Anal. Chem. **2002,** *74,* 1−9

Perspectives

Strategic Use of Affinity-Based Mass Spectrometry Techniques in the Drug Discovery Process

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Advances in biomolecular mass spectrometry (Bio-MS) have made this technique an invaluable tool for analytical chemists and biochemists alike. The applicability of Bio-MS approaches in drug discovery now encompasses in vitro, cellular, and in vivo pharmacological and clinical applications in an unprecedented expansion of utility. As a result, the role of Bio-MS in pharmaceutical discovery continues to proliferate for both structural and functional characterization of biomolecules. From target characterization to lead optimization, affinity techniques have been used to purify, probe, and enrich analytes of interest. Affinity selection employed prior to MS analysis can "edit" out extraneous noise and enable the researcher to examine only what is important. These affinity-based methods can be used as an alternative strategy when classical biochemical techniques are insufficient in advancing difficult projects. We have applied various affinity techniques in conjunction with mass spectrometry throughout the drug discovery process. This perspective will describe affinity-based mass spectrometry methodologies and related concepts, illustrated with original results.

The field of biomolecular mass spectrometry (Bio-MS) has made a lasting impact on protein biochemistry and molecular biology research by providing novel and direct means to study molecular properties and interactions. Biological scientists have found a great utility in commercial mass spectrometry tools that are increasingly available and readily applied. In addition, there continues to be a wealth of new technologies and experimental strategies being developed by Bio-MS research facilities that infuse into mainstream exploratory research.

From a drug discovery perspective, the key to achieving timely impact with new approaches is to forge an appropriate hypothesis to enable fast and efficient project execution. This may be achieved more efficiently by introducing affinity selectivity into the experimental approach, conveying the inherent advantages of both protein biochemistry and molecular specificity onto the Bio-MS experiment. Throughout all stages of the discovery process, from target identification through lead optimization, specificity obtained through the use of affinity Bio-MS approaches provides explicit insight into biochemical processes.

This report first summarizes the current significance of structural and functional MS, followed by affinity-based biochemical techniques, as they are commonly applied to biochemical research problems. The highlights and viewpoints presented are not intended to portray a comprehensive review; therefore, relevant references are provided for consultation. Examples are then given from our own work that demonstrate a variety of combinations of affinity selection with mass spectrometric detection, which illustrate a strategic approach for introducing selectivity and accuracy of analysis to virtually any complex biological mixture. Finally, these results are further delineated by establishing their significance in relation to the drug discovery timeline.

OVERVIEW OF BIOMOLECULAR MS

Over the past decade, matrix-assisted laser desorption/ionization[1,2] (MALDI) and electrospray ionization[3−5] (ESI) coupled with mass spectrometry have been developed and refined into highly versatile experimental tools. The inherent flexibility of MS techniques is continuously enhanced through augmentation of mass detection configurations[6−10] (e.g., QQQ, IT, TOF, FT-ICR),

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10.1021/ac010569y CCC: $22.00
Published on Web 12/06/2001

Table 1. Highlighted Structural and Functional Bio-MS Applications

Structural MS	
application	methodology
protein identification; expression; up/down regulation	proteomic MS; ICAT; sequence tags; accurate mass; database search
signal transduction products; pathway elucidation	phospho-Ser/Thr/Tyr detection techniques; parent ion scan; neutral loss
protein−protein gas-phase complex and multimeric association	noncovalent electrospray MS; soft-ionization conditions
protein folding and allosteric interactions	H/D exchange; isotopic mass distribution; FT-MS

Functional MS	
application	methodology
protein−antibody recognition by epitope mapping	affinity capture; scanning peptide library; peptide excision/extraction; differential chemical modification
ligand fishing and mechanistic elucidation	affinity selection from biological matrixes (cells/tissues)
protein−protein solution-phase complex and multi-protein complexes	tagged gene expression with immunoprecipitation
solution-phase binding measurements	SEC-MS; PUF-MS; ACE-MS; SPR-MS; FAC-MS

experimental functionality[11−14] (e.g., scanning techniques and data-dependent experiments), and powerful data interpretation and software applications[15−22] (e.g., automated data analysis and database searching). MS can be combined with traditional biochemical protocols or multidimensional separation techniques that significantly improve the selectivity and specificity of the experimental approach for use in increasingly complex pharmaceutical applications. The true advantage of Bio-MS for the detection of molecular ions is conferred by the direct measurement of analytes, giving an unequivocal readout. Bio-MS can therefore be used to validate a standard biochemical assay or screen by linking the molecular species with a secondary readout, or it can serve as the primary screen itself.

The precedent in the literature is rich for the broad utility of Bio-MS and is supported by a variety of detailed and creative experimental approaches. Moreover, the scope of the field warrants a breakdown into two descriptive categories, referred to here as structural and functional MS. The following two sections and an overview depicted in Table 1 provide a summary of highlighted applications and methodological approaches in each of these areas.

