinylated tag in the presence of Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) catalyst, allowing for the selective enrichment of the tagged protein pool. Thus, AHA-containing proteins or peptides can be enriched and efficiently separated from the whole proteome. The AHA methodology, combined with bottom-up proteomics, enables scientists to detect, identify and quantify newly synthesized proteins (NSPs). As recently described, these properties have established AHA methodology as a tool of outstanding utility in biological discovery experiments [3, 5–7].

1.1 MS-based workflows using AHA

In recent years, there has been an increasing interest in labeling proteins without disrupting biological function [8]. Proposed labeling strategies have included imaging agents (e.g., fluorescence, MRI, positron emission tomography), affinity tags, or a second polymer or drug molecule [9]. One elegant labeling strategy includes introduction of a non-canonical amino acid bearing a functional group that possesses bio-orthogonal chemical reactivity into the protein [10]. In many chemical reporter strategies for labeling proteins, azide and alkyne moieties have assumed leading roles because they are absent from cells and tissues. A set of azide- and alkyne-functionalized unnatural amino acids to tag proteins and track cellular synthesis has been developed [11]. Incorporation of unnatural amino acids into proteins by chemical methods such as solid-phase synthesis [12], native chemical ligation [13], and *in vitro* translation protocols [14] has permitted characterization of protein folding pathways, enzymatic mechanisms, and ligand-receptor interactions. However, the most efficient way to introduce unnatural amino acids into recombinant proteins utilizes the unnatural substrate tolerance of the native translational apparatus. These analogs mimic the fate of their natural counterparts and can be used to track cellular metabolism. Based on that, Dieterich et al. synthesized Azidohomoalanine (AHA)[2]. It has been reported that AHA is an excellent methionine surrogate which is activated by the methionyl-tRNA synthetase of *Escherichia coli* and replaces methionine in proteins expressed in methionine-depleted bacterial cultures [15]. Additionally, methionine is an essential amino acid and thus is required to be

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supplemented for use with mammals. Studies using AHA have grown rapidly in number and influence [5, 16–18].

AHA does not interfere with cellular processes and takes advantage of the substrate promiscuity of the translational machinery[2], allowing it to be readily incorporated into proteins simply by adding AHA to media for cultured cells [5, 19], to food pellets or by injection pump for animals [6]. AHA has also been successfully incorporated in zebrafish, *C. elegans*, tadpoles, brain slices, and the rodent retina[3, 20–24]. Upon incorporation of AHA into NSPs, a biotin-alkyne can be covalently linked to the NSPs incorporating AHA using azide-alkyne click-chemistry [25]. Typically, enrichment has been performed on the protein level prior to digestion, but it has been reported that peptide enrichment significantly increased the number of NSPs identified [26]. In the improved protocol, trypsin digestion was performed on samples immediately after Trichloroacetic acid (TCA) precipitation, followed by peptide enrichment using biotin-streptavidin, a powerful tool which is widely used in biotechnology. This system is highly effective for purifying biotinylated molecules because of the remarkable strength of the noncovalent interaction between biotin and streptavidin [27]. However, the strength of biotin-streptavidin interaction can also become a liability resulting in poor recovery of biotinylated molecules. To improve recovery rates of the biotin-streptavidin system, an alternative strategy which exploits the weaker binding affinity of anti-biotin antibodies for biotin has been developed [28].

Over the past few decades, significant advances in proteomics technology have been made which have facilitated the identification and quantification of proteins in complex biological samples. In particular, the bottom-up strategy, in which proteins are digested into peptides and analyzed using a combination of chromatographic separation, mass spectrometry (MS) and automated database searching, has greatly increased the ability to describe complicated systems. Both off-line and in-line liquid chromatographic (LC) separation have been employed in AHA strategies.

1.2 The development of strategies using AHA

The cellular proteome determines cell identity, including state and function. Cells and organisms adapt to signals and stresses by changing protein expression. The proteins involved in each process, including those that are newly synthesized, modified, or degraded, must be identified in order to understand how cells execute these and other functions. In 2006, Dietrich et al [29] successfully identified 195 NSPs in HEK293T cells in a 2-hour window by developing biorthogonal non-canonical amino acid tagging (BONCAT), a labeling strategy that uses AHA and click chemistry for selective enrichment and identification of NSPs. In this strategy, newly synthesized, affinity-tagged proteins can be enriched from the pre-existing, unlabeled protein pool and identified by MS. The BONCAT strategy decreases sample complexity and allows more direct analysis of proteins synthesized in response to the stimuli. Additionally, this strategy can be applied in any cell type to measure the temporal and spatial characteristics of newly synthesized proteome.

