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MALDI-MS: A Powerful but Underutilized Mass Spectrometric Technique for Exosome Research

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Abstract : Exosomes have gained the attention of the scientific community because of their role in facilitating intercellular communication, which is critical in disease monitoring and drug delivery research. Exosome research has grown significantly in recent decades, with a focus on the development of various technologies for isolating and characterizing exosomes. Among these efforts is the use of matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS), which offers high-throughput direct analysis while also being cost and time effective. MALDI is used less frequently in exosome research than electrospray ionization due to the diverse population of extracellular vesicles and the impurity of isolated products, both of which necessitate chromatographic separation prior to MS analysis. However, MALDI-MS is a more appropriate instrument for the analytical approach to patient therapy, given it allows for fast and label-free analysis. There is a huge drive to explore MALDI-MS in exosome research because the technology holds great potential, most notably in biomarker discovery. With methods such as fingerprint analysis, OMICs profiling, and statistical analysis, the search for biomarkers could be much more efficient. In this review, we highlight the potential of MALDI-MS as a tool for investigating exosomes and some of the possible strategies that can be implemented based on prior research.

Keywords : MALDI, Mass Spectrometry, Exosomes, Extracellular Vesicles

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I. Introduction

Various technologies have been developed in the past decades that aim to facilitate the exploration of the emerging field of exosomes, which is becoming more prominent in the scientific community.[1,2] The role of exosomes in mediating intercellular communication[3,4] may be associated with numerous medical issues, including immune responses,[5] viral pathogenicity,[6] pregnancy,[7,8] cardiovascular diseases,[9] central nervous system-related diseases,[10] and tumor progression.[11] To function properly, exosomes transport their cargo containing molecules, such as proteins, metabolites, DNA, messenger RNA, and microRNA,

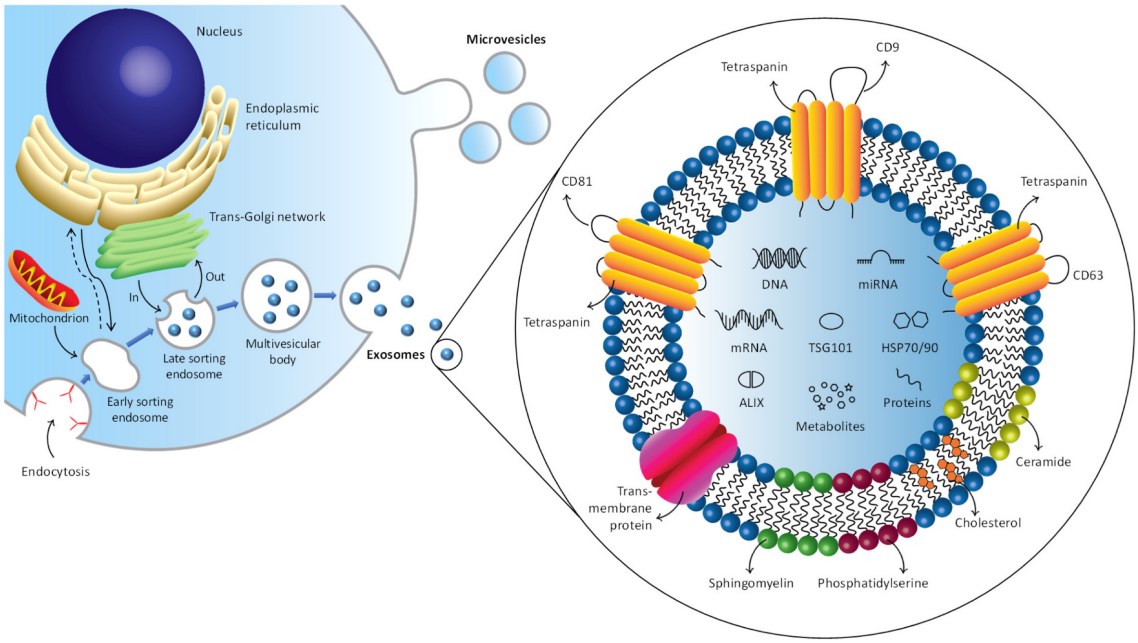

Figure 1. Biogenesis and structure of exosomes. Exosomes and ectosomes (microvesicles) are the two primary types of extracellular vesicles, which are distinguished by their origin and size. Exosomes (30-150 nm) are released into the extracellular microenvironment by MVB fusion to the plasma membrane, whereas microvesicles (up to 1 μm) are shed from the outward budding of the plasma membrane. Exosomes contain numerous biological components, including nucleic acids, proteins, lipids, and metabolites.

to specific cells to maintain cellular homeostasis.[12,13] Exosomes are not only being studied for their roles in disease diagnostics and therapeutics[14,15] but also as a tool for drug delivery.[16]

