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Targeting Out of Range Biomolecules: Chemical Labeling Strategies for Qualitative and Quantitative MALDI MS-based Detection

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ABSTRACT:

MALDI-MS is of crucial importance for detection and identification of relevant molecules involved in biological pathways allowing a better understanding of physiological processes advantageously related to diagnosis strategies. However, many relevant biomarkers remain undetectable due to several parameters including low molecular weight, weak abundance, MS ionization potential or localization in a complex environment. To overcome these limitations, labeling strategies were developed to target reactive organic moieties of biomolecules of interest. More recently, activity-based probes or antibodies were implemented to match the current need for in vivo biomolecule specific labeling. Furthermore, with the development of MALDI-MS imaging, matrix-free derivatization techniques are currently in the spotlight. MALDI-MS labeling requires the development of a panel of tags designed to target different functional groups promoting signal enhancement for many biomolecules. This review recapitulates recent derivatization methods and available labeling reagents while putting into perspective the importance of MALDI-MS labels in the biochemical research context.

KEYWORDS: MALDI-MS; Chemical Derivatization; MS-Signal enhancement; Biomolecule labeling, MALDI Imaging

ABBREVIATIONS:

2-AA: 2-aminobenzoic acid
ABP: Activity-Based Probe
AnCCA: (2E)-3-(anthracen-9-yl)-2-cyanoprop-2-enoic acid
AOMK: Acyloxymethylketone
BSA: Bovine serum albumine
CCM: Carboxyl-containing molecule
CHCA: α -Cyano-4-hydroxycinnamic acid
CHCE : α -Cyano-4-hydroxycinnamic methyl ester
DHB: Dihydroxybenzoic acid
DMBAH: 4-(dimethylamino)benzohydrazide
DNA: Deoxyribonucleic acid
DNPH: 2,4-dinitrophenylhydrazine
GABA: Gamma-aminobutyric acid
HCG: Human chorionic gonadotrophin
HTMBA: 4-(hydrazinecarbonyl)-N,N,N-trimethylbenzenaminium
IHC: Immunohistochemistry
ITO: Indium tan oxide
LDAI: Ligand-directed acylimidazole
LDT: Ligand-directed tosyl
LOD: Limit Of Detection
LOQ: Limit of quantification
MALDI-MS: Matrix-Assisted-Laser-Desorption-Ionization Mass Spectrometry
MALDI-MSI: Matrix-Assisted-Laser-Desorption-Ionization Mass Spectrometry
MOF: Metal-organic framework
MS: Mass spectrometry
mTRAQ: Mass tag for absolute and relative quantification
NHS: N-Hydroxysuccinimide
NpCCA: (E)-2-cyano-3-(naphthalen-2-yl)acrylic acid
NPs: Nanoparticles
P3DDT: Poly[3-dodecylthiophene]
PAL: Photoaffinity labeling
PAPAN: 2-phenyl-3-(p-aminophenyl) acrylonitrile
PBS: Phosphate Buffered saline
Pmg: Pyrenemethylguanidinium
PNDI(T2): Poly[naphthalene-diimide-bithiophene]
PTM: Post-translational modification
QAC: Quaternary ammonium containing compound
SALDI-MS: Surface-Assisted-Laser-Desorption-Ionization Mass Spectrometry
SID: Stable Isotopic Dilution
TFA: Trifluoroacetic acid
TAMSIM: Targeted Multiplex mass spectrometry imaging
THAP: 2'-4'-6'-Trihydroxyacetophenone
TMPA: N, N, N-trimethyl-2-(piperazin-1-yl)ethan-1-amminium iodide

INTRODUCTION

Among the main analytical techniques developed for biomolecule identification, mass spectrometry is the one that offers the greatest accuracy, sensitivity, specificity and speed. Introduced by Tanaka, Karas and Hillenkamp in the 1980s, Matrix-Assisted Laser Desorption Mass Spectrometry (MALDI-MS) is widely used as one of the most advanced and sensitive mass spectrometry technology.[1] Nevertheless, preparation of MALDI-MS sample requires a combined crystallization between a biomolecule of interest and a suitable matrix compound, promoting its ionization while protecting its molecular integrity. This process defines MALDI-MS as a soft ionization technology. Depending on the studied analyte nature, detection quality - in term of resolution and sensitivity - requires a careful attention to sample preparation methods. An inhomogeneous crystallization might lead to poor quality signal or loss of detection.[2][3] The evolution of MALDI-MS technology into imaging process (MALDI-MSI), as an alternative to immunohistochemistry techniques, is also of high interest. It offers sensitivity and specificity of mass spectrometry while enabling precise spatial localization of targeted analytes directly on tissue sections. Through the collection of multiple spectra at different coordinates across a tissue section coated with a matrix, it allows multiplexing of different inherent chemical features in a single experiment.[4]

However, some biologically relevant classes of biomolecule remain highly difficult to highlight using mass spectrometry, in particular membrane proteins, hydrophobic biomolecules or low molecular weight metabolites that might be key biomarkers in pathological processes. Analyte size, localization and environment, structure, or inner chemical properties represent as many issues for MALDI-MS analysis. As examples, analysis of small molecules is challenging due to the presence of matrix ions in this low mass range while analysis of bigger ones, such as proteins, faces weak ionization mainly due to a difficult desorption into the gas phase.

As detection and identification of both small and large biomolecules remains a key challenge in the understanding of numerous biological pathways, several strategies were implemented to overcome the issue. Appeared in the 1960s, chemical derivatization has been used as a very promising technique for Mass Spectrometry (MS) signal enhancement. From the use of trifluoroacetic acid (TFA) as acylating agent of natural amino acids, many strategies have ever been proposed since. The use of co-matrices in order to promote stronger laser absorption or a better inclusion into matrix crystals have been investigated. "Matrix-free" conditions have also been implemented, in particular the use of functionalized materials used as new drop-off supports while acting as matrix compounds.[1] Then, chemical reagents were designed to modify targeted analytes and help in their detection. Such compounds should ideally meet several requirements including fast, specific and reproducible labeling reaction as well as a complete stability under MS conditions. Besides, the use of a heavy version of the tag, usually enriched in deuterium or ¹³C atoms, may then provide an accurate quantification of the labeled compounds through Stable Isotopic Dilution (SID). Such method enables a mass/charge shift that can be characterized by MS while reducing drastically ionic discrimination.[5]