A classic way to quantitate steady-state proteome changes involves stable-isotope labeling of amino acids in cell culture (SILAC) [30]. But monitoring proteome response to stimuli, especially in primary cells, remains a challenge with SILAC[31]. Detection of early or

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immediate changes to protein expression are difficult using SILAC MS-based quantitation because only a small fraction of the proteome is labeled after pulse labeling and detecting incorporation of labels in the midst of high unlabeled background is challenging. However, this problem can be overcome by isolating the fraction of SILAC-labeled proteins. In 2012, Somasekharan et. al established a pulse labeling approach by combining click chemistry and SILAC to quantify NSPs regulated by Y-box binding protein-1 [32]. Since then, other strategies combining the strengths of SILAC and BONCAT have been developed including QuaNCAT [33], BONLAC [34] and BONCAT-pSILAC [35], in which the cells are labeled with AHA and SILAC amino acids at the same time. AHA allows selective enrichment, while SILAC amino acids make it possible to quantitate NSPs. However, labeling with multiple types of amino acids complicates experimental design. Similarly, in 2016 it was reported that isobaric labeling (iTRAQ) and AHA were combined to enrich and quantitate NSPs[18]. In the past decade, MS-based AHA strategies have been successfully applied in multiple biological model organisms including *Escherichia coli* [36, 37], C.elegans [24], tadpoles [38], mammalian cell line [26], primary cells [13], mouse [6] and *Arabidopsis thaliana*[16].

Traditionally, there has been one workflow employing heavy isotope labeled biotin through click reaction to reach the goal of NSPs quantitation [6]. To provide a more straightforward workflow to quantify NSPs, a new metabolic quantitative labeling strategy called Heavy Isotope Labeled Azidohomoalanine Quantification (HILAQ) has been developed. HILAQ enables NSP enrichment, confirmation and quantification using a single stable isotope labeled version of AHA. It has been demonstrated that HILAQ has higher sensitivity than QuaNCAT due to simplified labeling and data searching processes.

2. AHA methodologies to measure the immune response

The immune system is the body’s defense system against infections, invaders and tissue injury. The immune response involves a series of steps attacking organisms and substances that invade body systems. Immune disorders cause abnormally low activity or over-activity of the immune system. In cases of immune system over-activity, the body attacks and damages its own tissues (autoimmune diseases). Immune deficiency diseases decrease the body’s ability to fight invaders, causing vulnerability to infections. Understanding the mechanisms of immune system diseases in detail is essential for developing drugs to fight them.

Comparative proteomics analysis has provided in-depth information on the molecular mechanisms involved in immune response. Such strategies include differential 2D gel electrophoresis [39, 40], isobaric tags for relative and absolute quantitation (iTRAQ) [41], metabolic stable isotope labeling by amino acid in cell culture (SILAC)[42] and label-free quantitation [43]. These methods have successfully elucidated complex processes like host-pathogen interactions [44], secretary protein profile of infected macrophage [45], as well as biomarker discovery studies [46]. As an unbiased strategy applied to *in vivo* studies, SILAC has been demonstrated to be better than others. The great advantages of SILAC lie in its straight-forward implementation, quantitative accuracy [47], and reproducibility over chemical labeling or label-free quantification strategies [48, 49], which have propelled its implementation in proteomic research. However, SILAC needs to be performed on a fully

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labeled proteome, which usually take several weeks to achieve, so quantitating immediate or early changes during the process of immune response (such as from quiescent to activated status) with SILAC is problematic. Studies of proteome dynamics are facilitated by AHA strategies that enable separation of newly synthesized proteins from the preexisting protein pool. More importantly, AHA methodology permits an analysis of the primary protein synthesized in response to internal and external cues.

BONCAT and iTRAQ have been combined to quantitate the changes of the nascent proteome induced by inflammatory cytokines TNF-α and IL-1β [50]. In recent studies, QuaNCAT, in which SILAC and BONCAT are utilized together, has been employed to quantitate stimuli-induced proteome dynamics in primary $CD4^{+}$ T cells in two hours [19]. The dataset obtained from QuaNCAT specifically reflects how protein synthesis changed in response to external stimuli. To confirm whether the altered protein synthesis activity is due to transcriptional regulation or translational regulation, RNA sequencing (RNA-SEQ) was employed to provide a far more precise measurement of transcripts levels and their isoforms [51]. Intuitively, the degree of change of a gene's transcripts should represent similar alteration of protein synthesis. However, it has been extensively demonstrated that post transcriptional processing determines steady-state protein levels [52]. Comparing RNA-SEQ and NSPs datasets makes it possible to provide novel insights into the molecular basis underlying diseases.