Exosomes are extracellular vesicle subtypes derived from various cells and are commonly categorized by their size, which ranges from 30 to 150 nm in diameter.[17] Exosomes are produced within the endosomal membrane as intraluminal vesicles and are secreted into the extracellular environment after the fusion of multivesicular bodies (MVBs) with the cell surface.[18] The biogenesis of exosomes is illustrated in Figure 1. Early exosome research focused heavily on cultured cells, where numerous observations of exosome secretion were reported.[19-21] The prospect of using exosomes as biomarkers for diseases diagnosis has prompted researchers to investigate exosomes from various biological fluids, such as blood,[22] urine,[23] saliva,[24] breast milk,[25] amniotic fluid,[26] and seminal fluid.[27] However, biofluids contain a wide range of constituents and co-isolation of non-exosome contaminants, such as lipoproteins[28] and non-exosome proteins is likely.[29] Furthermore, determining the origin of exosomes, the physiochemical and biochemical commonalities between extracellular vesicles, and the heterogeneity of exosomes poses a challenge to the development of standardized methods for exosome isolation and analysis.[30]

Currently, ultracentrifugation is considered the gold standard for isolating exosomes,[31,32] with alternative techniques being polymer-based precipitation,[33] ultrafiltration,[34] and size exclusion chromatography (SEC).[35] Each approach has benefits and limitations that have major impacts on the final isolated products. For example, the use of ultracentrifugation has been shown to generate large amounts of exosomes, but the lengthy isolation procedure may be inefficient for practical use.[29] By contrast, ultrafiltration may reduce isolation time, but the risk for exosome loss due to membrane filter clogs requires the use of highly sensitive techniques for subsequent exosome characterization.[29] Exosome research has fortunately emerged alongside various complementary characterization methodologies, such as immunoblotting, nanoparticle tracking analysis, electron microscopy, and mass spectrometry (MS).[36,37] MS instrumentation has frequently been used in exosome studies for prognostic and diagnostic biomarker discovery.[38] The emergence of soft ionization techniques, such as matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), have significantly simplified MS analysis and produce less complicated spectrums for exosome component identification.

Both MALDI and ESI ionization techniques for MS emerged in the late 1980s and have been extensively used for biomolecule studies.[39,40] However, based on the results of electronic searches of the Scopus and PubMed online databases, exosome research is more frequently performed using ESI-MS analysis than MALDI ionization. As of

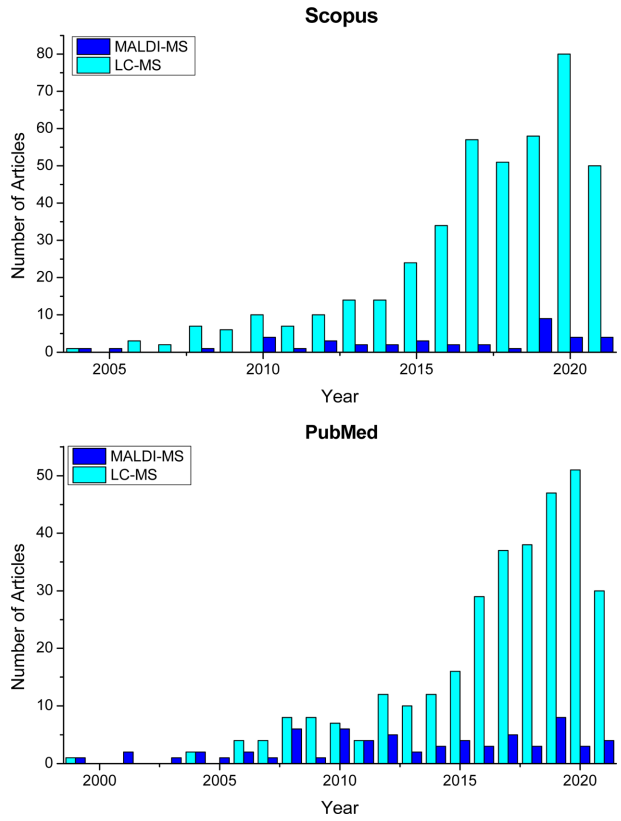

Figure 2. Comparison of MALDI-MS and LC-MS exosome articles based on an electronic search of the Scopus and PubMed online databases (search was performed on August 6, 2021).