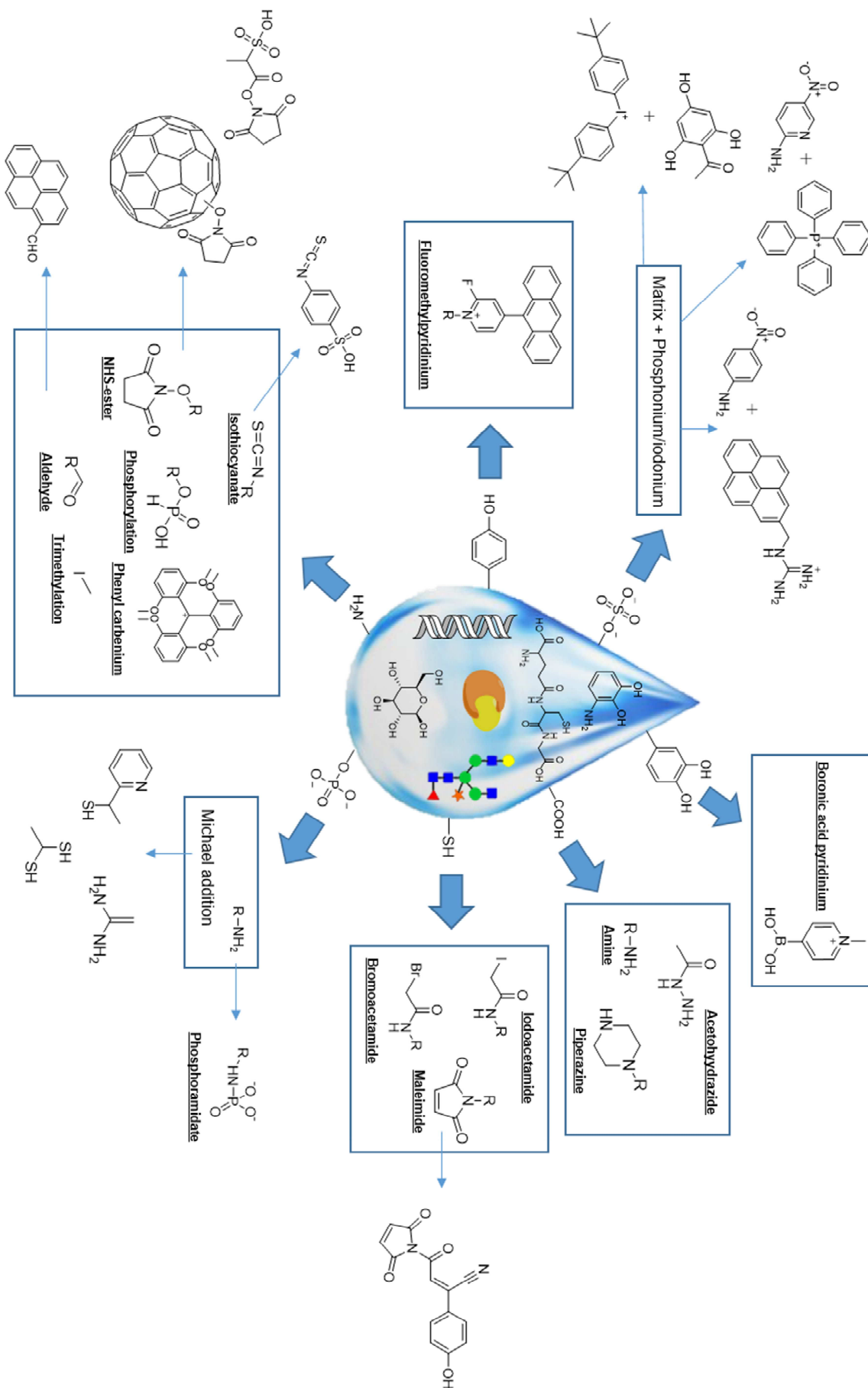


Figure 1. General chemical derivatization patterns

1. Analyte MS stabilization

One of the main issue in MALDI-MS analysis deals with the retention of molecular integrity during the ionization process in order to provide reliable mass spectrometry data. Numerous fragmentations may occur on fragile bioorganic moieties, jeopardizing MALDI-MS detection of such compounds. Several methodologies have been developed to overcome such issue.

1.1 Sialylated residues labeling

Protein glycosylation is one of the most common post-translational modification (PTM) as more than 50% of secreted and cellular proteins exhibit glycosylated moieties. Indeed, glycoproteins are of critical importance in several biological processes, such as protein folding and structural stability or protein-ligand interactions, but are also involved in cellular recognition and proliferation. Numerous pathologies induce overexpression of misfolded or aberrantly glycosylated proteins. Consequently, detection and quantification of such glycosylated residues may be a reliable way to promote early diagnosis, using these biomarkers to track disease evolution.[6] However, MALDI-MS analysis of glycoproteins is challenging for several reasons. The variable nature of glycosylated moieties associated to their MS-lability lead to a decrease of the ionization efficiency and a loss of molecular integrity during MS-analysis. Furthermore, the complexity of their structures induces multiple isomers hardly distinguishable without specific labeling. Finally, among all glycosidic bonds, sialyl ones are the most unstable, leading to the loss of sialylated moieties directly in the ionization source while inducing a strong negative charge retention through the carboxylic moiety.[7]

In the aim of promoting MALDI-MS analysis of intact sialylated species, chemical derivatization targeting the carboxyl group offered the advantage of stabilizing the sialyl bond by neutralizing negatively charged compounds (Fig.2). Moreover, as most of the labeling reagents contain aromatic moieties, it also increased biomolecule hydrophobicity, helping analyte desorption into gas phase. Several acylation strategies have been developed, such as esterification or the use of acetohydrazide moieties. However, such strategies suffered from major drawbacks such as easy reversion of ester to carboxylate form under alkaline conditions, or the presence of several by-products during the derivatization process. Most of all, they did not allow the differentiation between sialic acid isomers, involved in different biological pathways. In this vein, amidation has proven to be a key strategy to overcome these issues, as amides are completely stable under alkaline conditions.[8]

As α -2,3-linked and α -2,6-linked sialic acid species addressed different derivatization behaviours, nucleophilic amine-containing reagents have been designed to enable unambiguous differentiation of such isomers, called "linkage-specific derivatization". α -2,3-linked sialic acid compounds were used to spontaneously form intramolecular lactones under nucleophilic addition while α -2,6-linked compounds could lead to a direct and complete acylation. In a final step treatment, entitled lactone aminolysis, the lactone might be opened by using a different amine, enabling a specific labeling resulting in a characteristic mass/charge shift between sialylated isomers.[9] Experimentally, Wang et al. successfully analyzed sialylated glycans released from bovine fetuin, using methylamine isotopes. This dual isotopic labeling enabled neutral and sialylated moieties discrimination while indicating the number of sialic acids. Quantification has also been performed in order to characterize N-glycome alterations in serum from gastric cancer and healthy patients.[10]

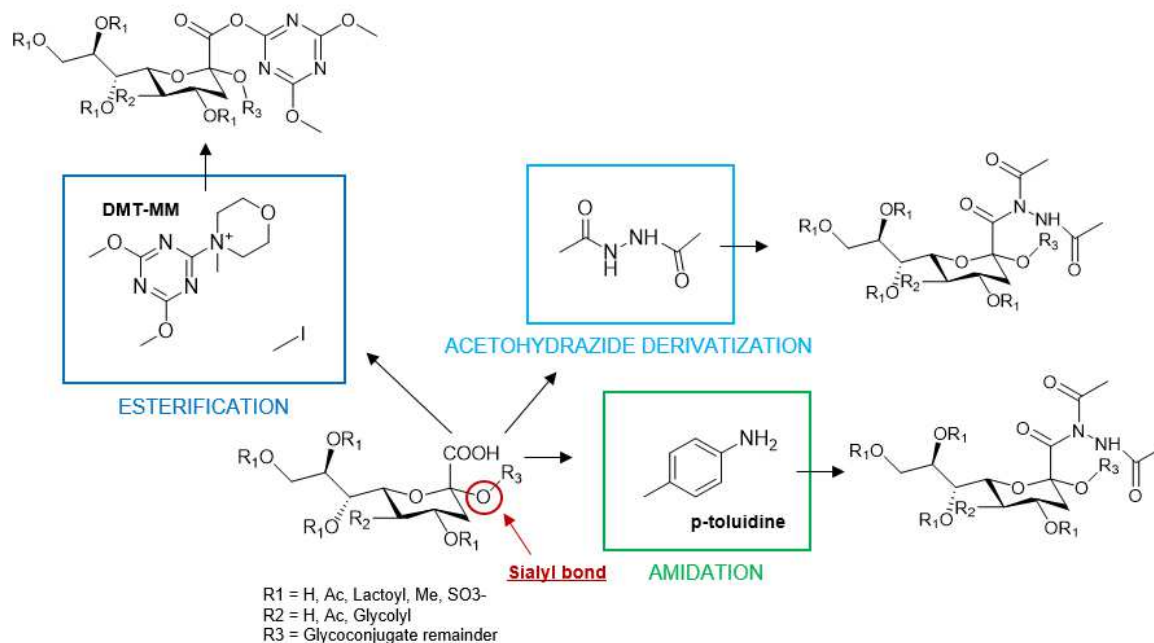


Figure 2. General procedures for sialic acid derivatization - [7]

1.2 Sulfoproteins labeling

Firstly discovered in 1950s by Bettelheim in bovine fibrinopeptide, sulfation is another common PTM, occurring mostly on tyrosine residues. Furthermore, O-sulfation is a key modulator for protein-protein interaction, embodying a biomarker of interest, involved in several pathologies and inflammatory processes. Consequently, detection of sulfoproteins and identification of sulfation sites is of importance for the elucidation of their role in such physiological pathways. Nevertheless due to extreme lability of the sulfoester function, the loss of the sulfated group during the ionization process results commonly in loss of structural information. Besides, as tyrosine sulfation may occur not only on soluble proteins but also on membrane proteins, detection of such PTM turns out even more challenging.[11]

Even if the sulfoester function may be in some case intramolecularly strengthened in presence of proximal basic residues, it remains necessary to stabilize it for MS detection of intact sulfoproteins. As non-covalent tag, quaternary ammonium derivatives, initially used as matrix enrichment, proved to be effective in negative ion mode while not being able to completely avoid desulfation. Phosphonium and iodonium salts appeared to be way more efficient regarding signal enhancement and sulfated species stabilization. Indeed, a co-matrix system composed of a combination of 2-Amino-5-nitropyridine and tetraphenylphosphonium bromide was used to efficiently detect heparin disaccharide, which contained three sulfated groups, mainly suppressing the desulfation phenomenon. Thus, such permanently positively charged compounds were likely to form strong ionic bonds with the negatively charged sulfo-moiety, stabilizing it and providing strong molecular signals. Following the same principle, Yamaguchi et al. demonstrated that 2,4,6-Trihydroxyacetophenone (THAP)/Bis(4-tert-butyl)phenyliodonium hexafluorophosphate co-matrix provided strongest protection against desulfation of cholecystoskinin analogs while limiting salt formation on carboxyl group. They were able to detect sulfated cholecystoskinin octapeptide down to 20 fmol.[12] Similarly, the latest strategy developed so far operates the stabilization with a pyrenemethylguanidinium (pmg) salt as non-covalent derivatizing reagent. Thus, such compounds provided a strong protection of sulfo-residues while enabling the detection of intact molecule in a positive ion mode as well as the obtention of specific modified fragments during MALDI-MS analysis, corresponding to a characteristic $\text{SO}_3\text{-pmg}$ loss. Furthermore, the addition of a sufficient amount of pmg equivalents lead to the determination of sulfation degree and the peptide molecular weight in a single experiment, in the study of human cholecystoskinin-33.[13]