Structural MS. Routine Bio-MS structural approaches include the identification of proteins, typically achieved through primary structure confirmation via molecular mass measurement. The structure of a protein can be further characterized by peptide mapping or peptide MS/MS sequencing.[23−27] In addition, primary structure adducts such as posttranslational modifications (PTMs) are deduced using distinctive experimental approaches. Tandem MS scanning combined with affinity-based MS techniques is employed to convey specificity for the analysis of protein phosphorylation or glycosylation.[28−35] For example, tandem MS scans can selectively detect precursor ions that generate a signature chemical fragment ion or a neutral chemical loss, both of which can be indicative of a particular modification. Additionally, affinity capture techniques such as metal ion affinity for phosphorylation and lectin affinity for glycosylation allow the researcher to enrich the sample for the specific PTM. Such strategies provide a simpler and more selective analysis of the structural modification.

Other advanced Bio-MS experimental configurations allow insight into secondary, tertiary, or quaternary protein structure. Noncovalent ESI-MS has demonstrated that, under the right set of conditions, intact protein complexes can be detected.[36−37] This has allowed insight into protein−ligand, protein−protein, and multimeric association states therein to be determined. Additionally, as new H/D exchange experimental strategies are applied, both secondary structural characteristics and protein folding or

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binding interactions can be elucidated by careful correlation with gas-phase and solution properties.[38−39] Finally, Bio-MS applied to protein expression profiling can be used to supply both the identity and relative quantity of expressed cellular constituents. Methodologies herein employ the standard peptide mapping and sequencing approach with an isotope tagging method that enables differential quantitative experimental comparisons.[17,40−42] This approach may be also be applied to a functional investigation of protein regulation in disease states, as well as biofeedback pathways related to signaling networks.

Functional MS. Basic experimental aspects of structural MS apply to functional MS, yet particular biochemical properties or consequences are under investigation in a functional approach. An example of a functional investigation is the study of the binding determinants of antibody−antigen interactions that make up the aspect of antigen recognition. This type of strong binding interaction may be elucidated using a number of various experimental approaches, known collectively as epitope mapping.[43−55] The power of Bio-MS for the identification of binding partners lies in its ability to directly characterize unknown ligands, which interact with and often provide a link to the biochemical significance of an orphan target.[56−57] In addition, MS can directly monitor the products or kinetics of substrate turnover or inhibition as a result of a functional assay.[58−61]

Affinity MS can additionally be used with tagged gene expression to determine the specificity and binding interface regions to provide insight into multi-protein complexes.[62−64] The isolation and characterization by MS of relevant multi-protein complexes can lead to regulatory or mechanistic understanding and the identity of interrelated biological targets. Finally, the properties of ligands binding to target molecules may be evaluated in detail using solution-phase binding methodologies for the determination and characterization of binding interactions[65−81] (e.g., size exclusion, ultrafiltration, surface plasmon resonance, capillary electrophoresis, or frontal chromatography coupled with MS). These approaches may provide insight into binding specificity, rank order of binding, dissociation constants, binding kinetics, interaction thermodynamics, competitive binding, or ligand displacement to investigate the active site of a protein target.

THE IMPORTANCE OF SPECIFICITY

Affinity for Purification or Isolation. Tagged protein expression is commonly employed to facilitate the purification of recombinant proteins. Expression constructs are often modified in the N- or C-terminal regions to incorporate peptide sequences or entire proteins that are high-affinity sequence determinants. Examples of affinity purification constructs include the following: His tags[82,83] to incorporate a hexahistidine sequence, purified using immobilized nickel affinity columns; small peptide antigens that are recognized by a specific antibody such as FLAG tags;[84] protein

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tags such as glutathione *S*-transferase fusion (GST),[85] purified by their respective molecular substrates that bind these proteins with high specificity; and protein antigens that contain antibody recognition sequences for subsequent immunoprecipitation by IgG, using a protein A support.[86] These approaches illustrate a strategy for recombinant protein expression to efficiently provide protein reagents for screening in a drug discovery environment. Mass spectrometric techniques can be readily applied to the rapid and automated characterization of modified or tagged protein expression constructs. Additionally, such mass spectrometric analyses often identify common impurities or side products involved in tagged protein expression, such as the α-*N*-gluconoyl modification on a His-tagged protein.[87]

Beyond their use for purification, tagged proteins offer a tether that may be further exploited for use in a particular assay design by taking advantage of the ability to immobilize the protein target on a surface. Traditional biochemical assays amenable to immobilized targets include the following: scintillation proximity assay[88] (SPA) to measure inhibition of binding of radiolabeled substrate; and reporter assays such as enzyme-linked immunosorbant assay[89] (ELISA) and electrochemiluminescence[90] (ECL) assay types, using antibodies containing a reporter enzyme or label, respectively. In addition, hyphenated affinity-based mass spectrometry or other related detector technologies (absorption, fluorescence) are often employed in inhibition or binding assays. These approaches often take advantage of the ability to immobilize targets to simplify the methodology and measure substrates or products of binding interactions. Techniques that take advantage of immobilized targets include SPR-MS[73] and FAC-MS[80] analysis.

