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Recent developments in microwave-assisted protein chemistries – can this be integrated into the drug discovery and validation process?

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Over the past 30 years, biotechnology has evolved as a multibillion dollar industry. A large sector of this industry is associated with translating human genetic information to discover, develop and commercialize bio-therapeutics. A broad range of disciplines across the Life Sciences is necessary for the selection of bio-molecules as biotechnological drug targets. Global and targeted proteomic approaches, as well as in-depth characterization of select proteins, are part of the process necessary for drug discovery and validation. Recently, microwave-assisted proteomic methodologies have emerged as useful tools for increasing the bio-catalysis and, therefore, throughput of analytical processes associated with protein characterization. Several microwave-assisted protocols that have become integrated into the drug discovery research process in the biotechnology industry are discussed.

Introduction

The role of proteomics and protein characterization in the field of biotechnological drug discovery can be categorized into two main areas: drug discovery and drug validation. In the discovery process, experiments such as investigations into protein–protein interaction partners [1,2] processing events [3,4] and post-translational modifications [5,6] are performed. During drug discovery, the aim is to identify either proteins or post-translational modifications that are key players in distinct cellular pathways; this type of analysis requires the characterization of highly complex material, typically in low abundance. In the highly competitive field of drug discovery, throughput is extremely important to remain at the forefront of scientific discovery.

On the validation side, late-stage research drug candidates and end-stage drug products require the application of a range of qualitative and quantitative analytical techniques to ensure correct translational and cellular processing. These methodologies include quantitation of material using amino acid analysis (AAA), verification of the intact molecular weight of proteins using mass spectrometry, protein characterization using proteolytic cleavage for peptide mass fingerprinting or MS/MS [7] or coupled separation and analytical techniques such as LC/MS/MS analysis [8] and N-terminal sequencing [9,10]. Figure 1 shows an overview of the analytical processes typically employed for the characterization of a protein during the drug discovery and validation phases of research.

The past decade has witnessed exponential growth in the capabilities of analytical instrumentation; chromatographic, mass spectrometric and bioinformatic tools have advanced significantly to establish new standards of sensitivity and throughput. Reverse-phase chromatography, for example, is now routinely capable of sub-nanoliter flow rates, allowing increased sensitivity for the analysis of low-level *in vivo* material. In addition, the introduction of new reverse-phase materials and technologies, for example, ultra high-pressure liquid chromatography (UPLC) [11] now provides superior reproducible separation of peptides and proteins, pushing the boundaries of dynamic range and sensitivity even further. Mass spectrometric instrumentation has also undergone significant improvements with the development of instrumentation providing increased resolution and faster scanning capabilities. The field has recently observed an era of new hybrid systems such as the LTQ-Orbitrap [12], the Q-TRAP [5,13] and the Synapt [14] mass analyzers, which allow versatile work flows and confident and high-throughput mass analysis of biological material. Through the coupling of these tools with powerful informatic programs for rapid and confident data analysis [15–18], a new bottleneck has been introduced in the process of protein characterization