2. Analyte ionization improvement

The success of an MS analysis relies not only on a molecular integrity preservation but also on an efficient molecular ionization. This ionization process depends on three main features: (i) biomolecule proton affinity, (ii) interaction with matrix compounds and (iii) laser absorption properties. In order to promote better ionization of hardly ionizable biomolecules, several families of tags have been designed. They display three main features : (i) the presence of a permanent charge within their structure, (ii) a high hydrophobicity, (iii) a strong absorption of laser energy.[5]

2.1 Derivatization of hardly ionizable biomolecules

Phosphorylation[14] or trimethylation are common derivatization strategies which target free amines. Widely used, trimethylation turned primary amines into tertiary amines, through the use of methyl iodide, inducing a higher proton affinity.[5] It enabled SID quantification using ¹³C labeled- or deuterated methyl iodide, providing at the same time specific patterns to discriminate analyte signals.[15] The use of specific functions, such as isothiocyanate or N-hydroxysuccinimide (NHS)-ester moieties, firstly enabled the covalent incorporation of permanently charged moieties on the targeted biomolecule, demonstrating the strong efficiency of permanent charge addition. Thus, reagents were developed to introduce a permanent positive charge. For example, tris(2,6-dimethoxy-phenyl)methyl carbenium, reacts with free amines by nucleophilic substitution of one of the methoxy-phenyl group, leading to the formation of cationic acridine derivatives. Such compounds were first tested on n-propylamine antibiotics derivatives, enabling their detection up to 25 fmol, before being challenged for aminoglycosides analysis, both pure and in complex mixture. Used against severe infections, their monitoring in biological fluids remained essential as aminoglycoside therapy goes together with high ototoxicity and nephrotoxicity. In this context, Kanamycin successfully detected four aminoglycoside derivatives in the nanomol range in a carbonate buffer.[16]

2.2 Carbohydrates issue

Carbohydrates turn to be hardly ionizable entities due to their complex structure coupled to a lack of aromatic compounds. Furthermore, the presence of numerous hydroxyl or amine functions, able to form hydrogen bonds, complicates the release of individual molecular ions during the desorption phenomenon.[16] To overcome this issue, permethylation strategy was developed. It consist in the conversion of hydroxyl groups into methyl ethers which induced an increase in sensitivity.[17] However, in the presence of carboxylic acid, methyl ester appeared to be a considerable by-product of the reaction, highlighting the need to target other reactive functions. In this way, arginine derivatives have been used for glycan reductive amination of aldehydes and ketones, enabling a 50-fold signal increase while profiling N-glycome in human serum and bovine milk.[18] As the use of amine containing reagents required the presence of high concentrations of reducing agent and further clean-up steps, oxime and hydrazide chemistry-based methods appeared as more promising derivatization techniques. Cationic hydrazide tags, such as Girard's T reagent or Girard's P reagent, gathered the advantages of an efficient reactivity under gentle conditions and the presence of a quaternary ammonium, increasing drastically MALDI-MS signals through permanent charge addition.[19] The latest example was the use of 4-(hydrazinecarbonyl)-N,N,N-trimethylbenzenaminium (HTMBA), developed by Zhang et al., who achieved a 19-fold signal enhancement while avoiding the formation of alkali adduct during labeled maltoheptaose analysis, compared to formally used hydrazide as neutral 4-(dimethylamino)benzohydrazide (DMBAH) derivative.[20] On another hand, Song et al. used hydroxylamine derivatives to perform steroid hormones derivatization by way of oxime formation, improving proton affinity thanks to an easily ionized tag. They successfully quantified steroids in human plasma until the nanomolar range.[21] (Fig.3).

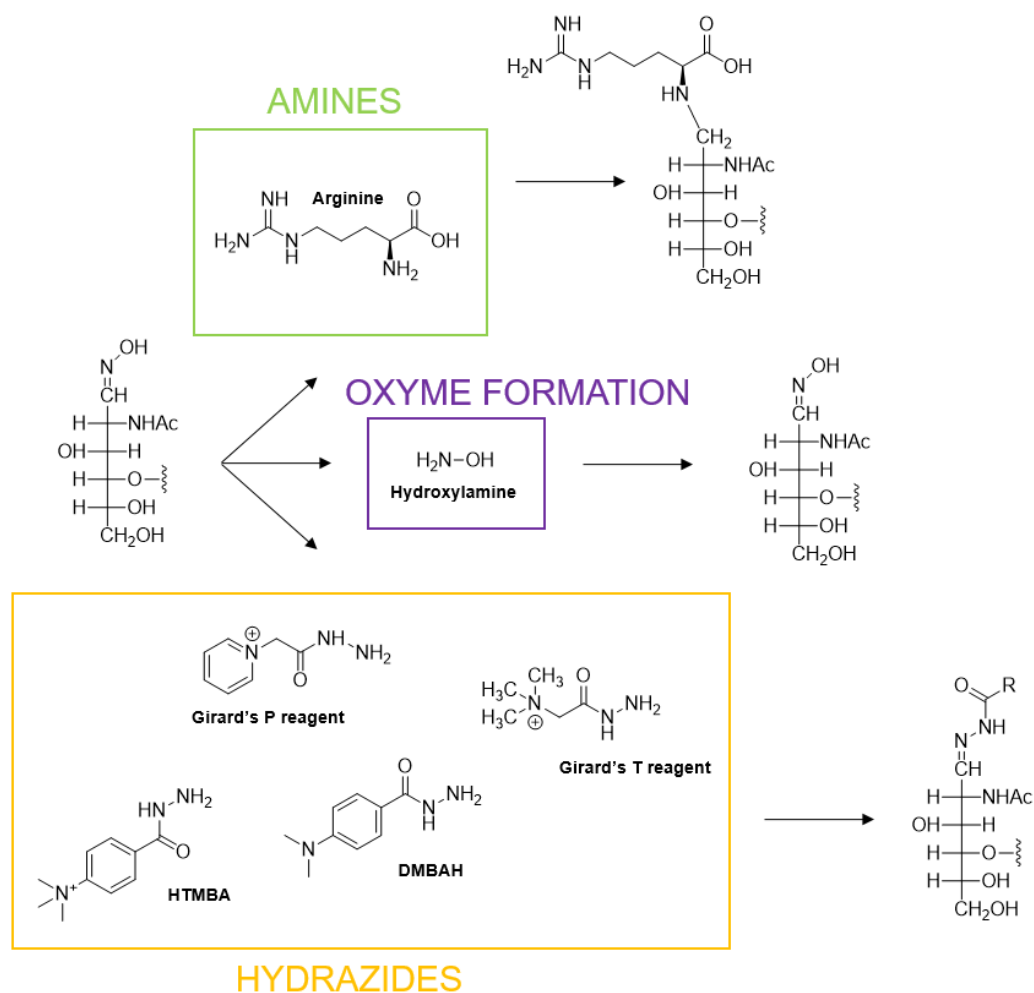


Figure 3. General procedure for glycan reductive amination - [18]

2.3 The case of phosphorylated species

Phosphorylation is a reversible post-translational modification of uttermost importance involved in numerous biological processes such as cellular homeostasis, signaling, translational and transcriptional regulation. Different types of protein phosphorylation are described in eukaryotic cells: serine, threonine and tyrosine O-phosphorylation, histidine, lysine, cysteine, aspartic acid and glutamic acid N-and S-phosphorylation. Nevertheless, phosphorylated proteins are difficult to detect in MS mainly due their weak abundance in biological media compared to their unphosphorylated form, a low ionization efficiency and rapid hydrolysis of the phosphoryl group in acidic conditions.[22]