Finally, immobilization techniques can be exploited to isolate molecules of interest from complex biological matrixes. Conventional affinity capture techniques using MS detection utilize immobilization on beads[65,66] (sometimes magnetic[47]) or other surfaces, such as direct analysis on laser desorption/ionization chips.[91−94] The benefit of immobilization is due to both enrichment of the substrate of interest and isolation from the matrix impurities. In this way, for example, an affinity MS approach can be used for in vivo analysis to isolate and measure ligands that are pharmacological, toxicological, disease, or drug-related biomarkers.

Elucidation of Binding Associations. The specificity of binding interactions can be investigated and characterized using affinity MS approaches. Multiple affinity MS approaches have been devised to characterize a unique binding interaction, such as antibody−antigen complexes, or to elucidate structure−activity relationships through inhibition or binding studies. The combination of antibodies and affinity-based mass spectrometry for mapping the epitope regions of protein antigens provides a classic illustration of MS applied to the characterization of binding interactions. Several creative MS-based epitope mapping strategies have developed over the years to characterize these antibody−antigen complexes (Figure 1). The scanning peptide library approach[43] has been utilized in our laboratory with affinity capture and MS detection to determine the sequences of linear epitopes.[95] Epitope extraction has also been applied to the affinity capture of linear antigenic determinants of proteins via proteolytic mapping followed by affinity MS, measuring either the presence[44−48] or absence[49,50] of antigen. A complementary technique includes epitope excision, which consists of limited proteolysis on an antibody−antigen complex, followed by antibody isolation and detection of the binding determinants.[51−54] The excision approach may be applicable to both linear and conformational epitopes.

Elucidating the antigenic determinants of an interaction involving conformational or discontinuous epitope is a challenge. A chemical labeling method utilizing differential modification has been demonstrated for mapping conformation-dependent binding interactions.[54−55]

We have employed the peptide scanning library approach to determine the epitope of a capture antibody used in a diagnostic assay. This monoclonal antibody recognizes an antigenic sequence contained in collagenase-degraded human type II collagen and, as such, may be useful for the diagnosis of osteoarthritis.[96] Utilizing a scanning peptide library approach, it was shown that affinity capture mass spectrometry utilizing ultrafiltration and LC−MS(/MS) detection could be used as an investigative tool for antibody−antigen interactions (Table 2). When compared to the results of solid-phase ELISA epitope mapping and solution-phase fluorescence polarization measurements, the affinity capture mass spectrometry method utilized was shown to be applicable for the analysis and identification of affinity complexes.[95] Similarly, combinatorial library analysis using various affinity-based MS approaches have been utilized to depict trends in the SAR of the structural determinants of synthetic chemical analogues.[66−70,74,75]

APPLICABILITY TO THE DRUG DISCOVERY PROCESS

Discovery Overview. The drug discovery time line encompasses processes from the earliest stage of idea generation all the way through to lead optimization of a druglike compound. Ideas that result in target identification have traditionally arisen through the observation of pathophysiological effects of disease states. However, in the postgenomic era, ideas for targeted discovery efforts can come from the analysis of functional genomics and gene families. Once potential targets have been identified, they are purified or expressed as recombinant proteins and are characterized for biophysical properties and activity. Additionally, validation of the preclinical, biological relevance of a given target must be verified in a functional setting, via

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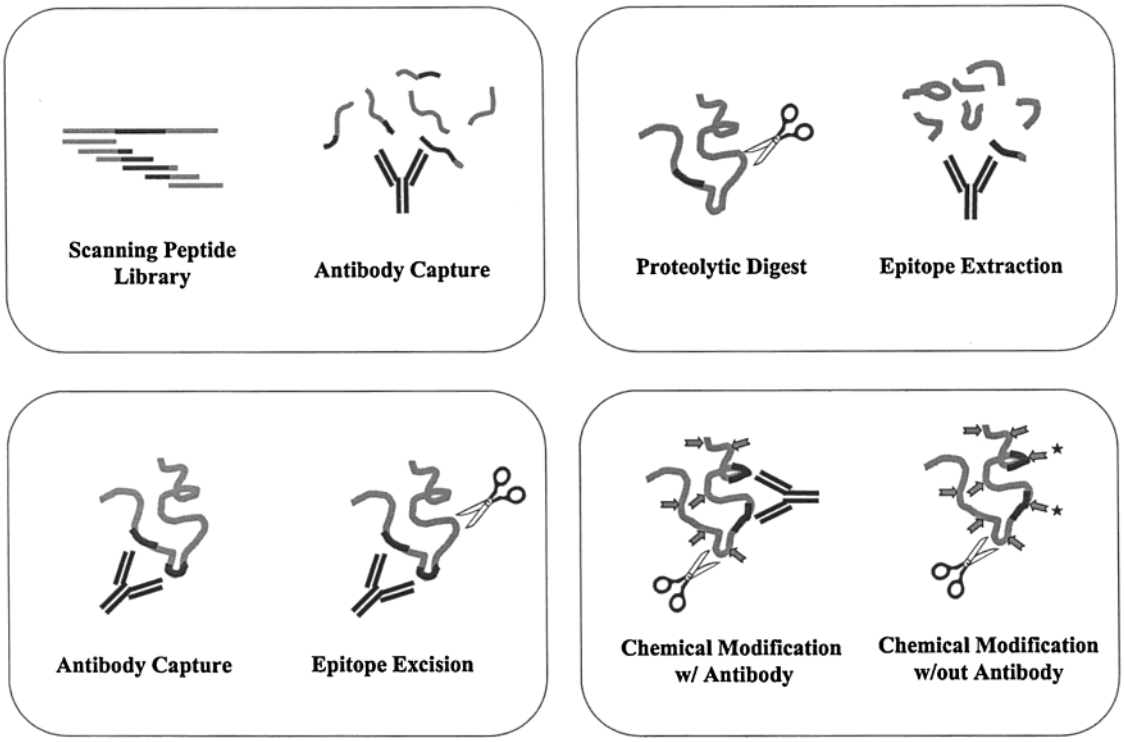