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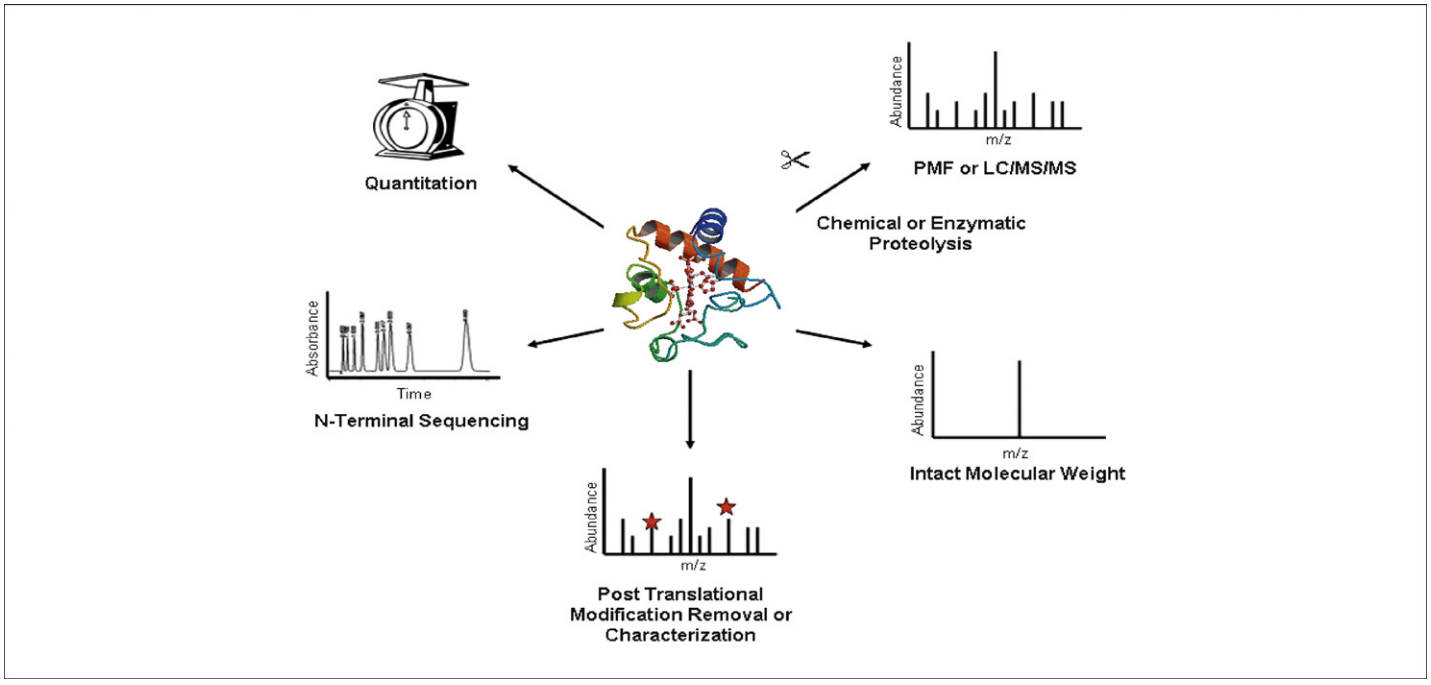

FIGURE 1

An overview of the analytical processes typically employed for the characterization of a protein during the drug discovery and validation phases of research.

and quantitation. These limitations are now typically defined by sample preparation time.

Recently, researchers in the biochemistry and biotechnology industries have taken a leaf out of the chemist's book; alternative catalyzing energies, such as microwave irradiation have been employed by inorganic and organic medicinal chemists for over two decades, resulting in accelerated and increasingly efficient reactions [19]. Currently, researchers are investigating and incorporating microwave-assisted methods into both the drug discovery process and drug development protocols to reduce sample preparation time and to explore alternative work flows for the characterization of recombinant proteins (for comprehensive reviews on microwave-assisted methods in the biosciences please refer to Lill *et al.* [20], Collins and Leadbeater [21] and Sandoval *et al.* [22]). In the following discussion, five areas of microwave-assisted protein characterization that have been incorporated into the drug discovery and characterization processes are reviewed.

Microwave-assisted protein hydrolysis for amino acid analysis

Quantitation of protein is performed at many points during the drug discovery phase. It is imperative that accurate quantitation of protein is performed for many *in vivo* and *in vitro* studies, including immunization of animals, X-ray crystallography studies for deciphering protein structure for small-molecule drug interaction and, hence, drug design, in addition to quantitation of protein for immunoassays, such as ELISAs, for screening high-affinity/avidity antibodies as potential biotherapeutics. In addition to quantitation of protein during the drug discovery phase, this is also an extremely important process during the development and validation phase where protein quantities for preclinical and clinical studies and also for final patient dosing need to be accurately measured as stipulated by the food and drug administration (FDA). Researchers have several options available for the accurate and precise quantitation of recombinant proteins, one of which is AAA. The initial process in AAA involves hydrolysis of protein into its constituent amino acids by breaking amide bonds, typically in the presence of 6N HCl at 110°C for 24 hours. Individual amino acids are subsequently subjected to either precolumn derivatization followed by reverse-phase chromatographic separation and UV or fluorescent analysis, or ion exchange separation followed by post-column derivatization and detection. One of the preliminary bioanalytical processes whereby microwave irradiation became routinely applied was for the hydrolysis of proteins for AAA. There are several comprehensive reports in the literature demonstrating the optimization of microwave-assisted acid hydrolysis for the quantitation of protein [23,24]. Microwave-assisted acid hydrolysis for protein quantitation has demonstrated results comparable to much lengthier traditional hydrolysis protocols using traditional ovens. Figure 2a illustrates the recoveries of amino acids from hydrolyzed beta-casein with theoretical amino acid recoveries for a conventional AAA hydrolysis protocol and a microwave-assisted protocol. Overall, comparable amino acid recoveries were observed between the two methods; however, the microwave-assisted method was complete within 10 min, more than two orders of magnitude faster than by employing conventional approaches [20].