Though numerous enrichment techniques have been developed,[39] the extraction of phosphorylated species did not improve neither MALDI-MS signal enhancement nor stabilization of the phosphoryl group. To overcome this issue, the main derivatization strategy proceeded in two steps. First phosphoserine or phosphothreonine were converted into dehydroalanine or dehydrobutyric acid under strong basic conditions, undergoing a β -elimination. Then, modified compounds reacted as Michael addition acceptors in a Michael addition using S- or N- nucleophiles such as ethanedithiol, ethylenediamine or mercaptoethylpyridine, linked to an MS tag such as trityl moieties, enabling ionization efficiency improvement and signal enhancement. However, this strategy presented major limitations including (i) the non-specificity of the β -elimination, as other biological moieties such as thiol-cysteine side chain or O-glycosylated protein are able to undergo such reaction, leading to non-

specific labeling and signal interferences, and (ii) the inability to perform β -elimination on phosphorylated tyrosine, which represented the majority of phosphorylation events.[22]

More recently, another methodology was implemented, involving the generation of phosphoramidate derivatives targeting primary amine containing molecules. Such compounds could bear affinity tag such as polyaromatic, guanidine or quaternary ammonium moieties, to promote simultaneous labeling of specific phosphorylated species, stabilization and MS signal enhancement. However, despite the versatile aspect of this strategy, this non-selective chemistry absolutely required the protection of any carboxyl group to avoid their acylation.[23]

Most of derivatization strategies rely on reagents which reacted with amino group, thus restricting their application range. Moreover, these chemical reagents are also sensitive towards other nucleophiles such as water or methanol, which could deactivate the tag.[5] This drove the emergence of other families of labeling agents with variable reactive functions targeting a broader set of biomolecules. NHS-ester or isothiocyanate derivatives remained the most widely used chemical patterns to target amines. For example, NHS-ester functionalized versions of different mass tags for relative and absolute quantification were used by Ito et al. to compare gamma-aminobutyric acid (GABA) level in different rat brain slices through absolute quantification (mTRAQ).[24] Lately, other derivatives such as cinnamaldehyde moieties emerged. 4-hydroxy-3-methoxycinnamaldehyde has been challenged for on-tissue chemical derivatization of amino-acids, dopamine or dipeptides from rat brain tissue sections, promoting a 20 to 235-fold signal increase.[25] Piperazine or hydrazine derivatives were the ones designed preferentially for carboxyl derivatization. N, N, N-trimethyl-2-(piperazin-1-yl)ethan-1-aminium iodide (TMPA) has been used by Sun et al. for carboxyl-containing metabolites (CCMs) imaging, such as tricarboxylic acid cycle intermediates, central components for cell energy production often implicated in tumoral development. TMPA induced signal enhancement through the presence of a quaternary ammonium and enabled the detection of 28 CCMs in rat brain tissue sections.[26] Last, maleimide, iodoacetamide or bromoacetamide moieties have been implemented to specifically label thiol-containing molecules, usually low polar, low basic and hardly ionizable.[27] (Fig.1) Zhou et al. and Harkin et al described the application of such chemical patterns for on-tissue derivatization in order to challenge MALDI-MS Imaging issues.[28],[29]

Besides the work performed to diversify the reacting moieties of labelling reagents, MS labels themselves were also upgraded and new tags were investigated. Usually sharing structural features with matrix compounds, such molecules incorporated aromatic or conjugated moieties as well-known coumarins or cyanines dyes. Kameyama's team successfully detected pure N-glycans from chicken ovalbumine, using hydrazide derivatives of cyanine dyes, until 200 amol, providing a 500-fold signal increase.[30] Lately, Watanabe's team used pyrene NHS-esters to study avidin-biotin interaction in PBS and identify biotin binding site on the carboxylase surface.[31]

3. Labeling enabling low molecular weight analytes detection

Targeting low molecular weight compounds is another major challenge in MALDI-MS analysis. Indeed, below a 1000 Da mass range, generated matrix ions disrupt MALDI-MS signal. Therefore, numerous small organic molecules such as amino-acids or biological metabolites remain undetectable. To address this issue, different labeling methods were developed to significantly increase analyte molecular weight, shifting them to higher mass range detection region. [32] However, starting from the statement that removing matrix compounds may be the ultimate solution to solve the problem, novel approaches have been investigated using inorganic materials, nanoparticles, or even specific organic molecules that improve sample ionization without leading to noisy signals. It was the birth of Matrix-Free Laser Desorption Ionization technology.

3.1 Increase of analyte molecular weight

One of the best way to drag the analyte out of the low m/z region is to increase its molecular weight. To this purpose, heavy chemical mass tags derived from fullerenes scaffold have been developed. These “**supermolecules**”, containing between 60 to 80 carbons, were able to add about 900 Da to the targeted compound, enabling its detection out of the m/z region saturated with matrix ions. Besides, fullerenes exhibited strong absorption properties, enhancing MALDI-MS signals of the attached analyte, with no additional matrix in some more favorable cases.[33] Limits of detection (LODs) from 300 to 70 fmol were achieved for the detection of amino-acids in urine, serum, plasma, whole blood and cerebrospinal fluid. Finally, analyte quantitation was easily accessible using carboxylic acid functionalized fullerenes, as internal standard. As it presents a similar MALDI-MS behaviour, namely similar analyte crystallization and desorption patterns, it minimized ionic discrimination.[34] Experimentally, such system enabled the quantification of polyamines in cells and mice feces samples until 0.68 to 1.48 pmol, using 1,7-heptanediamine or N, N'-bis(2-aminoethyl)-1,3-propanediamine as internal standard. [33] Another supermolecule family, namely cucurbituril, has been used as derivatization reagents for the detection of quaternary ammonium-containing low molecular weight compounds (QACs). Such compounds, composed of several glycouril units assembled in a ring-like arrangement through methylene bondings, were likely to form non-covalent complexes with quaternary ammonium-containing compounds, promoting their insertion into the supermolecule structure. QACs, as neurine, choline or phosphocholine, appeared to be key metabolites for lipid metabolism or neurotransmission, standing out the need for the evaluation of their distribution in significant biological tissues. Cucurbituril were also used by Chen et al. to get analytes out of the matrix-related signal area, successfully performing on-tissue chemical derivatization and MALDI-MS imaging of neurine, choline and phosphocholine in rat brain sections, enabling detection until the picomolar range.[35]

Following a comparable approach, peptides were used as analyte modifier. Such called “**peptidylation**”, it increases analyte molecular weight while improving its ionisation properties. Indeed, the peptide used as tag includes arginine or phenylalanine residues within its sequence, improving respectively proton affinity and laser absorption properties, providing the desired enhanced MALDI-MS response. This strategy was applied to a wide variety of low molecular weight biomolecules, such as cysteine, glutathione, increasing their LOD from 1 nmol to 10 fmol in human urine samples. The use of an heavy version of the labeling peptide enabled the SID quantification of cysteine concentration in urine samples from lung cancer patients, establishing an average concentration of 12 nmol per mol of creatinine.[36]

Finally, the use of high molecular weight matrices have been investigated for non-covalent derivatization of low molecular weight compounds. Mostly developed with the use of **porphyrins and phthalocyanines**, such compounds enabled the addition of at least 300 to 500Da to the targeted analyte, shifting it from the interfering region. Furthermore, the presence of endocyclic nitrogen atoms into the structure enabled easy substitution, usually with aromatic groups, improving molecule absorption properties. Lately, high molecular weight heteroaromatic oligomers have been under investigation as they displayed both, a greater vacuum stability and interference-free analysis. Besides, their polymeric backbone are easy-to-tune structure. However, in spite of the promising results obtained with thiophene or vinylene derivatives, their use seemed to be restricted to the

detection of amine-containing molecule.[32] Experimentally, Horatz et al. explored the potential of five semi-conducting thiophene derivatives, challenging them for the detection, both in pure and in complex biological samples, of different low molecular weight analyte. Poly[naphthalene-diimide-bithiophene] (PNDI(T2)) enabled detection of pure reserpine until 16 pmol while Poly[3-dodecylthiophene] (P3DDT) has been used for rat brain section imaging.[37]

3.2 Matrix-free laser desorption ionization techniques

Removing matrix assistance may be a drastic measure that may solve the problem of background interferences in the low m/z region while being detrimental for the detection of poorly ionizable molecules. To address this issue, different materials have been designed as new drop-off supports, assisting analyte ionization and substituting matrices and paving the way to Surface-Assisted Laser Desorption Ionisation mass spectrometry (SALDI-MS) technology.[38] These surfaces were usually composed of at least one inorganic layer, depending on the material properties required, with an optional final hydrophobic organic layer to improve analyte desorption into the gas phase, mainly through rapid localized surface heating.[1] The first generation of surfaces were structured of porous silicon, able to transfer UV-laser energy. More recently, silicon, carbon and metal-based materials became available, such as silicon micro or nanoparticles, graphene or structured carbon nanotubes and nanoparticles, metal oxide nanoparticles or even gold and silver nanoparticles. Experimentally, graphene oxide was used as one of the most cost-effective functionalizable surface. Through numerous hydroxyl groups, coupling with metal nanoparticles or semi-conductor materials promote strong affinity to various molecules such as thiols, phosphorylated compounds or aromatic molecules. As an example, it enabled detection of both benzo[a]dibenzo-p-dioxin, an environmental pollutant, until 15 fg and flavonoids until 1 pmol.[39] Furthermore, described as the most stable carbon-based material, graphdiyne presented a layer structure composed of multiple benzene rings connected by carbon-carbon triple bonds. Graphdiyne enabled the discovery of several biomarkers for liver cancer diagnosis while analyzing human serum samples.[40] However, some of these inorganic structures have to be coated to reach optimal desorption efficiency. This mainly consisted in converting the surface into a plasmonic material leading to better mass spectrometry performances. One example was the use of a mixture of AgNO_3 /pyrogallol, a trihydroxybenzenic compound. This coating, performed on hexagonal boron nitride sheets, demonstrated a 10-fold signal enhancement while studying ischemic brain damages by ex-vivo detection of small metabolites, including sulfatides. Applied to polystyrene surfaces, such modification enabled the detection of N-acetyl aspartic acid until the subnanomolar range which was impossible to achieve with the native polymer.[41].

