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Peptidomics for the discovery and characterization of neuropeptides and hormones

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Abstract

The discovery of neuropeptides as signaling molecules with paracrine or hormonal regulatory functions has led to trailblazing advances in physiology and fostered the characterization of numerous neuropeptide-binding G-protein coupled receptors (GPCRs) as potential drug targets. The impact on human health has been tremendous: approximately 30% of commercial drugs act via the GPCR pathway. However, about 25% of the GPCRs encoded by the mammalian genome still lack their pharmacological identity. Searching for the orphan GPCR endogenous ligands that likely are neuropeptides has proved to be a formidable task. Here we describe the mass spectrometry-based technologies and experimental strategies that have been successful in achieving high throughput characterization of endogenous peptides in nervous and endocrine systems.

Keywords

bioinformatics; endogenous peptides; sequencing; mass spectrometry; nervous system; quantitation

What is peptidomics?

Genome and transcriptome sequencing is upon us, so why we are still looking for ways to identify bioactive peptides in living systems? Bold genomic research efforts have provided extraordinary insights into the inventory of GPCR-receptor genes, the most coveted targets in drug development [1]. Gene association and knockout studies have illuminated the roles of various peptide genes in pathological conditions and diseases in animal models [2, 3]. Yet genetic investigations do not determine the actual peptide gene products, neuropeptides, and hormones that mediate vital body functions and complex behaviors. The immense challenge is due to the complexity of molecular readout; a single gene can produce many products as a result of single nucleotide polymorphisms, alternative gene splicing, post-translational processing of precursor proteins, and the addition of chemical post-translational

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modifications (PTMs) of cleaved peptides that oftentimes cannot be inferred from genomic data. Even annotating peptide coding genes and *in silico* neuropeptidome prediction requires specialized expertise and bioinformatics tools [4–6].

At the dawn of the new millennium, the term ‘peptidomics’ (see the workflow shown in Figure 1A) was formally adopted to describe a method for high throughput, direct measurement and structural characterization of the endogenous peptides present in a given biological sample (see detailed historical review by Schrader and co-authors [7]). In the intervening 15 years since the ground-breaking publications in the field, peptidomics has blossomed into a multitude of distinct approaches (Figure 1B), designed to accommodate a range of sample-related issues (chemical and anatomical complexity, difficulty of sampling, size and/or volume) and a lack of prior knowledge on the peptides expressed in the sample [8–10]. The quantitative capability of peptidomics has become more refined and reliable [11–13]. The use of bioanalytical methods, powered by mass spectrometry (MS) aided by liquid chromatography (LC) and bioinformatics, has steadily increased in the medical and life sciences. At the same time, technological refinement continues to push the boundaries of the limits of detection, resolution, mass accuracy, throughput, and efficiency of data processing.

Here we review the current state of MS-based peptidomic technologies and provide guidelines on their application, while also highlighting examples of how to make optimum methodological choices defined by the specific study goals and available resources. Our analysis of the literature covers peer-reviewed publications from the past two years, with a few important exceptions, and focuses exclusively on the analysis of endogenous bioactive peptides in nervous and endocrine systems. Other applications of peptidomic technologies are thoroughly reviewed elsewhere [11, 14–18].

Choosing your peptidomics *modus operandi*: a guide on methodological approaches

There are two main approaches to bioactive peptide discovery and functional characterization. The first, more traditional experimental pipeline targets a specific (or a few) compound(s) of interest within a biochemical pathway or neural circuit that have already been investigated using molecular probes or expression techniques. The success of this tactic greatly depends on *in silico* data mining and prior information (Figure 2A). The second, contrasting approach casts a broader net and aims to structurally characterize “all” soluble peptides present in detectable amounts in tissue or organs. One issue is that no current technology can actually measure “all” of the peptides present; yet even with this caveat, this untargeted strategy may lead to the discovery of unexpected molecules and is especially useful as an initial hypothesis-generating study (Figure 2B). Importantly, having a working knowledge of the available methods and selecting the appropriate approaches determines the success of the measurement and furthers investigative outcomes. This is a great way to chemically test a new, unexplored sample type that is abundant and accessible for peptide extraction procedures. In the following sections, we highlight applications and specific methods for both peptidomics strategies.