August 6, 2021, the keywords "exosome" and "mass spectrometry" produced 1030 hits in Scopus and 879 in PubMed. Adding the term "MALDI" to the preceding search resulted in 40 hits in Scopus and 60 in PubMed (Figure 2). Substituting the phrase "MALDI" with "liquid chromatography," which is the most typical setting for ESI-MS analysis, yielded 428 hits in Scopus and 290 in PubMed (Figure 2). The selection between the two ionization methods is closely related to methodological approaches, where ESI-MS being used in bottom-up proteomics[41] and MALDI-MS used in top-down proteomics.[42] However, exosomes comprise a wide range of components associated with biological functions and diseases,[2] and focusing on a single analytical method is not sufficiently comprehensive. Hence, there is a need for MALDI-MS in exosome research, as it allows for the discovery of more novel insights from different perspectives. In addition, MALDI-MS is not only capable of performing high-throughput label-free and direct analysis,[43] but it is also a cost- and time-efficient approach.

The strategies and applications of MALDI-MS analytical techniques in exosome research are the focus of this review. A brief introduction to MALDI-MS and exosomes is provided to assist with the understanding of these subjects in their respective settings. In addition, the current trends of exosome research that utilize MALDI-MS are summarized to demonstrate the ability of MALDI-MS to serve as a valuable tool in exosome research.

II. MALDI-MS

MS is an analytical tool for determining the mass-to-charge ratio (*m/z*) of ionized species. Initially developed in the early 1900s, it was not extensively employed for biological substances, such as proteins, until the late 1980s when MALDI[39] and ESI[40] were introduced. Both ionization methods are termed "soft-ionization," because they prevent extensive fragmentation of the molecules, resulting in limited loss of sample integrity.[44] MALDI not only has low fragmentation ionization, it also predominantly produces singly charged ions, which significantly reduces spectrum complexity.[45] Along with automated features that enable high throughput and rapid analysis, MALDI is frequently employed in macromolecule research.[46,47] The following sections provide a brief overview of the basic principles of MALDI-MS, as well as a brief history and important findings. MALDI technologies, such as matrices,[48-50] lasers,[51,52] and desorption/ionization processes,[53-55] have been comprehensively reviewed in other publications.

A. MALDI process

MALDI is an ionization method that uses a laser energy absorbing matrix to generate ions from large molecules with little fragmentation.[56] Soft ionization methods, such as MALDI and ESI, are required to ionize large molecules, such as biomolecules (e.g., DNA, proteins, and carbohydrates) and polymers, as these molecules are easily fragmented by other hard ionization methods. Electron ionization, thermal ionization, and inductively coupled plasma ionization are examples of hard ionization methods, which produce complicated spectrums that are difficult to interpret. MALDI mass spectrum generally display singly charged ions, which is useful for evaluating complex samples. Multiply charged ions are often needed for expanding the mass range on the mass spectrum or improving fragmentation for structural analysis.[57] Several examples of multiple charged ion studies using MALDI-MS have been reported.[58,59] MALDI involves the use of a matrix and a laser to facilitate a three-step process, which involves the incorporation of sample and matrix on a metal plate, the disintegration of the analyte-matrix solid into vacuum upon the laser beam ablation, and the ionization of analyte molecules through ion-molecule reactions in the hot plume of ablated gases.[60] The analyte ions are accelerated into a time-of-flight (TOF) tube, and their individual flight times are measured to calculate their *m/z* values. Typical MALDI-TOF-MS processes are illustrated in Figure 3.