3. AHA methodologies to measure secreted proteins

The proteins secreted by various cells (the secretome) play important roles in cellular communication, adhesion and migration. The secretory proteins mediate diverse physiological functions like differentiation, proliferation and immunity and often have a significant impact on disease progression, which has been investigated in various entities [53, 54]. Secretomes are valuable sources for biomarker searches since they reflect the various stages of pathological conditions in real time. Both qualitative and quantitative composition information of cellular secretomes is critical to understand the mechanisms of cellular interaction. This information can be used to identify disease biomarkers such as tissue-specific antigens or potential drug targets for clinical treatments [55].

Quantitative proteomics-based secretome analyses are well established and described for diverse cell culture models[56]. There are two general proteomics approaches to identify secreted proteins from the conditioned culture medium of a cell type of interest. In one approach, cells are grown in growth medium containing serum. This method usually includes extensive protein and/or peptide fractionation to detect low-abundance secreted proteins against a background of thousands of highly abundant serum proteins. However, this analysis is challenging because the secreted proteins are generally in far lower abundance than the serum proteins contained in cell culture media. Alternatively, secretome analysis can be performed in serum-free conditions with labeling strategies, or with a combination of both approaches, thereby reducing analytical interference and increasing the ability to detect secreted proteins [57]. Although serum starvation has been used to synchronize cells, cell behavior may be massively biased and proteins may be released into the medium by induced cell death and lysis, thereby distorting the protein composition of the

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secretome[58]. Therefore, alternative techniques for secretome analysis that allow the presence of serum in the medium would prove enormously useful.

In recent years, metabolic incorporation of AHA and pulse labeling of SILAC have been combined for reliable identification and quantification of secreted proteins using mass spectrometry[59]. AHA incorporation provides an unbiased way to enrich newly synthesized secreted proteins from serum-containing culture medium. The azide-containing NSPs can be covalently coupled to an alkyne-activated resin via click-chemistry, followed by stringent washes to remove serum and other abundant background proteins. AHA methodologies can reveal a part of the proteome that has been poorly understood, but biologically important. Furthermore, secretome analysis has been combined with transcriptome and full proteome analyses to produce an integrative analysis of the first 3 h of lipopolysaccharide-induced macrophage activation[60]. Novel mechanistic principles of processes specifically induced by lipopolysaccharides can be derived from this combination analysis, which will be very useful in probing the immediate effects of cellular stimuli and will provide mechanistic insight into cellular perturbation in multiple biological systems.

4. AHA methodologies to measure microbiomes

The microbiome, which is known as the collection of bacteria, viruses, and fungi that live in and on the human body, has recently been recognized as an important factor in human physiology and disease. There are on the order of 100 trillion microbes that make up the human microbiome, which is far more than the number of somatic and germ cells within the body [61]. Microbiomes are typically made up of vast numbers of different microbial species. Once established, the communities live in relative stability and share important traits, such as antibiotic resistance, by communicating across species [62]. Furthermore, the interactions between host and microbe shape and direct the development of the organisms involved. Environmental factors such as nutrition and stress also play a critical role in shaping the interactions [63]. Interestingly, the microbiomes are involved in host immunity, disease development and metabolism [64, 65]. In particular, the gut is a biological niche that is home to a diverse array of microbes that influence nearly all aspects of human biology through their interactions with their host.

Until recently, the properties of intestinal microbiome were largely unknown. Knowledge of the compositions of microbes in the microbiome is vital for understanding homeostasis and acquiring important information about the molecular insights of diseases. Cataloging the microbiome involves sequencing the DNA of the microorganisms present. Metagenomics provides a scan of the population of microorganisms present and the coverage of the individual genomes depends on the complexity of the community and the depth of the sequencing. It has been shown that the genetic predispositions of the host, together with abnormal composition of intestinal bacteria may be causing dysregulation of gut microbiome interactions and thus could be pathogenic factors in the development of Crohns disease [66]. Another study illustrated that gut microbiomes may play a critical role in the pathological process of type 1 diabetes[67]. Metagenomics provides a catalog of the organisms present, but does not provide any information about on-going biochemical interactions

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among the community. Metaproteomics, the analysis of the expressed proteins, is being used to better understand the on-going interactions of the microbial community [68].