Figure 1. Schematic of epitope mapping strategies using affinity-based MS techniques.

Table 2. Epitope Mapping Using Scanning Peptide Library Affinity Capture MS[a]

peptides	mass	capture	peptides	mass	capture
GPPGE	624.3	N	GPPGEKGEP	1035.5	N
PPGEK	695.4	N	PPGEKGEPG	1035.5	N
PGEKG	655.3	N	PGEKGEPGD	1053.5	N
GEKGE	687.3	N	GEKG*EPGDD*	1071.4	Y[a−c]
EKGEP	727.4	N	EKG*EPGDD*G	1071.4	Y[a−c]
KGEPG	655.3	N	KG*EPGDD*GP	1039.5	Y[a]
GEPGD	642.3	N	G*EPGDD*GPS	998.4	Y[a,b]
EPGDD	700.3	Y	*EPGDD*GPSG	998.4	Y[a,b]
PGDDG	628.2	N	PGDDGPSGA	940.4	N
GDDGP	628.2	N	GDDGPSGAE	972.4	N
DDGPS	658.3	N	DDGPSGAEG	972.4	N
DGPSG	600.3	N	DGPSGAEGP	954.4	N
GPSGA	556.3	N	GPSGAEGPP	936.4	N
PSGAE	628.3	N	PSGAEGPPG	936.4	N
SGAEG	588.3	N			
GAEGP	598.3	N			
AEGPP	638.3	N			
EGPPG	624.3	N			

[a]Peptides had a N-terminal acetyl group and a C-terminal amide group. Italicized residues represent the epitope. Peptide identification by [a]MW, [b]RT, or [c]MS/MS

mechanistic or pharmacological analysis. Massive chemical libraries are then screened against the target and hits are obtained for further development. Also at this stage, additional safety and metabolic assays are conducted in a preclinical setting to enhance the confidence in druglike compounds and ensure success in the clinical stages of development.

Directed synthetic efforts around active chemical matter are subsequently carried out to produce compounds that have improved efficacy against the targeted mechanism. Active chemical classes are optimized using structure−activity relationships, employing mechanistic, functional, biophysical, and structural investigations. Other physicochemical properties such as solubility, absorption, metabolic lability, distribution, and clearance are also optimized to increase efficacy of the drug. Finally, classes of compounds that are active in a chemical series may be ranked and prioritized in terms of their binding affinities to the target of interest, in cases where the binding strength is shown to be directly related to efficacy. We have applied various affinity techniques in conjunction with mass spectrometry in these stages of drug discovery for target identification, target characterization, target functional validation, mechanistic studies, and lead discovery and optimization.

Target Identification. The determination of targets with relevance in a disease state has historically been the providence of pharmacological and clinical detective work. Today, the exciting promise of mapping the proteome is within close reach due to new biochemical approaches that now complement molecular biological probes. Examples of complementary biochemical and molecular approaches include the following: (1) protein identification and functional characterization, linking the proteome[97,98] to the genome;[99−100] (2) protein expression profiling using relative quantitation and isotope-labeled affinity tags,[40−42] as a complement to cDNA or mRNA chip-based expression profiling;[101−102] and (3) determination of protein−protein interactions using protein isola-

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tion and affinity probes[63] or protein cross-linking,[64] as a complement to yeast−two hybrid interactions.[103−104] We have used an affinity MS approach to verify an interaction determined using the yeast−two hybrid method[105] for protein-signaling interactions in the cytoplasmic domain between the T cell receptor CD28 and the serine/threonine phosphatase PP2A.

The intracellular domain of CD28 was expressed as a GST fusion protein and used as a probe, while the catalytic domain of PP2A was expressed as a FLAG construct for ease of purification. Affinity capture MS methods were developed for the CD28−PP2A interaction to directly observe binding in vitro that could be subsequently applied to "ligand fishing" analyses in cell lysates. The two recombinant proteins were incubated together overnight, and the protein−protein complex was isolated using bead-bound glutathione. The protein−protein complex was pelleted, washed, digested with trypsin, and analyzed using MALDI-TOF analysis. Peptides were identified using accurate mass analyses that were sufficient to determine the presence of GST, CD28, and PP2A, using the Web-based protein identification tool, ProFound.[106]