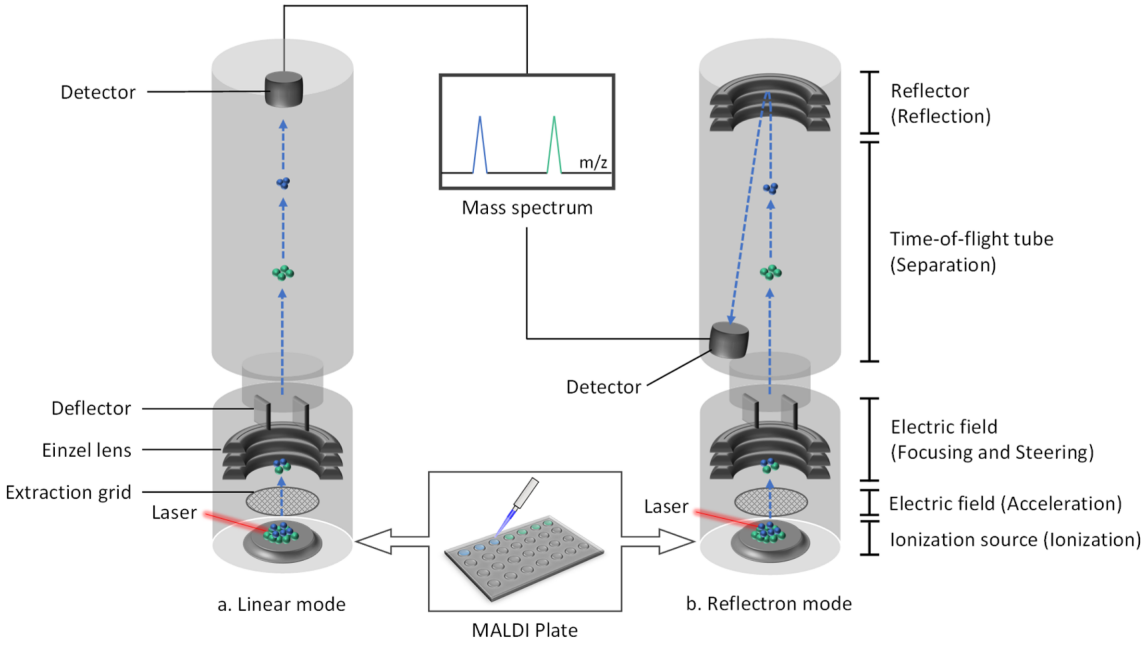

Figure 3. Schematic diagrams showing MALDI-TOF-MS processes for (a) linear and (b) reflectron modes. Linear mode is often used for samples with high molecular weight samples, whereas reflectron mode provides high resolution and mass accuracy for low molecular weight samples.

B. MALDI matrix selection

Matrix selection can significantly influence the resulting mass spectrum.[48,61] In general, there are three types of matrices available: organic, ionic liquid matrices,[49] and inorganic matrices.[62] Organic matrices are the most frequently used in MALDI-MS analysis because they are widely available and compatible with various analytes. The drawback of organic matrices is the generation of background interferences from matrix fragmentation and adduct formation, which makes elucidation in the low *m/z* region (< *m/z* 500) difficult.[63] Sinapinic acid,[64] 2,5-dihydroxybenzoic acid (DHB),[56] and α-cyano-4-hydroxycinnamic acid (CHCA)[65] are examples of typical organic matrices.[66] These three organic matrices have frequently been used in exosome research to identify biomarkers by profiling proteins and lipids.[37,67,68]

Small organic compounds containing aromatic rings with delocalized π electrons were initially proposed as matrices as they demonstrated significant resonance absorption at MALDI laser wavelengths, which are typically in the ultraviolet (UV) range (337 nm or 355 nm).[69] Other factors important to matrix selection include the ability to tolerate a high vacuum, the ability to produce analyte ions, the ability to segregate generated ions to avoid the formation of analyte clusters, and the ability to homogeneously co-crystallize with analytes.[48] The last two criteria are not required as ionization and detection is still possible, although the outcomes may be undesirable. The formation of analyte clusters, for example, may lead to the generation of dimers, which can complicate the acquired MALDI mass spectra. Matrix-analyte crystal homogeneity impacts "shot-to-shot" reproducibility, which is critical for quantitative analysis.[70,71] In addition, the method used to mix the matrix and sample may affect crystal homogeneity. The traditional dried droplet approach has been enhanced through advances in crystal uniformity that use forced droplet drying with the freeze-vacuum drying technique[72] and recrystallization of dried droplets with acetonitrile.[71] To the best of our knowledge, no studies have focused on comparing MALDI-MS sample preparation methods in exosome research. Table 1 highlights the exosome studies that utilize MALDI-MS, as well as the matrix and sample preparation procedures.

C. MALDI laser

The purpose of the MALDI laser is to irradiate the samples, which induces ablation and desorption on the analyte–matrix surface. Various MALDI laser configurations have been developed since the first application for MS in 1966, in which the laser beam was designed to strike the back of the samples and ablate the samples on the front side toward a TOF chamber.[73] In 1985, matrix-assisted lasers produced two peaks for the amino acids tryptophan and alanine only when simultaneously analyzed using 266 nm laser light from an Nd:YAG laser.[74] Tryptophan was shown to behave like a laser-absorbing matrix by assisting with the ionization of alanine, indicating that laser desorption ionization was matrix-assisted. Today, the Nd:YAG laser at

Table 1. Past exosome studies conducted using MALDI-MS.