All these structures induced a MALDI-MS response with an improved resolution and high sensitivity. Furthermore, in order to enhance sensitivity and specificity of the material, specific ligands or functions could be grafted on the surface to promote restrictive ionization. [38] Specific surface functionalization with nanoparticles has been described as the combination of two materials suitable for SALDI-MS while protecting the surface from degradation. The latest examples were the grafting of gold nanoparticles both on silicon surfaces and on porous TiO_2 film. Such strategy has been used for animal tissues (kidney and brain sections) and human fingerprint imaging. Firstly approved while detecting and quantifying glucose from 1 to 1000 ppm, the functionalization with gold nanoparticles provided a 45-fold sensitivity increase compared to classic silicon surfaces. Furthermore, the surface modification with hydrophilic and hydrophobic groups enables specific interaction between surface and sample, promoting selective ionization and precise localization of metabolites from sweat and sebaceous glands adhered to fingerprints.[42] On another hand, the addition of gold nanoparticles on TiO_2 surfaces has been used by Wang et al. for imaging of small molecules, such as amino-acids, alkaloids or dopamine, in both positive and negative ionization mode, promoting sensitivity increased until at least the picomolar range. The lowest values were reached for oxymatrine with a LOD of 0.2 amol and a limit of quantification (LOQ) of 0.4 amol. Besides, glucose level quantification has been achieved in rats from 0.5

to 9 mM.[43] Graphene surfaces were then particularly used for the analysis of non-polar compounds and TiO₂ nanoparticles for water insoluble metabolites while gold and silver nanoparticles bound specifically sulfur-containing compounds through easy formation of metal-S bonds. [38] The later support was easy to prepare and offered both high vacuum stability and good laser absorption, thanks to strong plasmonic properties, which granted a leading role in the development of matrix-free strategies. Kratochvil et al. used silver nanoparticles coating on indium tin oxide coated (ITO) glass slides for crude riboflavin detection, reaching a LOD of 3.10⁻⁸ g/L. Furthermore, as silver possesses two natural isotopes, detection of silver ions were facilitated, even in complex mixtures, due to their specific isotopic pattern.[44]

Recent complex metallic hybrid structures, entitled metallic organic framework (MOF), were designed to provide a better increase of small biomolecule LOD. Composed of metal ions clusters linked by organic ligands, these emerging materials were highly porous, leading to very versatile functional structures, while presenting strong laser absorption properties, promoting an easy proton transfer to the analyte.[45] Emerging as a new approach, MOF design appeared more valuable in the use of ligands sharing structural similarities with common matrices, as for 2,5-pyridinecarboxylic acid and 2,5-dihydroxyterephthalic acid with 2-picolinic acid and dihydroxybenzoic acid. Chen et al. developed zirconium-based structures for MALDI-MS detection of saccharides, amino-acids and nucleosides, obtaining very clean spectra with low background noise. A LOD of 1 pmol was reached while analyzing glutathione, maltose and raffinose while carbamazepine and atropine were respectively detected at 5 and 3 pmol.[46]

4. Use of reactive matrices

First, diverse aromatic compounds were explored to replace or assist usual matrix molecules without covalent analyte labeling. In the list, flavone, coumarindione, benzimidazole and porphyrin derivatives have been widely investigated for their ability to easily form matrix/analyte adducts, without producing any disrupting background signals.[47] Different strategies were particularly considered, on one hand, the use of porphyrin moieties to target alkylphenol ethoxylates, and on the other hand, the association of cinnamic derivatives and reagents presenting an aromatic scaffold such as anthracene or naphthalene. Porta and al. synthesized two α -Cyano-4-hydroxycinnamic acid (CHCA)-based derivatives, (E)-2-cyano-3-(naphthalen-2-yl)acrylic acid (NpCCA) and (2E)-3-(anthracen-9-yl)-2-cyanoprop-2-enoic acid (AnCCA), which yielded a 30% improvement of the signal to noise ratio while detecting pharmaceuticals.[48]

Meso-tetrakis(pentafluorophenyl)porphyrin was another compound whose structure, presenting several aromatic moieties, enables strong laser absorption and proton affinity. It was then successfully employed for ascorbic acid or citric acid detection, reaching 0.3 wt% as LOD. Finally, several naphthalene derivatives have been designed as "proton sponges" to improve MALDI-MSI experiments in negative ion mode, through analyte deprotonation. Such systems were highly alkaline base and were capable of easily deprotonating analyte. As an example, the 3-(4,5-bis(dimethylamino)naphthalen-1-yl)furan-2,5-dione was used to provide the ionic map of lactate and glutarate moieties in brain aggressive-tumor tissue.[48]

Lately, new compounds have been developed to enable a covalent labeling of the targeted analyte, providing a better selectivity regarding ionization and signal enhancement. Analogs of usual matrix compounds could react with the analyte after deposition on MALDI-MS target to yield a preferential ionization of the resulting conjugates. In this case, the same compound behaved both as a reagent to label analyte, and as a MALDI matrix, assisting analyte ionization.[32] As an example, Hronowski et al. used the 2-aminobenzoic acid (2-AA), for on-target derivatization of glycan reducing end, through glycan non reductive amination. Such strategy enhanced MALDI-MS signal in negative ionization mode due to the presence of an equilibrium, induced by the amination, between close-ring glycosylamine and open-ring structure which exhibited a better negative ion sensitivity. Compared to dihydroxybenzoic acid (DHB), 2-AA promoted a 300-fold increase regarding ions sensitivity, reaching an LOD of 40

ng instead of 125 ng and less sodium adducts.[49] In this vein, several amine-containing molecules have been primarily tested to be used as reactive matrices thanks to their high absorption/desorption efficiency while being easy-to use derivatizing reagents through the high nucleophilicity of amines. Challenged for carbonyl compounds modification through imination, tryptamine appeared to be the best compound, providing strong signals and intense peaks.[50] This type of in-situ labeling method was particularly useful for MALDI-MS imaging as no pretreatment of the sample was required, enabling a straightforward work on biological tissues. Indeed, Shariatgorji et al. used brominated triphenyl-pyrylium salts for on-tissue derivatization of endogenous catecholamine and serotonin neurotransmitters. While imaging rat brain sections, it promoted both signal improvement and a characteristic isotopic pattern, due to the presence of the bromine atom, enabling a clear differentiation with underivatized compounds.[51] This method also particularly helped the identification and quantification of peptides or metabolites into complex samples. As example, 2,4-dinitrophenylhydrazine (DNPH) is used for MALDI-MS detection of aldehydes converting them into corresponding hydrazones, enabling the detection of lipid oxidization products. DNPH favored the detection of octanal down to 130 pmol.[48] Furthermore, Hauser et al. developed pyrene-based tags for in-situ derivatization and detection of lactose in cow's milk, using three well-known chemical handles to create covalent bond, hydroxylamine, hydrazide or benzamide functions. First optimized on crude lactose, those compounds enabled a LOD from 1 to 10 pmol.[52]

Currently, despite the promising results obtained with such compounds, these reactive matrices often suffered from complicated synthesis pathways. Thus, in order to overcome this issue, new approaches, easier to handle, focused on the design of cinnamic and pyridinium derivatives, whose precursors were commercially available as common matrices.