The search was designed to exploit the resolution and mass accuracy afforded by the MALDI-TOF data. All spectra were internally calibrated using trypsin autolysis peaks to increase the mass accuracy and facilitate the measurement of unknown peptides (data not shown). This effectively decreases the mass tolerance and narrows the search window, which in turn tends to give better search results with higher confidence. Relevant control analyses using a GST fusion protein without the CD28 tail demonstrated the specificity of the CD28−PP2A binding interaction. Such an analysis represents a viable means of isolating protein−protein complexes and target identification. A growing precedent in the literature supports this approach for unknown protein identification using mass spectrometry and database searching.[17−22,97,98]

Target Characterization. The value of recombinant proteins as surrogate drug targets is dependent upon expression, purification, and characterization of the desired product. Protein identification is accomplished using molecular weight analysis, peptide mapping, and peptide sequencing.[23−27] Frequently, tags are added to targets for use as a tether after protein identification such that the target can be immobilized for use in biochemical assays as described above.[82−86] As an alternative to expressed tag constructs, chemical modification of the protein can be employed to equip the protein with an affinity tether. This, however, can lead to multiple modifications, some of which may render the protein inactive. To probe these sites of modification, we applied a comprehensive on-line technique: multidimensional liquid chromatography (MDLC) coupled to a mass spectrometer.

For this application, the protein target was chemically biotinylated by modification of the primary amine groups with Sulfo-NHS-LC-Biotin (Pierce). This reagent contains a sulfo-*N*-hydroxysuccinimide that reacts with amines to form a stable amide bond and is soluble in aqueous solvents. By controlling the conditions of the reaction, such as amount of reagent used, pH, and time, it may be possible to limit modification to the N-terminal primary amine. The NHS is coupled to biotin through a spacer arm that limits steric hindrance of the protein once it is immobilized, which is important when looking at interactions of other molecules with the tethered protein.

Using MDLC-MS, the biotinylated protein sorbitol dehydrogenase (SDH) was digested on an immobilized trypsin column, and the resulting peptides were passed over an immobilized monomeric avidin column, where only the biotinylated peptides are retained. The biotinylated peptides were eluted from the affinity column and trapped on a small C18 cartridge for desalting and buffer exchange. The biotinylated peptides were then chromatographically resolved by a microbore C18 column and the masses obtained by ESI-MS (Figure 2). This technique demonstrates the ability of on-line methods coupled with affinity methods to quickly identify sites of modification of proteins or peptides. This method is flexible and can be tailored to work with many systems.

Target Functional Validation. Affinity capture techniques can used to demonstrate the ability of the target to bind a known substrate, inhibitor, agonist, or antagonist. This is important in validating the functionality of a recombinant or purified target. When modifying or immobilizing protein targets, it is important to confirm that the target retains the capability of binding a known ligand. We have employed an off-line ultrafiltration affinity capture technique to characterize interactions with an immobilized target protein and its antagonist for subsequent use in lead optimization studies.[107]

The ligand-binding domain of estrogen receptor (ER) was chemically biotinylated and immobilized on streptavidin beads. The affinity capture procedure used an ultrafiltration membrane to perform an incubation of the following: (1) membrane plus agonist; (2) ER target plus agonist; and (3) bead-bound ER target with agonist, followed by extensive washing and elution. The results shown in Figure 3 indicate that the ER target was capable of binding 4-hydroxytamoxifen in the unbound and affinity bound states, signifying that this tether does not inhibit its ability to bind a known agonist. Steric hindrance of an immobilized protein target or active site modification may render a target useless. By employing this technique, we can quickly ascertain whether the protein target is viable in its immobilized format.

Mechanistic Studies. Once a target has been tested in vitro, it is still imperative to validate the mechanism in vivo. Because of their nature, in vivo pharmacology and clinical samples frequently contain many extraneous proteins and, therefore, are difficult to analyze. By utilizing immunoprecipitation techniques, the target or analyte of interest can be quickly purified and concentrated from the sample. We applied this strategy to analyze phosphorylation sites on the microtubule binding protein tau.[108] The aberrant phosphorylation of tau has been implicated in the pathophysiology of Alzheimer's disease.[109]