Origin of exosomes	Matrix	Sample preparation	Lasers	MS	Strategies	Biomarkers	Ref.
Cancer cells	CHCA, sinapinic acid	Dried droplet	-	TOF-MS	Proteomic profiling, statistical analysis	Distinct protein statistical patterns	67
Cancer cells	Sinapinic acid	Thin-layer	-	TOF-MS	Protein fingerprint analysis	Distinct protein fingerprints	121
Glioblastoma cell	CHCA	Dried droplet	355-nm Nd: YAG laser	TOF-MS	Peptide mass fingerprinting	6 proteins	135
Human B cell	DHB	Dried droplet	-	TOF-MS	Proteomic and lipidomic profiling	Multiple proteins and lipids	136
Mesothelioma cell	CHCA	Dried droplet	337-nm nitrogen laser	TOF-MS	Protein composition profiling	38 proteins	137
Oviduct fluid	DHB	Dried droplet	355-nm Nd: YAG	TOF-MS	Lipidomic profiling, statistical analysis	Phosphatidylcholines, phosphatidylethanol-amines, sphingomyelins	138
Plasma	DHB, CHCA, sinapinic acid	Thin-layer	337-nm nitrogen laser	TOF-MS	Statistical analysis	7 proteins	43
Plasma	9-aminoacridine	Dried droplet	337-nm nitrogen laser	Orbitrap-MS	Lipidomic profiling	Sphingomyelin, phosphoinositol, ceramide	81
Serum	CHCA	Layer-by layer	349-nm Nd: YLF laser	TOF-MS, FTICR-MS	Proteomic profiling	Platelet factor 4	37
Serum	CHCA	Dried droplet	355-nm	TOF/TOF-MS	Proteomic profiling	32 proteins	139
Serum	CHCA	Dried droplet	355-nm	TOF/TOF-MS	Proteomic profiling	38 proteins	140
Tear	CHCA	Dried droplet	360-nm	TOF-MS	Fingerprinting analysis, statistical analysis	Distinct fingerprints	126
Urine	DHB	Dried droplet	-	TOF/TOF-MS	Lipidomic profiling	Sphingolipids	68
Urine	Sinapinic acid	Dried droplet	-	TOF-MS	Proteomic profiling	Alpha 1-antitrypsin and H2B1K	123

wavelength of 355 nm is one of the most commonly used UV lasers in commercial applications of MALDI.[75]

Aside from UV lasers, other lasers developed for MALDI are visible lasers,[76,77] ultrafast lasers,[78] infrared (IR) lasers,[79] and two-lasers.[80] Each laser has certain benefits and drawbacks, but UV lasers appear to be more advantageous, which makes them the obvious choice in modern MALDI systems. Indeed, direct absorption of UV and IR lasers can result in the fragmentation of macromolecules, which may hinder the detection of molecular ions.[77] The use of visible lasers has been proposed as an alternative due to the transparency of macromolecules in the visible range.[76] However, additional constraints involving matrix compounds that must absorb laser energy and promote ionization when desorbed with analytes make visible lasers less efficient.[51]

MALDI-MS analyses of exosome research do not always specify laser type and wavelength, because the laser in most commercial MALDI-MS equipment is not interchangeable. Low-repetition-rate lasers, such as the nitrogen laser (~100 Hz), have been actively used in exosome research,[43,81] along with higher-repetition-rate lasers such as Nd:YAG (~10 kHz) and Nd:YLF (~5 kHz).[37] To the best of our knowledge, studies comparing the use of different lasers in exosome research have not been pursued. The types of lasers used in previous exosome studies are listed in Table 1.

III. Extracellular vesicles

Current extracellular vesicle research focuses on classification, isolation techniques, and understanding their biological role in disease progression and therapy.[82] Despite a considerable increase in publications and studies, extracellular vesicle research remains limited due to a lack of understanding resulting from the absences of a standard reference, limited replication of the findings, and a lack of examples that integrate the fundamental science with patient treatment.[82] Difficulties generating highly purified extracellular vesicles, performing single-particle detection, and imaging extracellular vesicles at a reliable resolution hinder extracellular vesicle research from reaching the next step of clinical application.[82,83]

Extracellular vesicles are often classified into two types: exosomes and ectosomes.[84] Exosomes, as previously stated, are of endosomal origin and average 100 nm in size, whereas ectosomes, also known as microvesicles, are larger vesicles (up to 1 μm) produced by the direct outward budding of the plasma membrane (Figure 1).[83] The following sections focus on exosomes, specifically their biogenesis and general biological functions. In addition, common isolation methods are briefly discussed to set a base for the subsequent topic. Comprehensive reviews of exosome biogenesis[83], intercellular communication[4], homeostasis[85], biomarkers[23,38], isolation techniques[36], and potential applications[14,15] have been discussed in other publications.

A. Exosome biogenesis

Exosomes are produced in the endosomal membrane as intraluminal vesicles beginning with the double invagination (inward budding) of the plasma membrane. The final steps involve the creation of MVB-containing intraluminal vesicles before the release of the exosome into the extracellular milieu. The first invagination is an endocytosis process that occurs at the plasma membrane and produces a cup-shaped pocket that holds cell-surface and extracellular proteins. Following the separation of the invaginated structures from the membrane, early sorting endosomes (ESEs) are formed and in certain cases, may merge with ESEs produced by constituents of the endoplasmic reticulum, mitochondria, and the trans-Golgi network. ESEs later mature into late-sorting endosomes (LSEs), where the second invagination occurs, resulting in the formation of intraluminal vesicles. Further modification may occur here, although the final formation of the intraluminal vesicle is the introduction of cytoplasmic constituents into the LSE or their release into the cytoplasm. In addition, the size of the intraluminal vesicles varies depending on the volume of invagination and the proteins originally introduced from the cell surfaces. LSE develops into MVB, which include intraluminal vesicles in their final form, ready to fuse with the plasma membrane. MVB fusion results in intraluminal vesicles (or exosomes, in this case) being released into the extracellular space through the exocytosis process.[83]