The use of feces as a means to assess health is growing. It was reported that mutant DNA, which can be an indicator of disease, has been isolated from the feces of patients with colorectal cancer and adenomas [69]. However, the sensitivity of a fecal DNA test was found to be poor for diagnosis since cancer was not detected in more than 40% of the population tested [70], suggesting that a DNA test may not be appropriate for clinical diagnosis. Analysis of proteins present could reflect biomolecules that directly influence cellular biochemistry and may provide additional insights into functional genome. The search for biomarkers has stimulated many groups to look at the potential of proteomics to reveal new biomarkers [71, 72]. New technologies are beginning to reveal important aspects of host-microbe interactions and disease biomarkers. It has been demonstrated that AHA can be incorporated into the mouse proteome by feeding for 4 days food incorporating AHA without interfering with gross behavior or physical appearance [6]. In addition, the administration of AHA did not substantially change the proteome of developing mice [73]. After pulse labeling, AHA methodology could be a powerful tool to investigate the mouse microbiome. AHA methodologies are not limited to mouse and can be expanded to study the microbiome of various model organisms.

AHA methodologies could be used in fecal proteomics. Investigation of fecal samples or biopsies also make it possible to directly analyze human samples and may provide insights into the mechanisms of diseases. Feces could be considered like other “body fluids” and cells and microbes present in feces labeled with AHA upon collection to determine the proteins present. Furthermore, fecal samples offer several advantages for the future clinical application of disease biomarker detection. Stool collection is non-invasive and can be easily collected. The collection also can be undertaken at home with a simple collection kit without the need for trained staff or a formal hospital visit. However, since there are many practical and ethical problems in performing microbiome study in humans, researchers may choose to use model organisms instead. With AHA methodologies, the appearance of NSPs in rodent stool samples could be quantitatively analyzed. Such analysis can help to illustrate potential biomarkers and molecular mechanisms underlying diseases.

Another potential application of AHA methodologies is the measurement of the local response of host proteomics. Numerous studies have analyzed the response of epithelial cells to induced stress or microbial load, which has provided valuable insights into structural, functional and physiological changes. Along with these studies, many aspects of epithelial integrity, innate immunity and recognition of either pathogenic or beneficial microbiota have been found to be controlled through proteins presented in epithelial cells [74, 75]. Despite these studies, host response essentially remains a large black box. One of the biggest advantages of AHA methodologies is that protein synthesis can be identified separately from static proteome, which decreases the complexity of the sample and potentially enables the identification of proteins expressed at low levels.

Systemic reaction is also an important aspect of host health. Previous studies have shown that there are dramatic physiological effects on host wellbeing when a host organism is

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subject to absence of its normal microbiota [76]. It is worth noting that the ability of microbiota to affect a host organism is not limited to the peripheral cells that are in direct contact, but extends to the cells of the acquired immune system and even some unknown pathways in more complicated organisms. Signaling information can be passed through microbe-host interaction and cell-cell communication to trigger an inflammatory response and activate tissue repair [77]. NSPs identification and quantitation by AHA methodology in an animal disease model will be a powerful tool to monitor proteome dynamics and provide comprehensive information for systemic reaction.

5. AHA methodologies in proteostasis studies

The proteome is a dynamic entity, tightly regulated by protein synthesis and degradation to maintain protein homeostasis (proteostasis) in cells, tissues, and organisms. The maintenance of cellular proteostasis requires a balance of protein synthesis, trafficking and degradation. The acquisition of a new steady-state level of any protein is the outcome of the change in its synthesis rate and degradation rate. Proteostasis plays an important role in almost every cellular event. Under proper maintenance of proteostasis, cells are allowed to grow, proliferate, respond to environmental changes and defend cells from being attacked by pathogens [78]. Many diseases, including neurodegenerative, auto-immune and cardiovascular diseases [79–81], are caused by abnormal proteostasis. As diseases progress, both protein synthesis and degradation can be altered to become faster or slower. Traditional proteomics has been used to find potential therapeutic targets by comparing the quantities of proteins in two different conditions. However, it does not address or provide information about the dynamics of the proteomes in different biological states. There has been an increasing demand for technologies to identify protein production and degradation separately.