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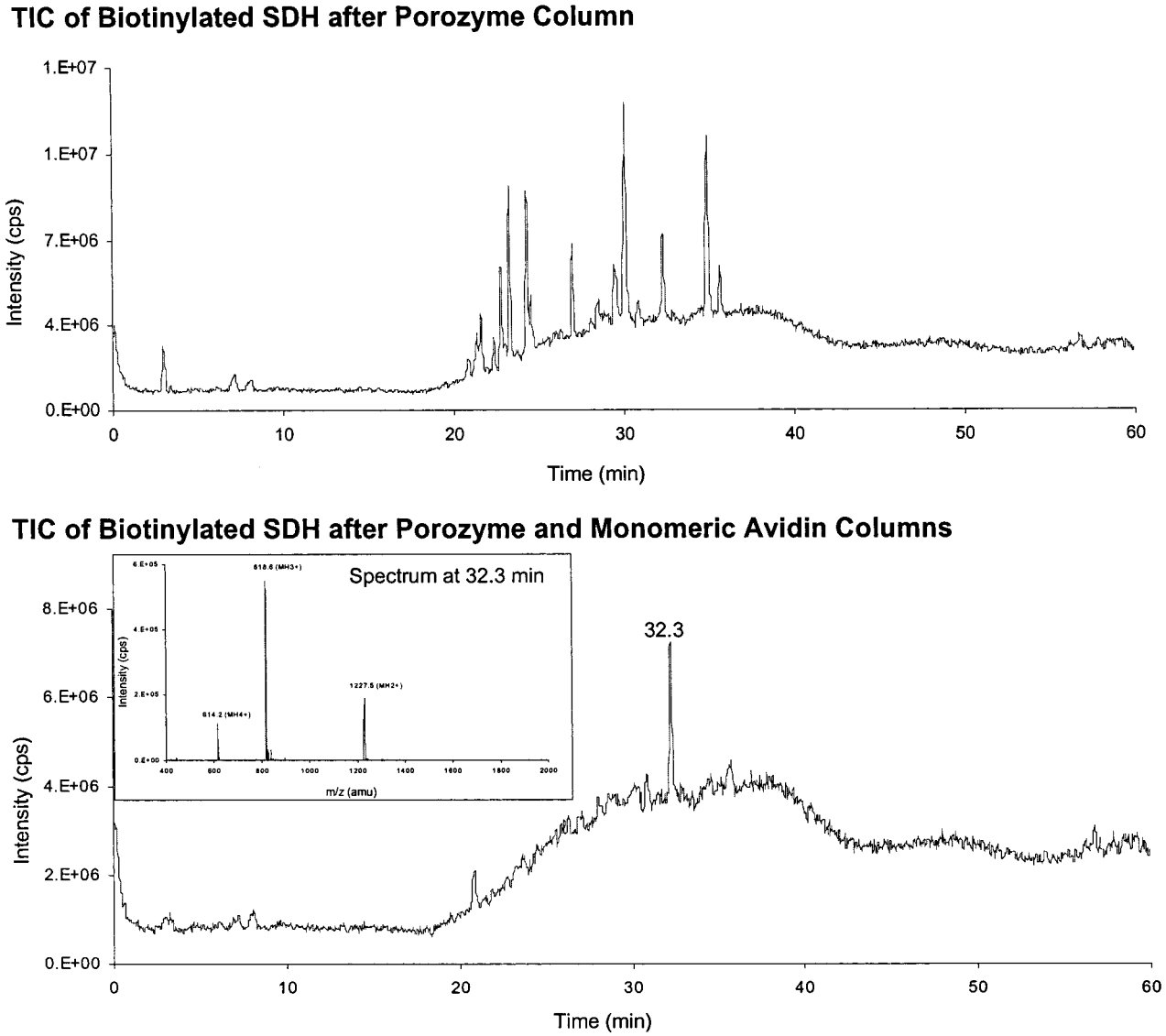

Figure 2. LC−MS chromatograms of biotinylated SDH. The top panel is the total trypsin digest. The bottom panel is the digest after passing over the affinity column. The inset spectrum is that of the single peak which agrees well with the calculated MH^+ of the biotinylated N-terminal tryptic peptide of SDH.

Phosphorylation of tau by recombinant cdk5 was investigated by first purifying tau from a mixture of proteins using immunoprecipitation with a polyclonal antibody, followed by tryptic digestion, isolation of phosphorylated peptides by immobilized metal affinity column (IMAC), LC−MS, and LC−MS/MS analysis (Figure 4). The combined immunoprecipitation and IMAC affinity approach effectively isolated the phosphopeptides from the complex mixture, and subsequent LC−MS/MS analysis identified site-specific phosphorylation states (e.g., see Figure 4). Phosphorylation of tau by cdk5 on Ser202, Thr205, and Ser404 (using the numbering of the longest human brain tau isoform) was detected. Phosphorylation at these sites has previously been shown to occur on tau isolated from the brain of Alzeimer's disease patients.[109]

Lead Discovery and Optimization. As targets and pharmacological models are validated, the discovery process becomes focused on lead generation and optimization. Affinity mass spectrometry techniques can be applied to drug discovery as an alternate, universal binding technique for lead identification or characterization. In one such technique, frontal affinity chromatography[110] (FAC), compounds are continuously infused over a column containing the immobilized target, which does not require a change in mobile phase for separation and elution. When combined with mass spectrometry as a means of detection,[80] the MS acts as a multichannel detector that can be used to identify unknown binding components to a target if they elute after the void marker. The mass spectrometer can also be utilized in ion monitoring or reaction monitoring mode to convey specificity and tolerance for a wide range of buffer conditions.