Commercial microwave-assisted hydrolysis kits are available; for example, the Protein Hydrolysis System from CEM Corporation (Fig. 2b) which is supplied with a complete hydrolysis accessory with vessel and vacuum pump and fiber optic temperature control, all of which are compatible with the Discover Microwave unit.

Microwave-assisted N-linked deglycosylation for intact molecular weight measurement of immunoglobulins

The majority of biotherapeutic proteins till date are monoclonal antibodies (Mabs). The past decade has seen the evolution of

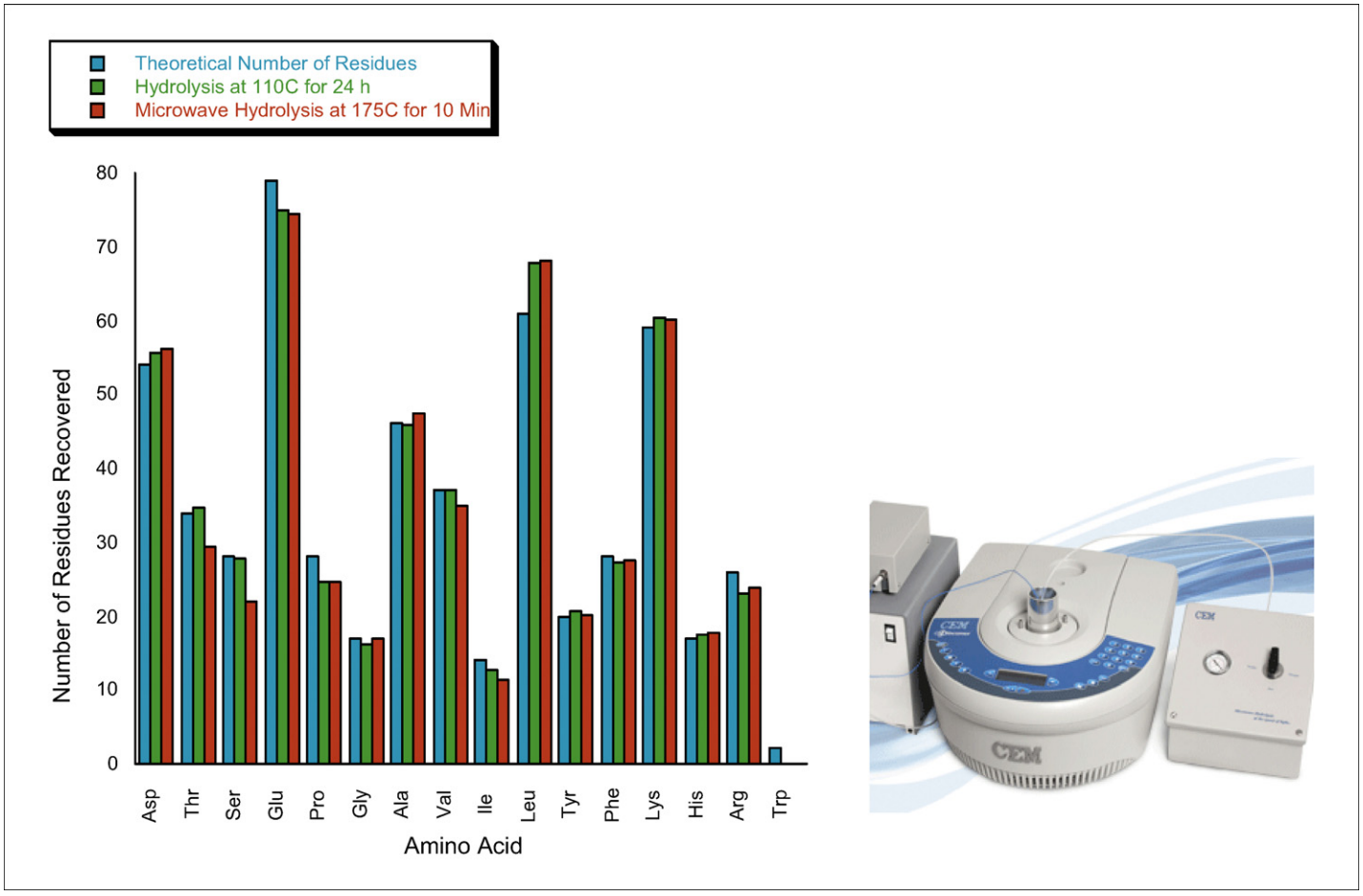

FIGURE 2

(a) A comparison of amino acid recoveries after hydrolysis using microwave-mediated hydrolysis and conventional oven-based hydrolysis methods for the protein beta-casein. **(b)** The Discover Research System is a specially designed microwave instrument for controlling acid hydrolysis conditions. The fiber optic temperature probe option allows precise, direct temperature measurement and control of acid hydrolysis procedures. The 45-mL vapor-phase hydrolysis vessel facilitates processing of up to 10 (300 μL) samples at one time. The valve panel allows connection to a vacuum and nitrogen source. The sealed sample vessel is alternately vacuum evacuated and purged with nitrogen. Hydrolysis is performed under inert, anaerobic conditions to prevent oxidative degradation of proteins or peptides.

antibody engineering to produce phage-display-mediated affinity-maturated antibodies [25], bi-specific antibodies [26,27], armed antibodies [28] and alternative immunoglobulin configurations for biomedical applications [29]. A common post-translational modification (PTM) on the heavy chain of antibodies, typically at or around Asn 297, is the presence of an N-linked oligosaccharide moiety. This moiety is typically a G0 (Asn 297-GlcNac-GlcNac-Mannose-[Mannose-GlcNac]$_2$) variant or the mannose-capped G1 oligosaccharide (Fig. 3).