4.1 Use of pyridinium compounds

Neurotransmitters are small signalling molecules involved in several neurological and psychiatric diseases. As key biomarkers, several imaging technologies, such as immunohistochemistry or positron emission tomography, have been used to assess their abundance and spatial distribution. However, most of these strategies induce the impossibility to discriminate different metabolite subtypes, such as catecholamines, a low spatial resolution or an important tissue waste. To work directly on tissue without any pretreatment, fluoromethylpyridinium matrices have been designed to react with phenol or primary amine containing molecules, through fluorine substitution. In case of multifunctionalized compounds, such as catecholamines, several fluoropyridinium moieties could be attached to a single molecule. They were the perfect example of molecule combining aromatics absorption properties, promoting matrix-like behaviour, and a quaternary ammonium, playing the role of signal enhancer tag thanks to its permanent charge. This strategy has been used for MALDI-MS imaging of neurotransmitters, including catecholamines and serotonin derivatives as well as their metabolites, directly onto brain slices.[53] Another pyridinium derivative, the 4-(N-methyl)pyridinium boronic acid, was developed for on tissue direct detection of endogenous catecholamines, through five-membered boronate ester formation. Working as reactive matrix for polyhydroxy-metabolites, boronate derivatives enabled detection, while promoting a unique isotopic pattern, thanks to the presence of boron atom. Noteworthy, this enabled a specific detection of epinephrine derivatives and dopamine in porcine adrenal gland sections.[54]

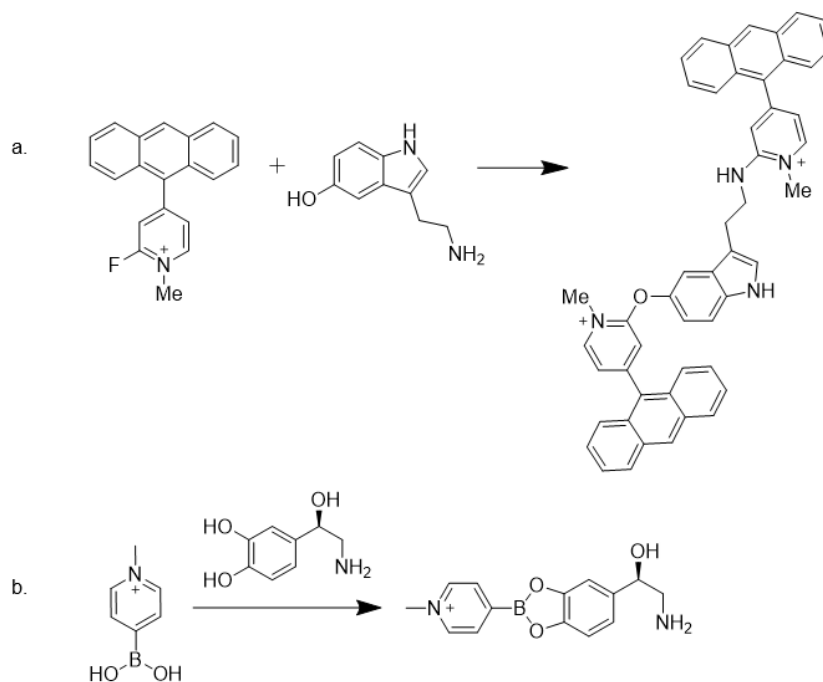


Figure 4. a. General serotonin derivatization procedure using fluoromethylpyridinium compound b. General catecholamines derivatization procedure using boronate derivative of pyridinium compound

4.2 Use of CHCA tags

α -Cyano-4-hydroxycinnamic acid, a common matrix in MALDI-MS, have also been investigated as a derivatizing reagent to improve detection of specific analytes. As example, Fülöp's team successfully labeled free thiols displayed by glutathione or reduced insulin, with a maleimide-functionalized CHCA moiety. It allowed biomolecular imaging on porcine and mouse tissues, without the need of additional assisting matrix.[27] Even better detection could be obtained by combining CHCA covalent labeling with the use of α -Cyano-4-hydroxycinnamic ester (CHCE) as matrix. Indeed, CHCE does not play the role of a "good" matrix since it usually induces poor MALDI-MS response. Somehow, discriminating analytical conditions could be created by the enhancement of labeled analyte signals while wiping out the signals of untagged molecules. Besides, the simultaneous use of a deuterated counterpart of the CHCA tag, as an internal standard, enables a highly sensitive detection (down to the amol) and SID quantification of the targeted analyte, without any further enrichment step. Application to the development of new pharmacological assays, V1A receptor/ HO-LVA ligand model, high affinity measurement could be achieved by running competitive binding assays in the sub-nanomolar range.[55]

Finally, another cyanocinnamic derivative, namely 2-phenyl-3-(p-aminophenyl) acrylonitrile (PAPAN), has been developed for sensitive and selective detection of glycans, through terminal aldehyde modification. Additionally to the strong absorption properties provided by the cyanocinnamic core, it is worth noticing that PAPAN significantly suppressed peptide ionization, enabling selective detection of N-glycans released from tryptic digestion of glycoproteins without any purification steps. Compared to formally used DHB, PAPAN induced a 100-fold signal enhancement, enabling to reach 5 fmol as LOD while detecting pure maltohexaose. Then, while analyzing ovalbumin tryptic digest, 19 N-glycans were successfully detected with no peptide signal whereas no glycans were detected using DHB. Finally, the same pattern could be observed while analyzing human serum samples where PAPAN induced the detection of 37 N-glycans without interferences where 9 were detected using DHB.[56]

5. Use of antibodies

Extensively used as biochemical analysis reagents, antibodies are especially involved in immunohistochemistry (IHC) assays. Thus, displaying unmatched selectivity for a specific target, antibodies are naturally attractive to be coupled to mass spectrometry, providing an easy-to-handle method with the sensitivity and multiplexing capacities of MS. Then, entire proteomes could be investigated in a single experiment, greatly improving diagnostic for various human diseases. Besides, with the development of MALDI-MSI, various biomolecules such as protein complexes, small metabolites, oligonucleotides or sugars have been detected and localized whereas it was formally impossible using classical immunochemical methods relying on fluorophores which may not allow the detection of high number of analytes in a single experiment.[57]

Antibody-coupled mass spectrometry experiment necessitates antibodies conjugated to laser-cleavable mass tags, mostly through a lysine side chain. After formation of an immune complex between the conjugated antibody and its target, laser induced the rupture of the photo-cleavable linker, usually thioether or ortho-nitrobenzyl linkages, to release a MS-tag, most of the time a trityl analog generating stable and easy detected trityl carbocations (Fig 5).[58] Biotin-conjugated antibodies against prostate specific antigens were used by Lorey et al. to detect immunocaptured proteins from human plasma using triphenylmethyl-(trityl) tagged avidin. After the cleavage of the thioester bond, the trityl cation was successfully detected down to 6 pM, namely 60 amol of antigen captured on the spot. [59] The synthesis of a range of tags analogues with different molecular weights, opened the way to multiplexing analysis and simultaneous detection of different antigens. This technology, entitled Targeting Multiplex Mass Spectrometry Imaging or TAMSIM, has successfully demonstrated its ability to detect untraceable samples, usually hardly ionizable, such as therapeutic agents, drugs or even high molecular weight biomolecules, mainly through the detection of reporter ions. It was particularly interesting for biomolecules exceeding 50kDa, formally undetectable using MALDI-MS, compelling to proteolytic digestion prior to analysis. As example, cellular localization of several biomarkers such as synaptophysin, calcitonin, insulin or somatostatin in neuroendocrine cells of Langerhans islets could be obtained, from human pancreatic tissue sections, without the need for an external matrix.[60]

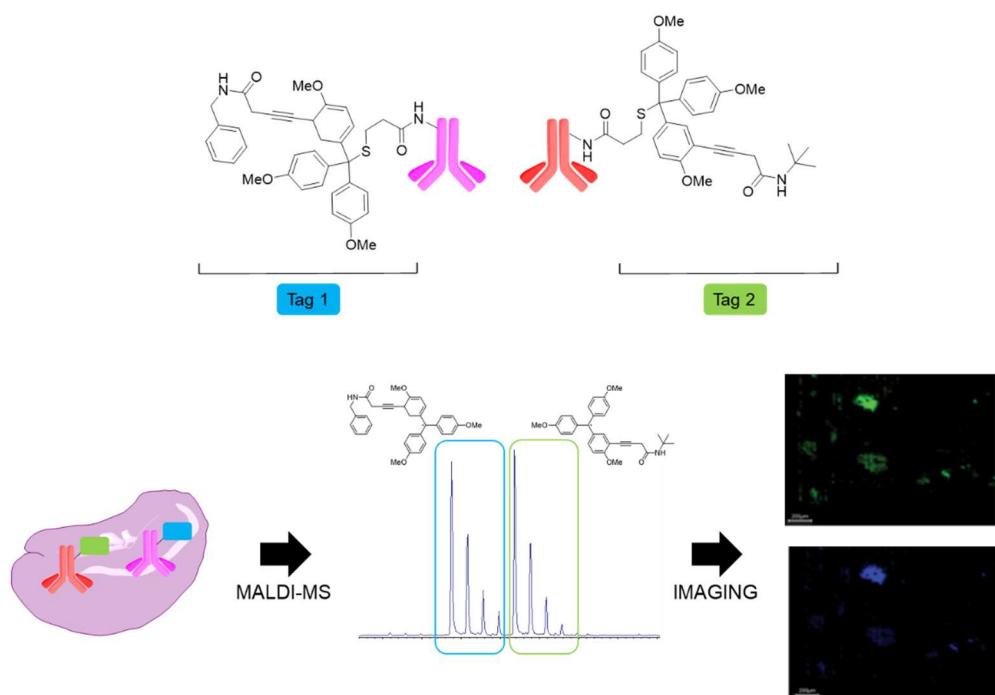


Figure 5. General procedure of TAMSIM experiment - [60]