Since Dieterich et.al introduced AHA to identify NSPs in 2006, AHA methodologies have been extensively used to monitor protein dynamics through *de novo* protein synthesis in multiple systems including immune cell response to external or internal cues [82], mouse development [83], as well as in native plant tissue under stress [84]. This methodology could be expanded in various types of future studies.

Excess proteins are not only a burden for cells, but also a waste of cellular energy. The degradation of NSPs is an extremely important component of proteostasis as well as the removal of aged or damaged proteins. Protein degradation is mediated by two major pathways, the ubiquitin-proteasome pathway and lysosomal proteolysis. Autophagy is a highly conserved intracellular degradation system that delivers cytoplasmic constituents to the lysosome. Under normal conditions, autophagy occurs constitutively at basal levels, possibly reflecting its role in the degradation of long-lived proteins and the removal of damaged cellular organelles [85]. Under stress such as starvation, enhanced autophagy flux promotes dynamic recycling of the basic biomolecules such as amino acids [86]. Autophagy is a dynamic process and has been implicated in pathological conditions including neurodegenerative diseases, cancer, and inflammatory diseases [87, 88]. Modulation of autophagy has become a potentially interesting therapeutic target in human diseases, which makes it essential to provide evidence and information for altered autophagy. AHA has been used in recent studies to trace the autophagic flux [89]. After a click reaction between an azide

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and an alkyne, the azide-containing proteins can be detected with an alkyne-tagged fluorescent dye, coupled with flow cytometry. In this way, global protein degradation can be quantitatively detected during autophagic flux by calculating fluorescence intensity. To provide more details, MS-based AHA quantification strategies could be used to measure individual proteins.

Over the years, it has remained controversial as to whether and to what extent the autophagic process is transcriptionally regulated. A number of nuclear transcription factors have been demonstrated to play crucial roles in autophagy regulation as either activators or repressors, mainly through transcriptional regulation [90–92]. Therefore, it is reasonable to speculate that *de novo* protein synthesis is implicated in both the basal and inducible autophagic processes. BONCAT and iTRAQ-based quantitative proteomics methods have been combined for the specific identification of *de novo* protein synthesis during autophagy induced by amino acid starvation[18]. 711 NSPs have been characterized and analyzed during starvation mediated autophagy.

It has been reported that a substantial fraction of cellular proteins are rapidly degraded and replaced with newly synthesized copies[93], minimizing accumulation of damaged protein to ensure a functional proteome throughout a cell's lifetime. Mcshane et.al conducted a kinetic analysis of protein stability in a cell line and in primary cells by performing pulse labeling of AHA in triple-SILAC labeled cell culture to distinguish degradation of "young" (NSPs) and "old"(pre-existing) proteins [94]. The results showed that proteins are less stable in the first few hours of their life and stabilize with age. This finding is consistent with previous observations, such as data that shows that post-mitotic cells cannot be completely labeled with SILAC despite a long labeling time [95]. Additionally, AHA-pulse chasing enables specific enrichment of newly synthesized proteins with no apparent impact on protein stability. A handful of proteins with limited turnover and long life spans (persisting for months or even years) has been illustrated by several studies [96–98]. One group of long-lived proteins, like crystalline and collagen, can be explained by their existence in a metabolically inactive environment that lack protein synthesis and degradation machineries. The other group, which stays in metabolically active cells with intact protein synthesis, degradation, and quality control mechanisms, might be sources of vulnerability in the mammalian proteome. Profiling of changes in long-lived proteins may play an important role in understanding disease cause and progression. A study of system-wide identification of proteins with exceptional lifespans in the rat brain using ^{15}N pulse labeling has shown that some nucleoporins (Nups) have incredibly long life spans in an aging mouse model. More specifically, the high stability of Nup96 is due to its deposition in a stable complex [99]. This pulse chase strategy can be expanded to numerous disease models. Alternatively, using AHA in a pulse chase workflow will be a more cost effective and straight-forward way to perform these studies due to shorter labeling times of AHA in animals, as well as simplified data analysis.

6. AHA methodologies in the clinic

The ability to metabolically incorporate AHA in cell lines, primary cells, brain slice, and animal models for NSPs analysis, together with its sensitivity and capacity for quantitation

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(i.e., heavy isotope labeled AHA), makes AHA methodologies a promising tool for various biological studies. With the development of other technologies, AHA methodologies could also be a potential powerful tool for clinical studies and diagnosis.