Several proteins involved in the formation and biogenesis of exosomes can be found on the surface of the exosome membrane or within exosomes. Exosome surface proteins include tetraspanin proteins (e.g., CD9, CD81, and CD63),[86] while proteins contained in exosomes include apoptosis-linked gene 2-interacting protein X (ALIX) and tumor susceptibility gene 101 (TSG101), all of which are frequently used as markers for exosome characterization.[87] Other types of proteins found in exosomes are classified as chaperone proteins (e.g., Hsp70 and Hsp90), enzymes (e.g., GAPDH and ATPase), signaling proteins (e.g., EGFR and HIF-1α), and exoskeletal proteins (e.g., actin and tubulin).[88] Certain exosome proteins are cell-specific, such as MHC-I/II on the surface of dendritic cell-derived exosomes and PD-L1 on the surface of cancer cell-derived exosomes.[83] Exosome membrane structures are primarily built by lipids, including phospholipids, spingolipids, and cholesterol that act to increase exosome flexibility and rigidity, as well as protect the exosome contents from various stimuli in the circulating fluids.[89] Other lipids, such as fatty acids[90] and eicosanoids,[91] are contained inside and transported by exosomes and are subjected to intercellular communication rather than the structural components of exosomes. Exosomes also include nucleic acid-based components such as DNA and RNA (e.g., messenger RNA and microRNA), which may affect the expression of genetic information in recipient cells.[92]

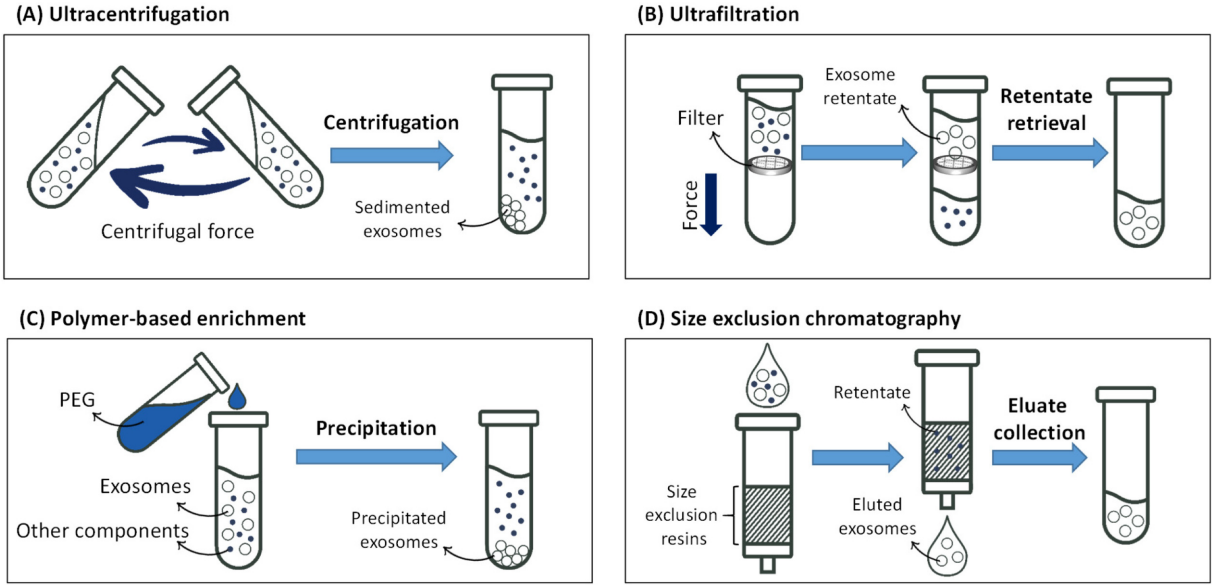

Figure 4. Typical exosome enrichment approaches of (A) ultracentrifugation, (B) ultrafiltration, (C) polymer-based enrichment, and (D) size exclusion chromatography. The most studied method for isolating exosomes is ultracentrifugation.