Intact MW analysis of antibodies may be performed for evidence that a single moiety is present and that the observed mass of the antibody matches that of the theoretical. Intact MW measurements also serve to pinpoint postprocessing artifacts common to antibodies such as the loss of C-terminal lysine or deamidation. Deconvolution of the mass to charge envelope of antibodies is severely complicated by the presence of carbohydrate because of the diverse nature of the sugar molecules. To reduce heterogeneity before intact MW measurement N-linked deglycosylation is often performed, typically via hydrolysis with the enzyme PNGase F.

Depending on the nature of the carbohydrate and perhaps the accessibility of the sugar to the enzyme, deglycosylation with PNGase F can take from hours to days. Further complications may arise when the antibody is conjugated to a small molecule. The typical minimum time for complete enzymatic deglycosylation of standard unconjugated glycoproteins in the literature is 2 hours and at least 24 hours for conjugated moieties [30]. Recently, microwave irradiation has been shown to decrease significantly the time necessary for PNGase F mediated deglycosylation for both conjugated and nonconjugated immunoglobulin drug products and other glycoproteins. This reduction in sample preparation time before intact MW analysis has had an impact on the total time for characterization in that now a reliable MW for a potential drug product may be obtained only in hours.

In addition to enzyme-mediated oligosaccharide removal, an additional microwave-assisted protocol has been described, whereby proteins are partially hydrolyzed in TFA allowing release of the component monosaccharides for direct monosaccharide analysis. This method is complimentary to the PNGase F-mediated protocol, as it is not suited for the analysis of the intact protein but rather the oligosaccharide composition and is a process more suited to the product validation stage rather than as an initial research tool [31].