Tissue diagnosis is particularly important for the determination of cancer grades, and especially for surgical margin evaluation during surgery excision of cancer patients. For decades, diagnosis of tissue samples in the clinical environment has been performed by skilled pathologists using light microscopy techniques [100]. Of late, MS imaging (MSI) has gained increased attention from the biological and medical communities as a powerful approach for tissue imaging and diagnosis. MALDI imaging has provided great hope for tissue analysis by MS, but it requires matrix application to tissue samples and analysis under vacuum conditions [101]. Several ambient ionization MS techniques have been developed for rapid molecular diagnosis of cancer tissues and have shown exceptional potential for clinical use[102]. These ambient ionization MS strategies allow sample analysis in an open environment at atmospheric pressure. More recently, an automated, biocompatible and disposable handheld device called the MasSpec Pen have been developed for direct, real-time nondestructive sampling and molecular diagnosis of tissues[103].

For clinical use of AHA, a harmless method of introducing AHA in clinical samples needs to be developed. Traditional human cell cultures are limited in their representation of *in vivo* responses due to lack of an appropriate micro-environmental context of the responding cell types. With the development of newer culture approaches there have been several workflows like three-dimensional (3D) cultures, organoids, or organs-on-a-chip which partly succeed to better replicate the tissue microenvironment. Human organ cultures (HOCs) offer a simple approach that may better to address these issues. HOCs can preserve cellular responses that may be lost in cell cultures and can reveal positional effects on cellular responses. HOCs can be used to study stem cells within their *niche,* and demonstrate altered response and signaling pathways in diseases [104]. A combination of HOCs and AHA-related strategies can provide sensitive detection and accurate measurement of proteome changes caused by pathological processes and may also be used for clinical diagnosis in biopsies. A potential advantage is the ability to measure active protein synthesis which is information that may have diagnostic value. It has been reported that AHA can be incorporated into mouse by injection without substantially changing the proteome of developing mouse and AHA incorporation has no apparent impact on protein stability[73]. It is possible to perform AHA injection into targeted tissue *in situ*, followed by collection of a piece of the tissue and detection of abnormal NSPs activity with mass spectrometry. Such a strategy may help to uncover subtle, but vital changes in the proteome that may be useful to understand disease potential.

MS-based AHA strategies constitute a sensitive, multiplexed method with potential applicability to the high-throughput analysis of clinical samples. Furthermore, its unique ability to identify NSPs increases the value of AHA related strategies as a powerful diagnosis tool.

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7. Concluding remarks

Unnatural amino acids have emerged as a powerful tool for chemical biology. Since it was found that AHA can be accepted by cellular translational machinery as a methionine analog and metabolically incorporated into proteins, MS-based AHA related methodologies have been rapidly developing and have been used in various biological studies including the immune response, secretome, microbiomes and protein homeostasis. Since AHA labeled NSPs can be enriched though a click reaction, there is less concern about labeling efficiency, which is sometimes an issue in traditional metabolic stable isotope labeling strategies. Furthermore, AHA-based identification and quantification strategies could be carried out in mice, which is an ideal organism to explore drug effects and disease mechanisms prior to clinical application. Outstanding multiplexing ability, sensitivity, and selectivity make AHA strategies invaluable tools in NSP identification, quantification in simple or complicated systems and it may have a promising future in medical diagnosis.

8. Expert commentary

Over the past few years, shotgun proteomics strategies combined with labeling of proteins using stable isotope labeled amino acids on whole proteomes has enabled the discovery of protein factors involved in biological processes and pathogenesis. Since AHA was introduced and combined with mass spectrometry, new strategies in proteomics have emerged. MS-based AHA strategies overcome the deficiency of traditional whole-proteome labeling analysis, which has a strict requirement of high labeling efficiency that limits its application. AHA strategies decrease sample complexity and increase detection sensitivity through selective enrichment of the newly synthesized part of whole proteome, and also saves time by reducing the *in vivo* labeling period from weeks/months to hours/days in cells/animals.

While there are many advantages to AHA, there are some limitations. AHA is known to be incorporated into NSPs less efficiently than methionine. Although previous studies showed that AHA labeling has no substantial adverse effect on protein synthesis or degradation, it is possible that methionine starvation and AHA labeling have some effect on certain signaling pathways. AHA labeling is not applicable to methionine-free proteins. Although methionine-free proteins comprise only 1.02% of the human proteome, an additional 5.08% of proteins have a single N-terminal methionine which can be readily removed post-translationally [3]. HILAQ can be used to analyze at least 94% of the proteome.