FAC-MS operates under dynamic equilibrium processes such that affinity dissociation constants can be readily measured using a simple titration analysis. In this way, the binding capacity of the immobilized target (B_t) and binding affinity constants of the compounds (K_d) are attainable using the relationship between the elution volume to the amount of ligand adsorbed to the target.[80] This approach can be extended for use in detecting mixtures of multiple binders to a single target, where relative binding and

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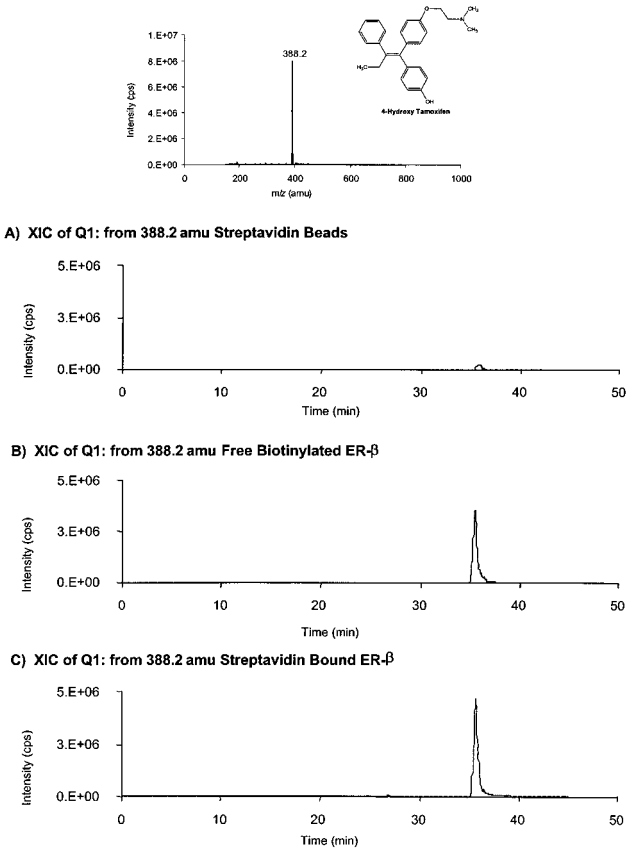

Figure 3. ESI-MS spectrum of 4-hydroxytamoxifen with structure shown in the top panel. Extracted ion chromatogram of the three samples spiked with 4-hydroxytamoxifen are shown normalized, for (A) streptavidin beads alone, (B) free, biotinylated ER LBD, and (C) immobilized ER LBD.

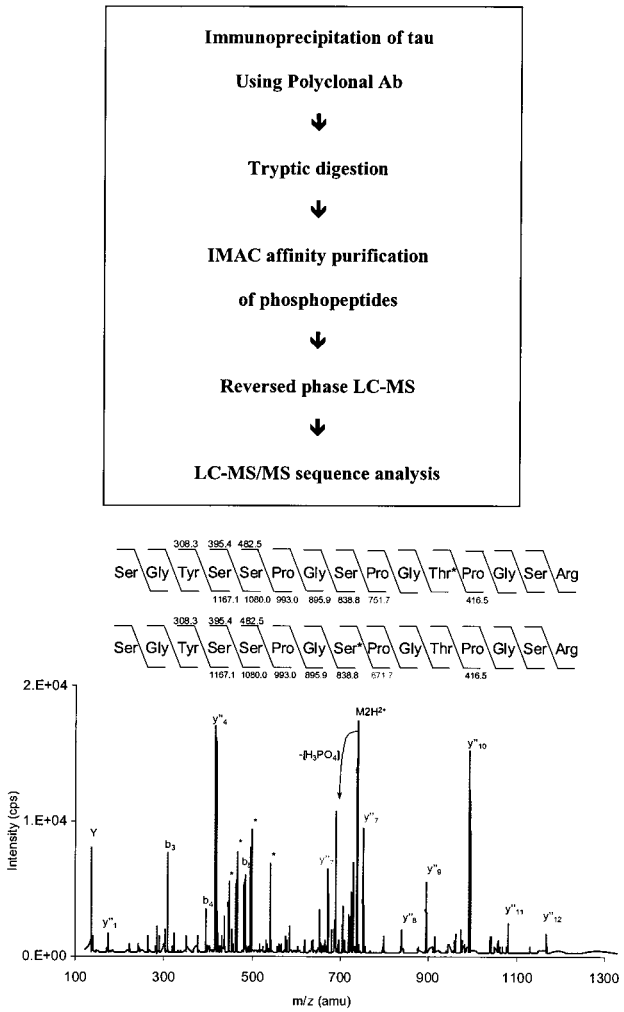

Figure 4. Flow diagram for the affinity isolation and of τ phosphopeptides for LC−MS analysis. LC−MS/MS of the *m*/*z* 737.4 ion is illustrated with representative sequences above the spectrum. Peptides are detected with either the Thr phosphorylated or the Ser phosphorylated and the b and y″ ions expected from each. The peaks labeled with * are doubly charged y″ ions.

rank ordering of ligands is demonstrated (e.g., see Figure 5). This kind of information can assist in the identification and prioritization of chemical material for lead development.

Another FAC-MS assay format uses a positive control as an indicator compound for "hit" discovery, which can readily distinguish multiple weak binders from strong binders in a mixture. In the case of multiple weak binders, the elution time of the indicator will decrease, indicating a reduction in the binding capacity of the target on column. Likewise, a stronger binder will effect the elution of the indicator; however, in this case, an additional displacement or roll-up effect is also detected[108,111] (data not shown). The downward slope of the displacement front mirrors the expected elution front of the competitive binding hit. Hence, a screen can be configured to select for hits of higher affinity than that of the indicator by selecting an indicator with a known K_d to serve as a "cutoff" value.