The newest advance regarding the use of antibody probes was their conjugation with different nanoparticles, providing an inherent signal enhancement while enabling unambiguous detection. Initially, gold and silver nanoparticles (NPs) have been investigated to play the role of ions reservoir for signal amplification. Indeed, thanks to their strong plasmonic properties, enabling high energy absorption properties, these NPs could generate abundant characteristic ions (Au^+ , Ag^+) under laser irradiation, providing excellent MS signals.[61] Such method was used to detect HCG in urine sample down to 5 amol, demonstrating a 7.10^4 times signal amplification with gold NPs. Imaging experiments have also been performed, successfully detecting megalin and podocin in rat kidney tissue sections. These results also highlighted the importance of the NPs size to increase signal sensitivity while increasing analyte LOD.[62] Working on complex biological samples, gold NPs surfaces could be modified using different chemicals to reduce non-specific binding while promoting solubility of NPs in biological buffers. Tam et al. used carboxylated functionalized polyethylene glycol (PEG) capped gold NPs in order to analyze cervical cancer biomarker, the protein E7, which plays a critical role in the formation of malignant tumor. Human Papilloma virus (HPV) induced cervical cancer is one of the most lethal cancer as HPV infection since diagnosis remains difficult. Indeed, formally used HPV Deoxyribo nucleic acid (DNA) tests only provided 70% of positive rate for a single test while being unable to differentiate high risk and low risk subtypes. Here, the use of gold NPs as mass tags provided high sensitivity and minimal non-specific binding. They reached a LOD inferior to 35 amol while detecting recombinant HPV protein spiked in buffer, inducing a 100-fold increase, and a LOD of 2 zmol/cell in HeLa cell lysate.[63]

Wang et al. used gold NP probes for exosome imaging. These extracellular vesicles are promising indicators for early cancer diagnosis as they bear several protein biomarkers which play significant role in tumoral development, such as intercellular communication or alteration of cell function and state. As exosomes are large biomolecules, their detection using MALDI-MS usually suffered from limited detection and low sensitivity. To overcome such issue, gold NPs were used as ion reservoir while using organic oligomers as signal amplifiers. Such particles were used to quantitatively image CD9 and CD44 at exosome surface. While being tested on exosomes from different breast cancer subtypes, CD9 and CD44 were successfully quantified while imaging 50 exosome particles.[64]

Antibodies conjugates to silica NPs (SiNPs) have also been investigated for MALDI-MS signal enhancement. Wang et al. used SiNPs for insulin detection which was captured by antibody-conjugated SiNPs, promoting simultaneously enrichment and ionization improvement. Such method enabled insulin quantitation until 0.1 nM[65], much better than classical immunoassays.

6. ABP technology

As a complement to antibodies approach, Activity-Based-Probes (ABP) have been designed to enable a covalent and selective labeling of active biomolecules, in vivo, bypassing the need for further treatment or enrichment. Even if different structures for such probes exist, ABPs share a common skeleton composed of (i) a reactive warhead which acts as target specific ligand (i.e. enzyme substrate analog, protein ligand or inhibitor), (ii) a reporter tag used to improve MALDI-MS detection, (iii) a spacer linking those two parts. The main difference with antibodies is the use of a specific warhead that targets the active site of the biomolecule of interest. Indeed, such probes were designed to interact selectively with biomolecule active forms, enabling specific activity detection and/or quantification. Besides, among the 3000 genes encoded proteins in the human genome, only 500 are currently considered as usable drug targets. The 2500 remaining are usually considered as useless targets, mainly due to a lack of characterization regarding their biological role. ABP strategy may provide a unique insight to decipher protein function and interaction, allowing biomarker discovery, substrate identification and thus, the emergence of new therapies.[66]

6.1 Covalent warhead

As targeting enzymes is of first interest, designed ABP might covalently react with the target through one of the nucleophilic residues (e.g. Ser, Cys, Lys,...) within the active site. Among the high variety of endogenous enzymes, ABP targeting proteases were the most likely to be considered, especially serine hydrolases as they represent one third of the proteolytic enzymatic genome. Besides, they play crucial biological roles, aiming blood coagulation, wound healing, cell signaling or immune response and apoptosis. As a reactive warhead could be designed to mimic enzyme natural substrate or inhibitor, fluorosphosphate probes were generally used to target such enzymes. Such compounds were able to interact with a wide range of serine hydrolases, through nucleophilic substitution of the fluorine atom by the serine-derived hydroxyl function, while remaining inactive facing cysteine- or metallo-proteases.[67] This approach enabled the discovery and study of several serine hydrolases, such as S-formylglutathione hydrolase, leading to decipher their involvement in different pathological pathways linked to lung cancer, melanoma or breast cancer. Thanks to their ability to differentiate active and inactive enzyme, the use of ABPs also enabled enzyme activity characterization as numerous pathological behaviors were linked to enzymatic disorders. In this way, it was demonstrated that, among different cancer cell lines, it was possible to distinguish different states of invasiveness thanks to the identification of specific serine hydrolases as well as promoting a better understanding of their enzymatic activity mechanisms.[68] Such probes, linked *in vivo* using so known click-chemistry strategy, lead to generate 8-poly(amidoamine)-dendrimer containing multiple trityl moieties designed for MALDI-MS signal enhancement (Fig. 6a). Used in MALDI-MS imaging, J. Yang et al. achieved an almost three orders of magnitude signal improvement while detecting serine hydrolases directly onto rat brain slices, without external addition of matrix.[69]

Cysteine proteases are also enzymes of high interest for pathological processes, as they are involved in several cardiovascular, tumoral or inflammatory disorders. More precisely, cathepsins B have been investigated as key diagnostic and prognostic biomarkers for aggressive pathologies development including cancers and arthritis-related diseases.[68] Even if various electrophilic inhibitors have been designed for cysteine proteases, including fluoromethylketones or vinyl sulfones, acyloxymethylketone probes (Fig. 6b) proved to display the highest selectivity and reactivity towards those enzymes. Such ABP were firstly synthesized for active cathepsin B detection in mouse pancreas and spinal cord. More particularly, as well as being a biomarker of the pathology progression, cathepsin B might also be a predictive marker to chemotherapy response. Lately, Chowdhury et al. developed new prodrug-inspired peptidic structures as chemical probes and study cathepsin B behaviour in different cell lysates. Such systems are based on the design of a peptidic chemical warhead able to be degraded by cathepsin B, thanks to high affinity and specificity for such enzyme, promoting probe activation and release of a reporter tag, an amino-methylcoumarin derivative.[70]

Finally, recent approaches described the use of metal complexes as new reporter ions for MALDI-MS imaging. Conjugated to a targeting element, such as a peptide ligand, they provided a specific isotopic pattern facilitating data processing and ion identification. Han et al. developed a photocleavable Ru(III) polypyridine complex as mass tag for integrin $\alpha\beta_3$ detection. Their expression was associated to metastasis and tumor vascularisation, in hypopharynx tumor tissues. The cyclic antagonist RGDfK peptide was used to specifically bind integrin $\alpha\beta_3$. Then, upon laser irradiation, a polypyridyl ruthenium cation was generated, enabling integrin overexpression area localization in the tumor, without any external matrix.[71]