Untargeted high throughput peptide exploration

Peptidomics is powered by a hyphenated technique (Figure 1A), liquid chromatography (LC)-tandem mass spectrometry (MS/MS), which can handle chemically complex mixtures and the wide dynamic range of concentrations typically found in biological samples. Chromatographic methods most often include reversed-phase high-performance liquid chromatography (RP-HPLC), or orthogonal, two-dimensional LC. Downstream sequencing MS platforms measure and fragment a set number of ions, usually from the most abundant peptides, eluting from the chromatographic column in a small time frame, which are then temporarily excluded from analysis to allow detection of less abundant peptides within the same time frame. The method is known as data-dependent acquisition (DDA); its effectiveness is influenced by the LC separation, and the sensitivity, spectral rate, and resolution of the mass spectrometer. The resulting data are a combination of intact peptide profiles in the sample and their respective fragmentation spectra (e.g., MS/MS), both needed for follow-up bioinformatics-guided peptide identification (Figure 3). Another separation technique, capillary electrophoresis (CE), is less frequently used as a front-end approach in peptidomics applications because of fewer available commercial systems; CE-MS does allow the down-scaling of the measurement to smaller-volume or low-abundant samples [19, 20]. When the required pre-analytical conditions are met and essential resources are available (Figure 2B), robust characterization of the peptide complement in most peptidergic tissues, peptide extracts, and biological fluids generates a wealth of information for further investigation. Numerous model and socio-economically important organisms have benefited from high-throughput queries of their peptidomes [4, 5, 21–29], providing molecular detail to link peptides to environmental factors, nutrition, physiological states and behaviors. Discovery peptidomics usually involves larger samples, often comprised of many individual samples, which ensures detectable levels of low-abundant peptides and a broader prohormone coverage of detected peptides. Pooling hundreds of individual samples, such as insect brains [4, 28], neural ganglia [23], or defined mammalian brain regions [30–32], is common in these types of untargeted peptidomics studies.

Neuroendocrine tissue and select types of neuronal populations often contain high local concentrations of neuropeptides and thus are suitable for direct analysis either by matrix-assisted laser desorption/ionization (MALDI) MS profiling [33] or MALDI MS imaging (MSI) with minimal sample manipulation [34–38]. Mass spectrometers with standard or interchangeable MALDI ion sources usually have MS/MS capabilities, which allow automatic or manual sequencing of peptides in solid tissue samples [39, 40]. MALDI MSI has an added benefit of being capable of mapping peptides to specific loci within the tissue section [34, 38]. Combined with *in situ* or immunohistological analyses of selected neuropeptides, direct MS measurements reveal neuropeptide distribution patterns in organs or tissues of interest [27]. Although direct analysis of peptides in tissue is convenient and effective, it has been applied to only a limited range of animal models. The approach has been used more frequently to investigate invertebrates due to the relative simplicity of their nervous and endocrine system organization.

Targeted peptide characterization

This approach relies on the same high sensitivity, high throughput MS-based technology described above, but focuses on specific peptides [41, 42] or peptide families [43–45] that are usually implicated in biological pathways of interest to the researchers (Figure 2A). Identification of PTM sites on known peptides is another example of a targeted peptidomics approach [46].

An unusual PTM is the enzymatic single amino acid d-isomerization in a peptide, as has been observed in frog skin antimicrobial peptides, spider, mollusk and mammalian venom toxins, mollusk neuroexcitatory peptides, and crustacean neurohormones (comprehensively reviewed here [47]). Compared with the all-l-amino acid epimer, a d-amino acid in the peptide can confer distinct and dramatically enhanced bioactivity, as is the case with the newly characterized GdFFD peptide from the marine mollusk *Aplysia californica* [48]. Targeting d-amino acid-containing peptides for investigation has been notoriously difficult via molecular techniques, or even MS, due to the lack of a sequence change or mass defect associated with this PTM. However, new multidisciplinary peptidomics methods promise to facilitate the discovery of other putative d-amino acid-containing peptides in many animal models by measuring the distinct molecular fragmentation patterns among peptide diastereomers with MS/MS [48–50].

Differential and quantitative peptidomics

It has become evident that prohormone levels do not always correlate with their coding mRNA levels. Although microarray techniques can be useful analytical tools, they do not provide definitive information on neuropeptide dynamics in perturbed biological systems. The demand for peptide-relevant, in-depth quantitative assays has stimulated the development of quantitative MS approaches (reviewed by Romanova and co-authors [11]). Differential peptidomics compares qualitatively or quantitatively detectable peptides (Figure 2) between experimental sample groups, either to test or generate hypotheses on the functional connections of detected peptides, or to correlate peptide levels to the biological paradigms under investigation [10]; these investigations can be performed on a global scale [30–32, 51–53] or selectively [54]. The intensity of the observed intact peptide ions, or frequency of fragmentation events during chromatographic separation, serves as a basis for peptide level comparisons. In either case, the approach requires careful experimental design and thoughtful interpretation of results.