AHA strategies have been used to identify synthesis activity and to compare two biological conditions in various biological model organisms including *Escherichia coli* [36, 37], C. elegans [24], tadpoles [38], mammalian cell line [26], primary cells [13], mouse [6] and *Arabidopsis thaliana*[16]. Theoretically, AHA strategies can be expanded to any system, since AHA is recognized by cellular translational machinery which has similar characteristics in either eukaryotic or prokaryotic cells. It also offers the possibility of assessing the temporal and spatial dynamics of certain subcellular compartments, organelles and protein-protein interaction networks in combination with subcellular fractionation, and immunopurification of protein complexes. It is not known whether AHA intake or injection would have an

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adverse effect in human beings, but it may nevertheless develop some role for clinical diagnoses in clinical settings.

9. Five-year review

MS-based AHA strategies have been used with significant success in the last decade through decreased sample complexity and improved sensitivity. Their ability to distinguish the newly synthesized proteome from pre-existing static proteome demonstrates their great potential as a powerful proteomic tool in high-throughput discovery studies. AHA methodologies will continue to be employed in secretome research since it allows serum to remain in the medium. MS-based AHA strategies also provide a novel way to expand the NSPs analysis to primary cells and animals by overcoming the need to completely label the proteome, as required with traditional stable isotope labeling strategies. Since comparative proteomics plays an important role in biomarker discovery, MS-based AHA strategies will provide an alternative strategy for studies in this field. The quest for detection of low abundant proteins and subtle, early onset changes in pathological conditions using MS-based AHA strategies will require technological advances in high performance, high-resolution mass spectrometers to detect proteins with greater specificity and dynamic range. In combination with improved sample preparation and fractionation strategies, MS-based AHA strategies will allow researchers to go further and deeper in various studies and to translate the knowledge into clinical use.

10. Key issues

- Azidohomoalanine (AHA), an analog of methionine, can be naturally incorporated into newly synthesized proteins (NSPs) by cellular translational machineries. The azide moiety in AHA can specifically react with an alkyne-bearing biotinylated tag in the presence of CuAAC catalyst, allowing subsequent selective enrichment of the modified protein pool using avidin affinity purification. This strategy, called biorthogonal non-canonical amino acid tagging (BONCAT), decreases sample complexity and allows direct analysis of proteins synthesized in response to the stimuli.
- BONLAC has been combined with other proteomic approaches such as SILAC and iTRAQ for relative quantification to directly compare the newly synthesized proteomes. Recently, with the synthesis of heavy isotope labeled AHA, heavy isotope labeled AHA (HILAQ) has shown higher sensitivity and comparable accuracy.
- AHA-based methodologies have been applied to various model organisms including *Escherichia coli* [36, 37], C. elegans [24], tadpoles [38], mammalian cell line [26], primary cells [13], mouse [6] and *Arabidopsis thaliana*[16].
- Pathogens can attack organisms and cause disease initiating an immune response. Understanding the mechanism of this response in detail is essential for developing drugs for different immune system disease. Without proteome fully labeled, it is difficult to do quantification using SILAC because the levels of

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incorporated label are low and thus signal to noise is problematic [105]. Studies of proteome dynamics would be facilitated by AHA strategies that enable separation of newly synthesized proteins from the preexisting protein pool.

- The proteins secreted by various cells (the secretomes) are incredible sources for biomarker searches, and a novel route to discovery since they reflect the various stages of pathological conditions in real time. The presence of serum has been a limitation for the analysis of secretomes. When using serum in media, the secreted proteins are mostly of low abundance and will be obscured by highly abundant serum proteins. When not using serum, cell behavior may be massively biased and proteins may be released into the medium by cell death and lysis, thereby distorting the protein composition of the secretome. AHA incorporation can provide an unbiased method to better identify newly synthesized secreted proteins by selective enrichment from serum-containing culture medium.
- The interactions between host and microbe shape and direct the development of organisms involved. The influenced microbiomes are involved in host immunity, disease development and metabolism. Until recently, the functions and components of intestinal microbiome were largely unknown. To reveal important aspects of host-microbe interactions microbiome functions and disease biomarkers, new technologies has emerged for discovery studies. AHA methodologies can be performed in fecal proteomics to reveal new biomarkers. Another potential application of AHA methodologies is the local response of host proteomics and host systemic reaction.
- The proteome is a dynamic entity, tightly regulated by protein synthesis and degradation to maintain protein homeostasis (proteostasis) in cells, tissues, and organisms. Many diseases, including neurodegenerative, auto-immune and cardiovascular diseases, are caused by abnormal proteostasis. Proteomic strategies have been used in protein degradation studies. Using AHA for pulse chase workflows will be a more cost effective and straight-forward way to perform such studies because of the shorter labeling time of AHA in animals, as well as simplified data analysis.