6.2 Non-covalent warhead

Alternatively, when no residue was available in the active site, for example in the case of Zn-metalloproteases, the spacer designed to generate adapted ABPs could either be activated by an external stimuli or attacked by a proximal nucleophile in the target active site. In the case of the absence of a nucleophilic residue enlisted in the catalytic mechanism, previously described probes were useless as no covalent linkage could be formed through the warhead. The main strategy implemented to overcome this issue relies on the use of photoactivatable moieties as spacer to covalently bind the target. Thanks to this technology, entitled photoaffinity labeling (PAL), several protein structures, functions and conformational changes have been elucidated while enabling the discovery of new potential therapeutic binding sites. In this case, the photoaffinity moiety, was used to generate, under UV irradiation, highly reactive species such as nitrenes, carbenes or free radical species. The generated intermediate was then able to generate a stable and covalent bond with the targeted protein, "capturing" it. Lately, diaziridine derivatives, benzophenone and tetrazole were amongst the most used photoaffinity moieties, thanks to the short half-life reactive intermediates generated and the low energy necessary to create them.[72]

This technology was lifesaving regarding Zn-metalloproteases targeting. Indeed, the catalytic mechanism relied on a zinc atom activating a water molecule that launches the hydrolysis process, preventing from a possible covalent binding through enzymatic activity. Consequently, the presence of a photocrosslinker targeting a proximal nucleophile was essential. Using generally benzophenone as the crosslinking moiety, hydroxamate, carboxylate and lately phosphonate ABPs have been developed, as strong zinc chelating agent, to selectively and specifically target metalloproteases active forms. Experimentally, such probes (Fig. 6c), developed by Saghatelian et al., enabled the sighting of tumorigenesis key biomarkers such as neprilysin, a membrane-associated metalloprotease, up-regulated in invasive melanoma cells.[68]

As PAL might reach its limit regarding its use in vivo, new chemical moieties were investigated to be used as reactive spacer. In this vein, three main chemical patterns have been synthesized respectively based on tosyl, acylimidazole and sulfonyl derivatives (Fig. 6d), providing protein labeling in living cells. Such spacers could react, through nucleophilic substitution or acylation, with a proximal nucleophilic amino-acid (e.g lysine, threonine, tyrosine). The tag was then covalently introduced directly into the active-binding pocket of the protein while preserving its natural function. Once the covalent bond formed, the ligand part of the probe was released from the active site, enabling traceless protein labeling. "Ligand-Directed Tosyl" (LDT) chemistry was the first to be implemented for antibiotic binding enzyme FKBP12 targeting, enabling rapamycin metabolism monitoring.[73] Improving such probes in terms of labeling kinetics and nucleophile specificity, "Ligand-Directed Acylimidazole" (LDAI) chemistry opened the way for membrane-bound protein labeling. It enabled the determination of protein half-life and degradation pathways for various types of transmembrane proteins such as folate receptor, bradykinin receptor or carbonic anhydrase 12, a Zn-metalloenzyme overexpressed in tumor cells.[74] Lately, N-sulfonyl pyridone derivatives were synthesized, displaying a fastest labeling kinetic and highest amino acid specificity. Indeed, such derivatives were poorly reactive in biological conditions and the labeling reaction was highly driven by the proximity effect generated by the ABP warhead-protein interactions. This compound was challenged for carbonic anhydrase 2.[75]

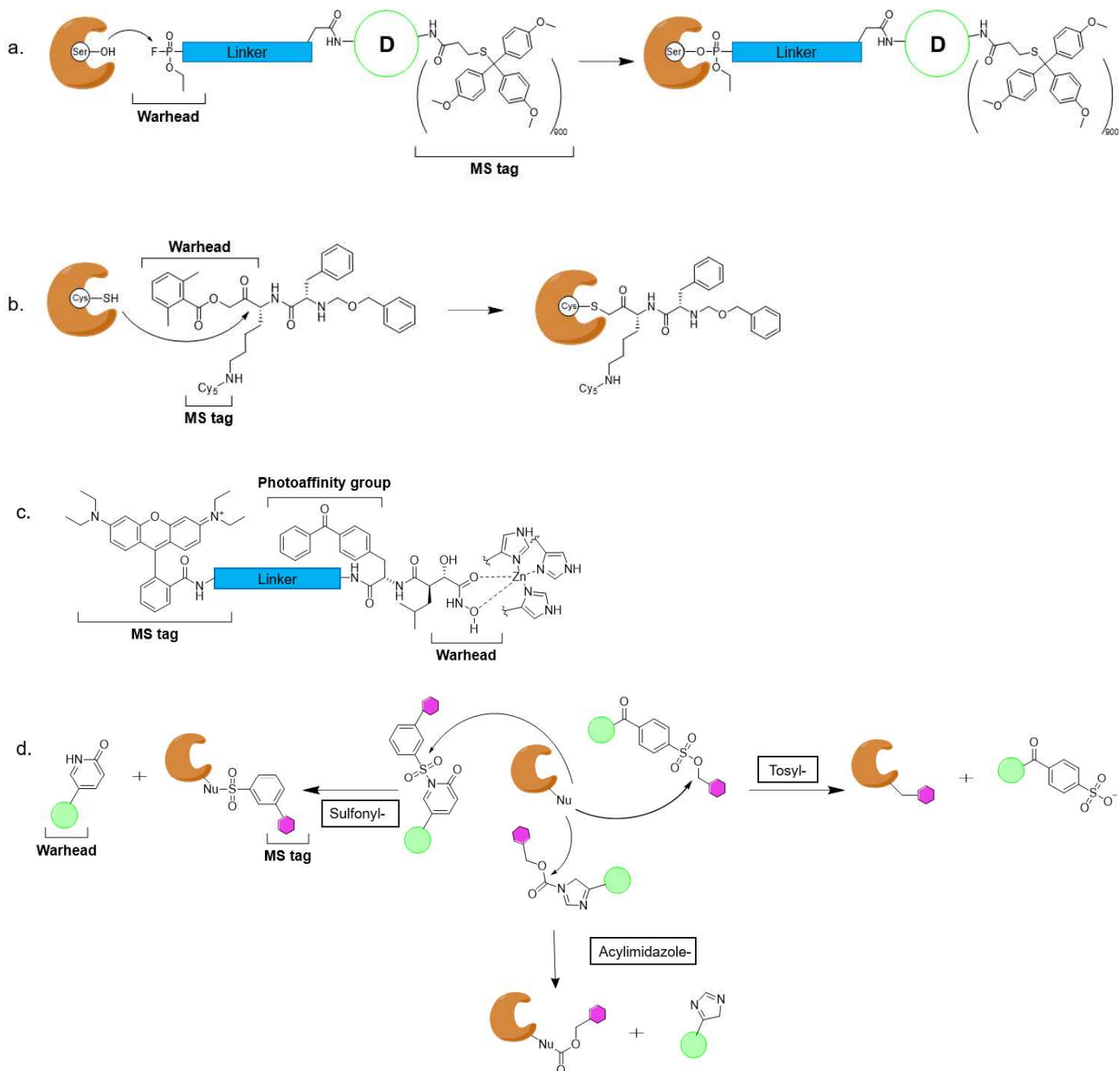


Figure 6. **a.** Structure of a fluorophosphate probe targeting serine hydrolases - [69] **b.** Mechanism-based labeling of cysteine protease using AOMK probe **c.** Structure of an hydroxamate probe targeting metalloprotease - [68] **d.** Chemical patterns and mechanisms involved in enzymatic traceless affinity labeling

CONCLUSION

Mass spectrometry has to tackle the new challenge of the in-deep understanding of biological systems while dealing with their dynamic complexity. Reaching a sensitivity able to match the low concentration of relevant biomolecules is a prerequisite but not sufficient. When possible, analysis has to be performed in the biological environment and not only the quantity but also the localization of biomolecule of interest, its active or inactive state, and the presence at the same time in the same environment of many other partners, has to be addressed to decipher complex physiological or physiopathological pathways.

In this context, only the development of spatially and temporally defined MS analyses allowing imaging techniques could afford exhaustive answers. To address these requirements, MALDI-MS should be supported by a range of dedicated reagents to label the biomarkers in an in vivo context. They benefit from well-known conjugation reactions (e.g. NHS esters, Michael acceptors)

but novel biorthogonal chemistries allowing a highly specific labelling could be implemented for this type of applications involving for example, biochemical or enzymatic transformations to generate unique functional moieties on the targeted biomolecule to be tagged.

Besides, when the tagging reaction itself is not selective enough, targeting elements have to be added to the reagent, the paramount of them being antibodies or highly specific ligands in the case of ABPs. In this later case, the chemical design of warheads or linker gathering the targeting role and triggering a specific labeling reaction, is still a challenge to be addressed.

Of course, the tag itself is of importance as it should allow an easy detection at subnanomolar concentrations but should ideally be derivatized to yield collections of various m/z to unlock the possibility of simultaneous detection in the case of multiplexing. This is limited with metal ions, on the contrary of organic cations such as trityl derivatives which could be tuned easily. At last, absolute quantification could also be included in the workflow by using isotopically different analogues of the tags. At the moment, no system combines all the desired features and combination of techniques has to be deployed to answer each biological questions.

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