Although it is tempting to associate differential peptide profiles or levels in the samples to peptide expression in the phenotype of interest, they may not reflect true *in vivo* expression status. First, only peptides that remain stable under the tissue collection protocols used, and are soluble in the extraction media, retained under the selected separation condition, and detectable with the chosen MS approach, can be assessed by peptidomics. Second, perturbation of the biological system leading to *in vivo* peptide changes may also affect *in vitro* detection of peptides, irrespective of the perturbing event, simply by altering the balance of molecular homeostasis within the sample. This can lead to changes in the solubility, charge balance, and ultimately, the ionization efficiency of different ion species during the MS measurement. In other words, at the measurement stage, we deal with a

subset of biomolecules that are only as representative of their physiological state as our sampling protocol allows. Recent developments in sampling approaches, such as focused microwave irradiation and heat stabilization [55], minimize post-mortem protein degradation products and thus, may provide information that more closely reflects the *in vivo* peptidome. Additionally, the measurement strategy has a profound effect on experimental outcome. The versatile method of MS-based structural characterization, DDA, may restrict global quantitative analysis because it relies on real-time decisions about which precursor ions from a survey scan should be directed for fragmentation, and so its performance declines as sample complexity increases. Experimenting with alternative methods, the Li group [53] conducted a proof-of-principle study using data-independent acquisition (DIA) to quantify feeding-related peptides from crab. A brute force method, DIA [56], which acquires fragmentation spectra independently of precursor ion information, has gained acceptance for both identification of tryptic peptides and targeted quantification of proteins. However, quantitation of endogenous neuropeptides by DIA remains challenging. Finally, processing of MS data for quantitation is critical and should take multiple variables into account, ranging from simple instrument performance drift over time to the presence of multiple charge state ions for the same peptide, peptide co-elution, partial labeling with chemical or isotopic tags (if used), high background noise, small numbers, and natural variability between biological replicates. In our opinion, integrated studies where differential peptidomics data are validated by independent approaches deliver the most biologically relevant discoveries [57–59].

Recently, differential comparisons have been used to probe unknown mechanisms in the physiological response to environmental [51], developmental [60] or pharmacological perturbation [31, 61, 62], disease states [36, 63–65], phenotypes [39, 66], or even various sample preparation methods crucial for successful neuropeptide detection [67, 68]. It is safe to say that relative or differential MS quantitation has already become routine due to the availability of commercial reagents, sensitive analytical instrumentation, and software. With this said, absolute quantitation of endogenous peptides continues to challenge the bioanalytical community. Just as with radioimmunoassay and ELISA, absolute MS quantitation is most effectively performed for selected and known neuropeptides for which synthetic or isotopically labeled standards are available [54, 69–71].

Probing cellular diversity by single-cell peptidomics

Locating a specific cell from within a relatively uniform cell population to determine its chemical content presents a demanding bioanalytical task. MS measurement at the single-cell level can identify neuropeptides co-localized within the cell soma, even if they are encoded by different co-expressed genes. Single-cell peptidomics is most effective when working with well-characterized neuronal circuits that underlie defined behaviors or physiological functions in suitable neurobiological models having accessible neurons or other cells of interest, which rarely includes mammals. Historically, the most common application of single-cell peptidomics has been geared towards determination of the actual peptide products of a cloned gene or transcript in functionally characterized cells. Detecting multiple predicted peptides by their molecular mass often is sufficient to assign peptides to a certain prohormone, in the same way as the peptide mass fingerprint method is widely used

in proteomics for protein identification. This approach allows matching of the individual cell peptide profiles to electrophysiological activity [72], localization of molecular probes [73], or both [74, 75]. A unique two-step strategy hyphenates MS to targeted chemical analysis of immunocytochemical-selected peptidergic neurons containing selected biomarkers of interest [76]. While robust, single-cell peptidomics is difficult to mainstream for comprehensive chemical analysis, primarily due to technical challenges in adapting chemically complex nanoliter-volume samples to compatible nanoseparation platforms and hyphenating those with MS [77]. Therefore, direct targeted MALDI MS continues to be the most successful approach for single-cell measurement. We have reviewed numerous inspiring examples during the past decade elsewhere [77].