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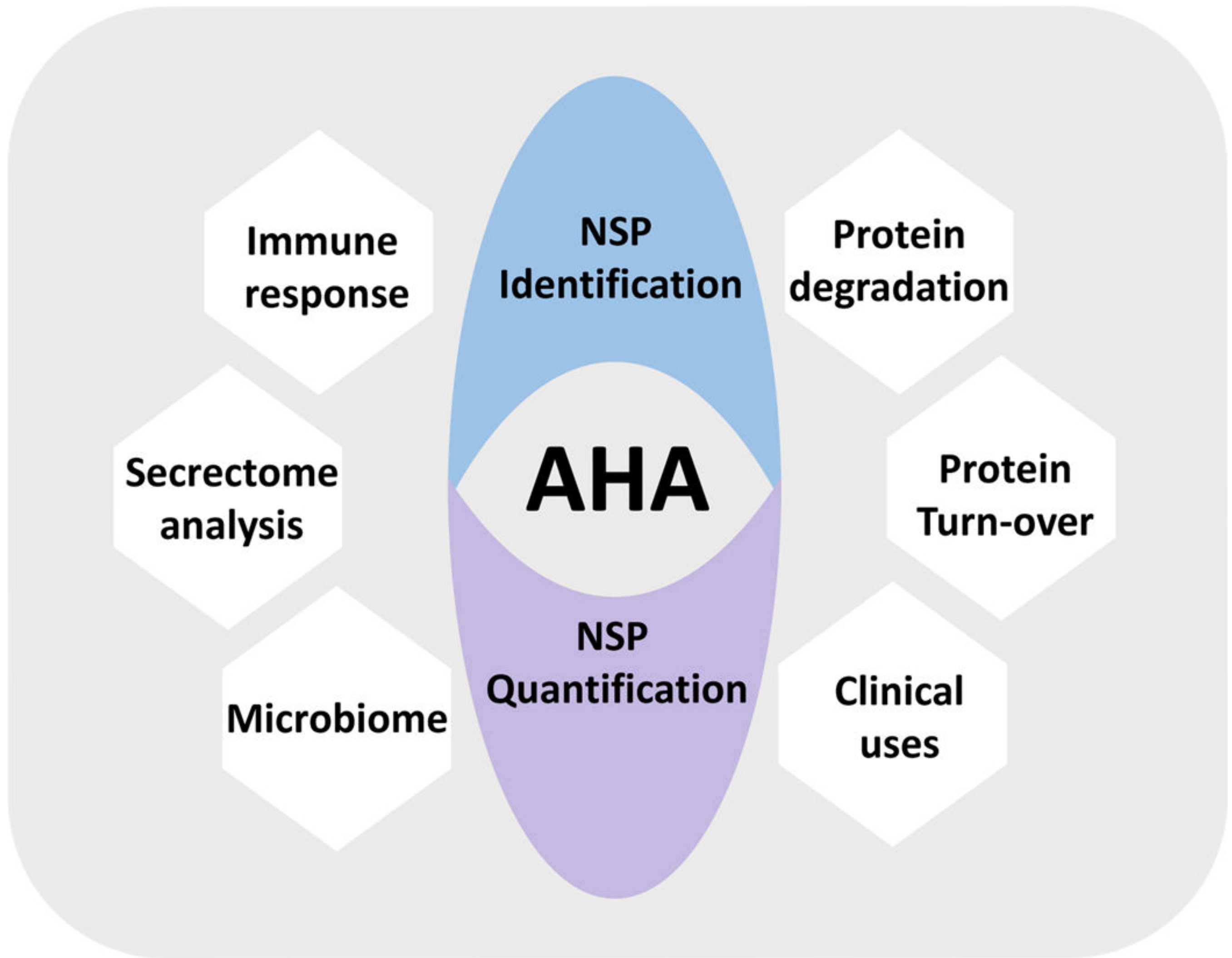

Figure 1:
AHA methodologies and its potential applications in immune response measurement, secrectome analysis, microbiomes, protein degradation, protein turn over and clinical uses