Applied to lead optimization, FAC-MS offers the advantage of a multidimensional readout for the simultaneous detection of binding affinity trends for components contained in a mixture. Frontal analysis facilitates resolution of multiple binders and the rank order for a wide range of binding affinities and class-specific structural trends. In the case of mixtures, displacement analysis also occurs and is visualized as a roll-up effect in the elution profiles, indicative of competition for a common binding site.

An evaluation of this technique was carried out using the polyol pathway target sorbitol dehydrogenase (SDH) to evaluate the applicability of FAC-MS for lead optimization and structure−activity profiling.[111] Biotinyl-SDH was characterized as described above, and approximately 15 pmol of protein was immobilized onto a 0.5-μL column packed with 200-μm streptavidin beads. Binding affinity constants were obtained for individual compounds using both direct and indirect titration analyses. K_d values were measured for selected compounds covering an affinity range of 2.2 μM to 1.3 nM, where the results correlated well with existing inhibition values. Rank ordering of multiple binders to SDH was demonstrated for three different subsets of ligands that were in good agreement with the order of K_d values (Figure 5). Competition for a common binding site was observed in the rank ordering of these mixtures that is visualized as an overconcentration or roll-up effect in the elution fronts. Compound class-related nonbinders were also evaluated and shown not to bind with the SDH target, and isomers of differing affinities were resolved in the

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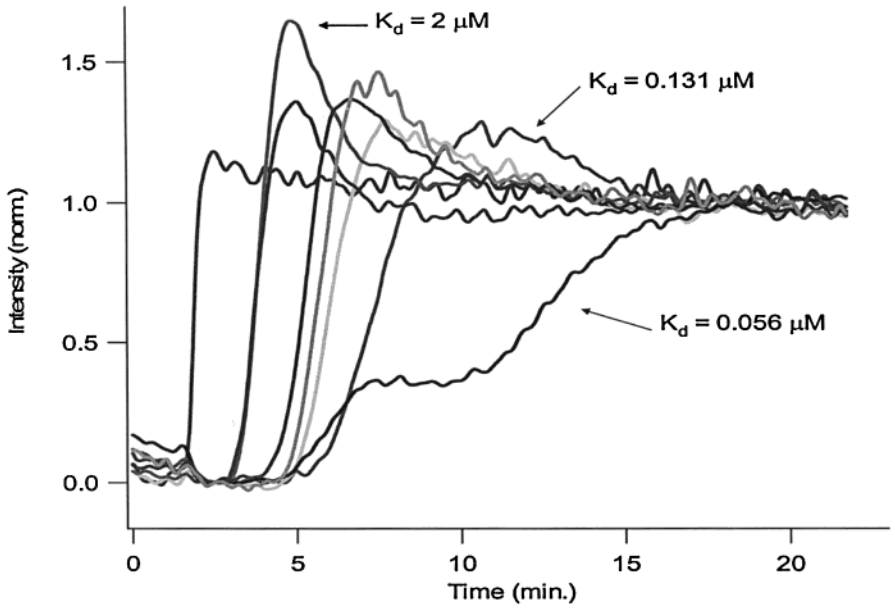

Figure 5. FAC-MS profiles of compounds eluting from the SDH column as measured using single ion monitoring of molecular ions. A nonbinding compound elutes first as the void marker because it shows no affinity to the target. The elution order of eight components analyzed as a mixture reflects their relative binding strengths, as confirmed by IC_{50} and K_d values.

correct rank order by applying MS/MS detection. Overall, these results demonstrated the utility offered by FAC-MS for application in both lead discovery and optimization.

SUMMARY

The power of affinity-based mass spectrometry techniques is formidable. It can be used at various stages in the drug discovery process to identify new targets, characterize modification sites on proteins, elucidate the mechanistic pathways implicated in disease states, access the ability of a target to bind ligands, and measure ligand affinities for a target. Affinity techniques such as those described offer novel approaches to discovery problems. When combined with mass spectrometry, they offer high-resolution structural and biophysical insights. In addition, affinity MS approaches are poised to make a significant impact in microseparations and multidimensional approaches for defining the structure and function of the proteome.

Taken separately, biomolecular mass spectrometry and affinity-based biochemical techniques are currently widely employed. Yet in combination, these approaches provide the potential to make contributions to promoting new directions in drug discovery. The future direction of such research might lead to a universal expectation for mechanistic evaluation of targets at the molecular level or introduce the routine emergence of affinity MS as a primary screen for lead generation and optimization. Additionally, the elucidation of signaling and biofeedback pathways coupled with affinity MS methods for selective detection will play an increasingly important role in biomarker discovery and diagnostics in both preclinical and clinical settings.

ACKNOWLEDGMENT

The authors thank our many colleagues for scientific collaborations and discussions over the past several years, as recognized in references 95, 96, 105, 107, 108, and 111. Additional acknowledgment is extended to Drs. David Schriemer, Nora Chan, and Darren Lewis for a successful collaboration.

Received for review May 24, 2001. Accepted October 2, 2001.

AC010569Y