Microfluidic platforms for peptidomics of cellular releasates

Given the tremendous chemical diversity of neurons and neuroendocrine cells, it is logical to focus on peptides released in response to physiological stimulation to gain insights into the mechanisms of intercellular communication. *In vitro* neuronal networks present the opportunity to collect and characterize intercellular signaling peptides released by the neurons upon physiologically relevant stimulation, but small dimensions as well as a high degree of dilution of released compounds in media has long limited the investigative process. The challenges can be partially circumvented by using microfluidic systems suited to the study of released neuropeptides [78, 79]. Microfluidic designs allow the user to selectively apply chemical stimulations to neurons maintained in these devices. The resulting neuropeptide releasates can be collected off-line and detected with MALDI-time-of-flight (TOF) MS [57, 78, 80]. An additional benefit of the off-line coupling of microfluidic devices with MS for the characterization of small-volume extracellular releasates is the capability for label-free, absolute quantitation of peptides [81].

Synergy of tools and resources in peptidomics research

While MS technology is often marketed as a complete solution for comprehensive protein/peptide characterization because of its unprecedented structural characterization capabilities, speed, sensitivity, and throughput, it often works best as a part of multifaceted or integrative characterization effort. A multifaceted approach can be defined as a combination of complementary separation and MS techniques having specific advantages for peptides of a certain mass range [82], or other characteristics or modifications. The synergetic effect may be achieved by combining, in one study, different ionization techniques for generating different subsets of peptide ions from the same sample [37], multiple complementary fragmentation methods (ETD, HCD, and CID) performed on liquid chromatographic time scales, which ultimately enhance peptide structure determination or PTM localization [82–84], or different mass analyzers [82, 85]. Liquid separation methods on the front end of a peptidomics pipeline offer additional leverage for improved peptide coverage. Respectively, various separation optimization strategies, such as multidimensional LC [29, 62, 86, 87] and alternative separation methods, have been actively explored in the peptidomics area [40, 88–90].

Assignment/identification of neuropeptides in peptidomics showcases this interdisciplinary synergy. Existing identification methods (Figure 3) are facilitated by the ever-growing

transcriptomic resources and protein databases obtained by translation from the coding sequences in public nucleic acid databases. However, a robust and widely popular identification approach via database search that relies on *in silico* data is effective for assigning peptides from known prohormones that are already in the utilized database, and when the deposited prohormone sequences are free from mistakes. Moreover, only peptides resulting from conventional enzymatic processing for which theoretical MS/MS spectra can be predicted may be found via database search. In contrast, *de novo* sequence tagging is independent of databases and thus offers more discovery power, even with partially correct tags. It is currently the only means for detecting mistakes in deposited protein sequences, and revealing single amino acid substitutions, PTMs, transcript variants, and homologous sequences. Overall, there are numerous interactions between peptidomics, genomics, and transcriptomics that ultimately drive neuropeptide discovery [5, 21, 25, 26, 28, 29].

Of utmost importance to accurate and effective peptide detection and identification in a peptidomics experiment are the bioinformatics tools used for interpretation of the MS and MS/MS spectra, employing a search engine that queries data against a protein database to find statistically validated peptide-spectrum matches (Figure 3). Due to the multitude of commercially available and open source programs, often built upon different mathematical algorithms for interpretation of the MS/MS data [91, 92], multilayered or combinatorial analysis of MS/MS data sets have proved to be beneficial [93, 94]. Likewise, quantitative assessment of peptide levels may not be executed without advanced bioinformatics algorithms for signal normalization, noise reduction, correct feature extraction (*m/z*, charge state, peak intensity/area, retention time), and data conversion from vendor proprietary formats to open formats that are compatible with stand-alone statistical tools.

Quo vadimus: Future perspectives and concluding remarks

Owing to the rapid and dramatic advancements within the past two decades, highly sophisticated mass spectrometric instrumentation and auxiliary resources have become useable and affordable to many laboratories. Because of this availability, neuropeptide discovery has skyrocketed in terms of the diversity of the biological systems investigated and the number of identified peptides reported by individual studies. However, enthusiasm about the long lists of peptides sometimes prevails over scientific wisdom and hinders thoughtful interpretation of chemically exciting results relevant to their biological significance. Peptidomics methods do not differentiate between naturally occurring peptides, post-mortem degradation products, and sampling artifacts: any peptide has a chance to be detected and identified. Reporting a few hundred chemically unique peptides from a few dozen prohormones is often a simple mathematical exercise that overlooks the fact that many of the detected peptides are sequentially truncated forms of mature peptides positioned between conventional cleavage sites on the prohormone. This is especially common to mammalian neuropeptidomes because of their astonishing complexity. Should these shorter forms be accounted for as independent peptides, or be considered as redundancies? While in a few cases, such as with angiotensin, the truncated forms have distinct and important biological roles, we feel that often, many of the truncated peptides reported in peptidomics studies are low-level peptides arising from sampling artifacts. We hope that in the coming

years the awareness of such factors becomes more widespread in the bioanalytical community.