B. Biological role of exosomes

The primary purpose of exosomes is to contain and deliver biological components that serve as information to recipient cells. Exosomes are considered mediators of various biological processes, including intercellular communication[3] and maintenance of cellular homeostasis.[12] Exosomes discharged into the extracellular environment by their parent cells merge their membrane contents with the recipient cell plasma membrane and deliver effectors, such as transcription molecules, oncogenes, and small and large non-coding regulatory RNAs.[93] In addition to their role in therapeutics, such as stem cell maintenance,[94] tissue repair,[95] and blood coagulation,[96] exosomes also affect disease pathogenesis, including tumor growth,[97] inflammation,[98] and immunosuppression.[99]

C. Exosome enrichment methods

The selected isolation method affects exosome recovery and can result in possible discrepancies in the identification of biological components associated with exosomes. The ultracentrifugation enrichment approach is currently considered the gold standard for isolating exosomes as it requires only one ultracentrifuge, no special or expensive consumables, and minimal skill for operation with no specialized sample preparation.[29] However, this method has several drawbacks, including co-sedimentation, aggregation formation, exosome rupture, and a prolonged enrichment process that could influence the results. Other typical exosome isolation methods include polymer-based enrichment,[33] ultrafiltration,[34] size exclusion chromatography,[32,35] and affinity bead or column isolations.[100] Figure 4 shows the schematic diagrams of four commonly used exosome isolation methods. Brief summary for each of the four exosome isolation methods is provided below.

a. Ultracentrifugation

Ultracentrifugation separates particles based on sedimentation rates using centrifugal force.[101] The word "ultra-" differentiates the method from standard centrifugation by employing centrifugal forces of more than 100,000 × g. An exosome isolation method using a lower centrifugal force has been employed with multiple cycles of centrifugation at 40,000 × g centrifugal force.[102] However, most exosome researchers eventually resort to ultracentrifugation. The standard ultracentrifugation procedure[103] uses two cycles of low-speed centrifugation (300 × g and 2000 × g), followed by mild-speed centrifugation (10,000 × g), and lastly, two rounds of ultra-speed centrifugation (100,000 × g).[102] The first three cycles pellet the cells, cell debris, and dead cells, whereas the subsequent rounds are meant for pelleting exosomes. Ultracentrifugation has been implemented in more than 56% of exosome studies,[104] despite having a few drawbacks, such as contamination and exosome losses.[29]

b. Ultrafiltration

Ultrafiltration is a size-based exosome isolation method that uses membrane filters with specific molecular weight cut-offs. The use of centrifugal force in ultrafiltration may result in the deformation and rupturing of large vesicles, which can influence the outcome.[29] Ultrafiltration techniques have been coupled with other separation methods, such as size exclusion chromatography[105,106] and precipitation-

based enrichment,[107] to improve the efficacy of exosome enrichment.[104-106] The ultrafiltration method was initially used to enrich exosomes from large volumes of medium (> 1 L), using a 500 kDa hollow fiber membrane employed under constant pressure[108] for clinical application.[103]

c. Polymer-based enrichment

Polymer-based enrichment or the exosome precipitation method modifies exosome solubility and dispersibility, allowing exosomes to precipitate in biological fluids.[29] ExoQuick and Total Exosome Isolation are examples of two commercial polymer-based isolation kits that use volume-excluding polymers, such polyethylene glycol, dextrans, and polyvinyls as reagents.[33] The method is adapted from the viral isolation method that uses a polyethylene glycol solution because viruses are biophysically similar to exosomes.[33,109] The ExoQuick protocol calls for only a few steps, beginning with the introduction of a polymer solution into the samples, followed by incubation for a specified duration, and finally, sample collection through centrifugation.[110] Many researchers have been drawn to this polymer-based enrichment method because of its ease of use, but researchers are always seeking alternatives due to its low purity and high cost.[33] This approach is continually being improved, and has resulted in superior versions of the method, such as the ExtraPEG procedure.[33]

d. Size exclusion chromatography

Like ultrafiltration, SEC utilizes size to separate exosomes from other components using solid-phase extraction (SPE)[111] or liquid chromatography (LC),[112] where larger particles elute first, followed by small vesicles and proteins.[113] The stationary phase, which can be a porous gel filtration polymer, is a critical component of SEC systems, unlike the mobile phase. A few examples of gel polymers are crosslinked dextrans, agarose, and polyacrylamide.[113] SEC is considered an ideal method for therapeutic applications and biomarker discovery because the integrity and characteristics of the exosomes are preserved.[114] SEC enrichment of exosomes has certain issues related to yield and purity. First, the purity is equivalent to ultracentrifugation combined with density gradient, which is considered high compared to other techniques.[115] Second, SEC combined with other approaches, such as ultrafiltration or ultracentrifugation, results in clean exosomes with considerably lower total protein content.[116] Finally, circulating proteins, such as albumin and apolipoproteins, continue to contaminate exosomes due to their abundance.[117]