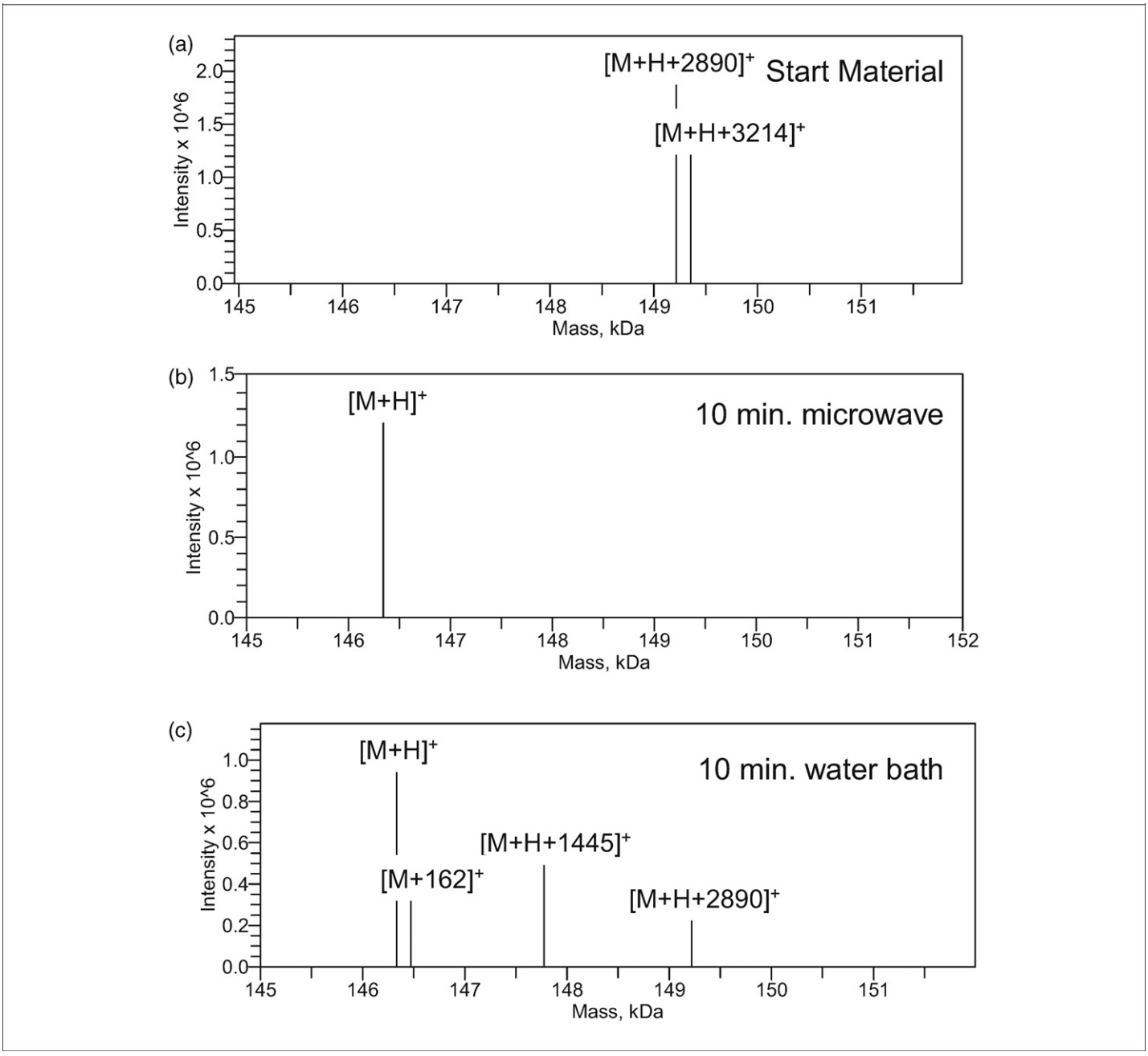

FIGURE 3

Deconvoluted mass spectra of **(a)** a nonreduced glycosylated commercial antibody drug product, **(b)** a nonreduced antibody after 10 min incubation in the microwave at 37°C with PNGase F at an enzyme:substrate ratio of 1:50 and **(c)** an antibody after incubation with enzyme for 10 min in a water bath at 37°C [30].

Microwave-assisted proteolytic digestions

The discovery of new molecules for development as drug candidates can start with several screening processes to determine novel proteins playing key roles in pathways associated with tumorigenesis, autoimmune disease and other maladies. One popular method for deciphering key players in an intracellular pathway is to perform an immunoprecipitation of a known protein (termed the 'bait') within the pathway, and to determine which other proteins are associated (interacting) with the bait protein, with the eventual goal of uncovering key molecules that can be intercepted by drug development [1,2]. The most commonly employed proteomic technique for the characterization of complex mixtures, as in the above case of identifying intracellular signaling pathways for discovery research, as well as validation of individual recombinant proteins, is the 'bottom-up' approach. The bottom-up approach entails the proteolytic cleavage of proteins into smaller peptides, typically employing an enzyme such as trypsin or endoproteinase Lys C, followed by mass spectrometric analysis [1,2]. Analysis of resultant proteolysis products typically involves either peptide mass fingerprinting (PMF) or, more commonly, LC–MS/MS. An efficient proteolytic digestion allows for the identification of low-level proteins with high sequence coverage in a short period of time.