Even with the recent technical advances, the functional characterization of novel peptidomes is still a slow and often multi-laboratory collaborative process, with newly discovered putative neuropeptides needing to be individually evaluated. There is a pressing need for reliable approaches that can focus the peptide functional annotation effort on a subset of the most probable candidate compounds. Existing differential and quantitative MS methods help with the selection process, but to become broadly accessible, they have to be streamlined. Nonetheless, we expect in the coming years that breakthroughs in MS technologies will allow absolute quantitation and easier implementation of complex peptidomic studies.

Thinking about peptidomics from the functional perspective emphasizes an idea that only secreted peptides are cell-to-cell signaling molecules. Although cellular releasates have been successfully probed by microdialysis and microfluidics in combination with MS, these approaches are still being optimized to provide more universal and dependable technical solutions to sampling and sample pre-concentration. Additional methods involve brain slice chambers to sample from specific release sites [95]. We envision wider implementation of lab-on-chip platforms, which may dramatically reduce the amount of releasate sample required, minimize sample manipulation, and shorten analysis times.

In conclusion, MS-based methods provide an effective toolset for the discovery and characterization of neuropeptides in biological samples. Due to its high sensitivity, multiplexed detection, small sample size, and compatibility with a wide array of sampling approaches and complimentary measurement techniques, MS allows broad scale quantitative and qualitative investigations, from the organismal to single-cell level. Ultimately, it is the flexibility of MS that enables the elucidation of inherent chemical and functional complexities within the nervous and endocrine systems.

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Highlights

- Alternative splicing, posttranslational processing challenge neuropeptide prediction
- Mass spectrometry sequences and quantifies neuropeptides directly in tissues and cells
- Synergy of mass spectrometry, genomics and bioinformatics drives neuropeptide discovery

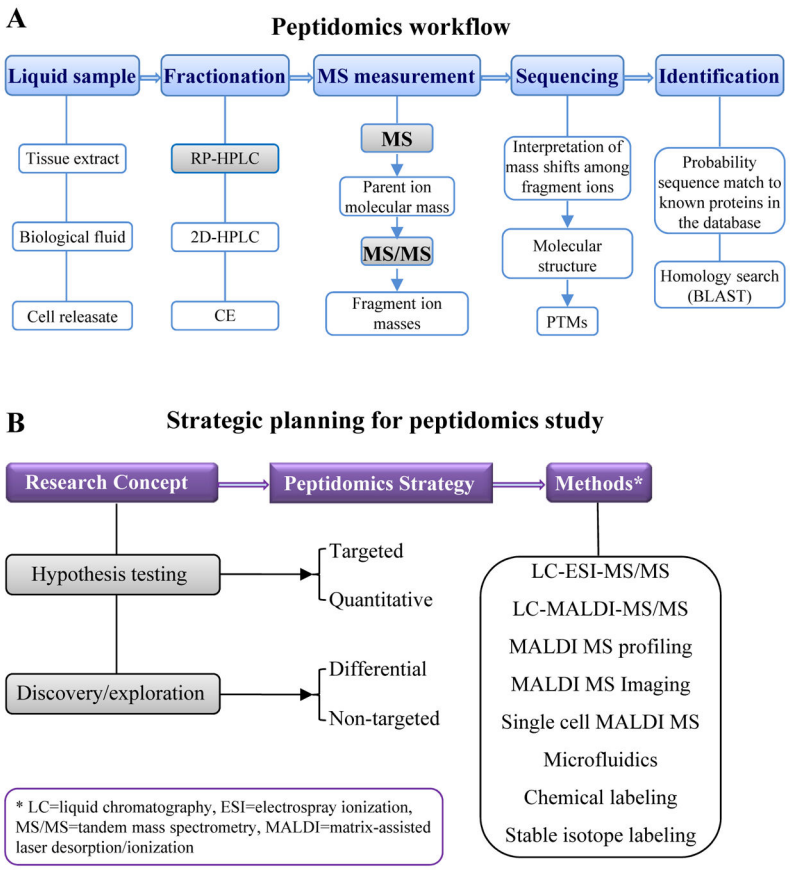

Figure 1.