In the case of recombinant proteins, sample amount is often not the limitation. During the drug discovery process, however, where the aim is to identify the proteins of interest, one is often dealing

TABLE 1

Percent coverage of sequence after proteolytic digestion with trypsin for two proteins in the thermocycler and microwave at 37°C.

Proteins	Thermocycler at 37°C				Microwave at 2 W, 37°C		
	5 min	30 min	60 min	8 hours (conventional method)	5 min	30 min	60 min
BSA	32	41	52	58	47	52	68
Myoglobin	33	62	65	62	59	76	85

Samples were analyzed by LC–MS/MS on an LTQ-Orbitrap mass spectrometer in data-dependent mode (fragmenting the top five most abundant ions from the Full MS scan). Data were analyzed using the search engine Mascot [15]. Average of three experiments.

with the identification and subsequent post-translational mapping of endogenous proteins that are present at low (subfemtomole) levels. As per our observations, microwave-assisted tryptic digestions result in higher peptide recovery and, hence, better sensitivity in a significantly shorter incubation time (5–30 min at 37°C) than traditional overnight water bath mediated incubations (Table 1).

It is hypothesized that the shorter incubation time in microwave-assisted enzymatic cleavages minimizes adhesion of peptides generated during proteolysis to the wall of the vial, resulting in a higher recovery of peptides. In this respect, microwave-assisted enzymatic digestion allows one to identify proteins, and/or their post-translational modifications when they are present at low levels, which would otherwise be impossible to identify using conventional incubation methods. Indeed, several steps in the drug development process benefit from microwave-assisted proteolysis. For example, during the validation of the integrity and purity of drug products, microwave-assisted enzymatic proteolysis methods have been introduced. Zhu *et al.*, for example, have developed a method for the rapid identification of therapeutic recombinant human monoclonal antibodies (rhuMAbs) using microwave-assisted trypsin and Lys-C digestions with PMF [32]. The microwave-assisted method is much faster than conventional incubation methods and has been demonstrated to be reproducible, robust and widely applicable.

Microwave-assisted acid hydrolysis for N-terminal sequencing

For nearly 50 years N-terminal sequencing has been carried out using the chemistries first described by Edman and Begg [9]. Edman degradation involves the sequential coupling of a free alpha amino group from the N-terminus of a protein with phenylisothiocyanate, cleavage of the coupled amino acid from the peptide backbone, and conversion to form a phenylthiohydantion derivative that is chromatographically separated and detected by UV. N-terminal sequence analysis is an important step in the characterization of biotherapeutics because of the information that is obtained relating to correct processing, ragged N-termini and internal cleavages. N-terminal sequencing by Edman degradation is often employed after the transfection of a recombinant protein into an alternative cell line to determine whether the protein has been correctly transcribed, translated and has been intracellularly processed. In the case of antibodies it is also employed as a means to determine monoclonality. Automated Edman degradation occurs sequentially from the N-terminus of a protein with each cycle taking up to 55 min. Although accelerated cycles have been developed [10], the process is time-consuming and costly and often a bottleneck in the characterization process for recombinant proteins.

Recently, several alternative N-terminal sequencing techniques have been reported. Both biochemical capture [33] and top-down proteomics [34] have been described as rapid means for characterizing N-termini; however, both are specific or limited in their functionality (i.e. the requirement of an appropriate predicted proteolysis site for biochemical capture, or limitations of current top-down methodologies on mid-size to large proteins). As of yet, no single method has come close to replacing Edman sequencing.

Zhong *et al.* described a technique to identify the termini of proteins using microwave-assisted acid hydrolysis (MAAH) [35]. MAAH consists of hydrolysis of the protein of interest in acid for a short duration (i.e. 2 min) in the microwave. MAAH is often effective in confirming the proper termini of a molecule within minutes, as opposed to hours by traditional Edman chemistries. MAAH appears to be dependent upon the tertiary structure of the molecule and the resulting accessibility of the termini, somewhat limiting its universal applicability [20]. One tremendous benefit of MAAH over Edman, however, is the ability to characterize blocked N-termini. In addition, valuable C-terminal information may be obtained (currently there is no single standardized method for C-terminal sequence elucidation) [20].

The entire process of MAAH, from microwave hydrolysis to bioinformatic analysis may be automated for high-throughput characterization. In our laboratory we consider MAAH to be a valuable 'first pass' methodology for the rapid verification of protein termini, thus reducing the quantity of drug discovery samples that require Edman degradation. For further information on this technique please refer to Refs. [20,35].