A simplified flow chart showing (A) a general peptidomics workflow and (B) a strategy that helps to explain the wide variety of mass spectrometry platforms and for which experiments to use them.

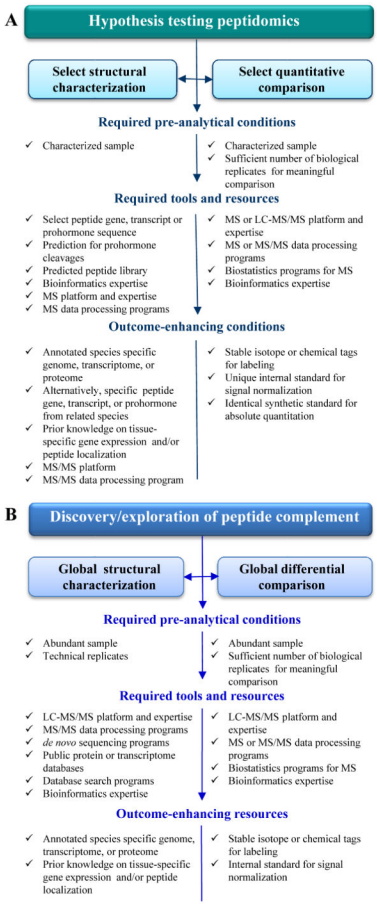

Figure 2.

Synergy of tools and resources in peptidomics research showing both (A) non-targeted approaches and (B) targeted approaches.

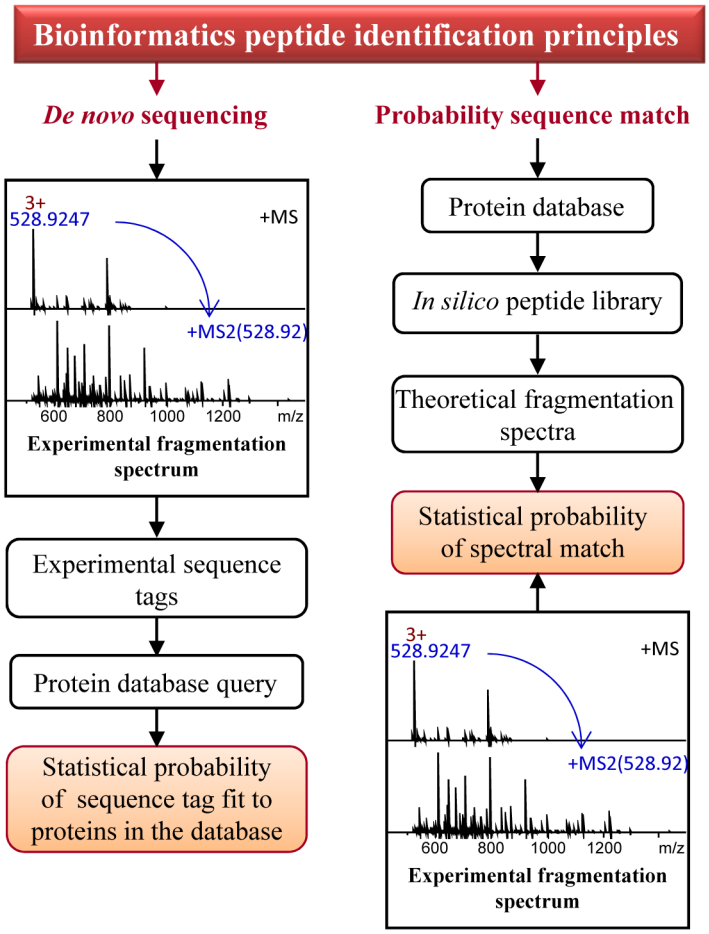

Figure 3.

Bioinformatics approaches for MS-based peptide identification. Two categories of approaches are distinct: the database search approach depends on generating theoretical spectra *in silico* from protein sequences in a database and querying experimental spectra against those to find the closest matches; the *de novo* tag approach infers peptide sequence directly from experimental MS/MS data by calculating mass shifts between series of peptide fragment ions, and then aligns the tag to protein in the database. Unassigned *de novo* tags can be used to interrogate EST depositories or databases of homologous species.