Microwave-assisted removal of pyroglutamyl N-terminal residues

Mabs have been used as therapeutic agents for the treatment of several malignant diseases over the past decade. As mentioned above, one of the initial steps in drug development involves the assessment of monoclonality and purity of antibodies, as well as correct N-terminal processing. Mass spectrometry has rapidly emerged as a popular tool in proteomics and despite the fact that MAAH allows for many more samples to be analyzed than with traditional techniques, for preclinical antibodies, Edman degradation remains the technology of choice. As Edman chemistries require a free N-terminus for the reaction to proceed, termini that are blocked need to be removed or deblocked. The heavy chains of murine antibodies are particularly prone to being N-terminally blocked by pyroglutamate that occurs due to cyclization of the glutamine residue by the enzyme glutamine cyclase. The pyroglutamyl group at the N-terminus renders the protein inaccessible to Edman chemistries and, therefore, the removal of this blocking group using pyroglutamate amino peptidase (PGAP) is required.

PGAP, isolated from *Pyrococcus furiosus*, is a thermo-stable protease and is active up to 90°C. Taking advantage of the high thermo-stability of this enzyme, microwave irradiation was assessed and the kinetics of this PGAP digestion were investigated. Microwave-assisted PGAP digestion was demonstrated to be more efficient than conventional heating methods and although the mechanism is not fully understood, the role of microwave-mediated PGAP digestion resulted in a higher yield (and therefore deblocking efficiency) compared to digestions performed in the thermocycler [22].

Beyond drug discovery

Drug discovery is just a small component of the bioanalytical processes that occur within the biotechnology industry. Quality control and validation of drug products requires rigorous protocols to monitor the integrity of the drug product and also to monitor the presence of contaminants. A common contaminant that is thoroughly tested for by the FDA in antibody-based therapeutics is the presence of the Staphylococcal Protein A, which is used to capture antibodies from complex cell lysates. Shimoni *et al.* have introduced a microwave-mediated method to monitor the concentration of Protein A in a multiproduct leached Protein A immunoassay. Here, a microtiter plate format was employed using an adapted MARS microwave system (CEM). This method was optimized to work with multiple Mabs and samples were diluted into dissociation buffer and heated in the microwave at 80°C for 10 min followed by a further dilution before an ELISA reading was performed to measure dissociated Protein A concentration levels. Complimentary methodologies are currently being investigated and developed for a whole range of analytical assays [36].

Conclusion

Can microwave-assisted proteomics be integrated into the drug discovery process?

The answer is yes. Microwave-assisted protocols are employed on a daily basis in traditional synthesis methods, proteolytic digestions and mapping of post-translational modifications. Many reactions can be performed at elevated temperatures in a water bath and do not necessarily require microwave irradiation for rapid execution. Side-by-side comparisons of some standard analytical reactions in both the microwave and at elevated temperatures in the water bath, have demonstrated this, including protein reduction and alkylation in solution, chemical digestions with CNBr and certain enzymatic reactions. There are, however, also several undisputed protein chemistries in which microwave-assistance is advantageous and where it has been incorporated into the drug discovery process for routine analysis, five such topics have been discussed within this article [20–22].

Many researchers feel limited by the instrumentation described in several microwave-assisted proteomic publications; instrumentation does not have to be the limiting factor when initiating such microwave-assisted studies. Many laboratories successfully utilize conventional domestic microwave ovens for these purposes. During high-throughput analysis, however, industrial systems are preferred because they are designed to allow power and temperature control, uniformity and reproducibility to minimize the 'hot spot' phenomenon observed with traditional domestic microwaves [20].

The exact mechanism of catalysis of microwave irradiation on proteins is a subject of controversy. Is the enhanced reaction time observed due solely to increased temperatures and a faster rate of achieving that desired temperature, or is the augmented agitation resulting from the dipolar rotation of molecules responsible for increased efficiency [21]? Once the mechanisms involved in microwave-assisted protein reactions are better understood, further utility of the technology will be possible. A literature search over the past five years revealed over 4000 articles associated with microwaves in general. Of those, only 21 articles were directly related to proteomics and the biotechnology industry. Microwave irradiation is just beginning to become a routine part of the drug discovery process, and there are many more fields, reactions and protein chemistries in which microwaves may prove beneficial in years to come.

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