IV. MALDI-MS strategies in exosome research

MALDI-MS is used in exosome research because of its compatibility with clinical applications due to the rapid and direct analytical capabilities.[118] Exosome research is currently focused on LC-MS, as many of the studies have been analytical in nature. Indeed, LC-MS is a valuable analytical tool; especially, LC combined with tandem mass spectrometry (LC-MS/MS) is currently the most widely used analytical methodology to characterize exosome proteins.[119] However, exosome research has garnered clinical attention[120] and a more appropriate instrument, such as MALDI-MS, is required to translate exosome strategies from basic science to patient care.[82]

Recent research has demonstrated the ability of MALDI-MS to examine intact exosomes without the requirement for exosome lysis or labeling.[121] Several interesting conclusions have been reached, including that exosome fingerprints help in differentiating parental cells. Three phases of intact melanoma cell lines (SBCI2, WM115, and WM239) could not be discriminated by mass spectral fingerprints; however, the exosomes derived from the corresponding cell lines were able to distinguishable.[121] This discovery suggests that each cell line secretes unique exosomes that can distinguish not just between cancer stages, but also between normal cells. The mass spectral fingerprints of exosomes derived from cell lines would be incredibly useful to analyze exosomes isolated from biofluids, as the exosome origin is generally unknown. Specific proteins that serve as melanoma biomarker proteins could only be identified using exosome mass spectral fingerprints.[121] Premelanosome protein, a melanoma diagnostic marker implicated in melanosome maturation from stages I and II, was not found in the mass spectral fingerprint of the WM115 melanoma cell, but was found in the associated exosomes.[119] Exosomes may improve crucial indicators for a more accurate diagnosis and prognosis, allowing for the appropriate treatment to be determined.

Using exosomes as a tool in cancer monitoring is appealing to cancer researchers because invasive cancer monitoring, such as tissue biopsy, can be avoided.[122] The term “liquid biopsy” is associated with exosome research[67] and OMICs profiling with MALDI-MS has led to the discovery of disease biomarkers.[66] Proteome profiling of urine exosomes resulted in the identification of fragmented alpha-1 antitrypsin and histone H2B1K peptides.[123] As a result, two peaks observed by MALDI-MS were identified as biomarkers to discriminate between urothelial carcinoma patients and healthy individuals.[123]

MALDI-MS analysis can be challenging, particularly when dealing with complex spectra containing peaks irrelevant to the experimental subjects. Statistical methods such as principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) help distinguish and highlight important peaks that respond to biological variables. Han *et al*. (2021) showed that PCA of MALDI-MS mass spectra from plasma-derived exosomes could discriminate between healthy controls and patients with osteosarcoma and lung cancer. The investigation was extended to osteosarcoma lung metastasis, which resulted

in the discovery of seven biomarkers that differentiate this disease from non-lung metastasis.[43] Many recent MALDI-MS strategies in exosome research have led to biomarker discovery and an essential component of these efforts is the selection of the method.

Table 1 summarizes the strategies for exosome research using MALDI-MS, including the matrix, sample preparation, lasers, and mass analyzers used in the respective studies. All studies used organic matrices, with DHB and CHCA being the most frequently used. The 9-aminoacridine matrix is specifically used for lipidomic analysis in the negative ion mode, which results in the detection of anionic lipid species.[81] The selection of the matrix reflects the OMICs profiling routine use, with DHB and CHCA matrices prevalent in proteomics,[124] while matrix selection in lipidomics is based on the specific lipid species being studied.[48]

Sample integration with the matrix is often performed using the dried droplet method. This procedure can be carried out by either reconstituting the loaded samples with the matrix solution or directly integrating the samples with the matrix before applying them to the MALDI plates. Reproducibility remains an issue because the standard dried droplet approach cannot resolve hot-spot formation.[70, 71, 125] Thus, improving the surface uniformity of MALDI sample spots for exosome research should be a focus. The laser and mass analyzer used in the respective studies may have been limited by available resources, although the use of TOF/TOF MS and Fourier transform ion cyclotron resonance MS can elucidate the molecules and provide high-resolution mass spectra, respectively. However, TOF-MS is sufficient for most strategies.

A. Fingerprint analysis

MALDI-MS fingerprint analysis uses variation in spectrum profiles and major peaks to distinguish between exosomes. The peaks reflect a variety of biological components, particularly when the study is conducted on intact exosomes.[121] The majority of MALDI-MS fingerprint analyses result in the identification of proteins or their fragments that may serve as disease biomarkers.[43,67] Although mass spectral fingerprint analysis has been performed on exosomes derived from cultured cells,[37,121] only a few studies have been conducted on exosomes derived from biofluids.[126] This may result from the complexity of biofluids and wide variation in exosomes, which complicates the mass spectrum. Hence, a MALDI-MS spectral fingerprint database for exosomes derived from cells may allow the origin of exosomes recovered from biofluids to be determined. To the best of our knowledge, no spectral database for exosomes derived from cell lines has been developed such as SpectraBank for microbial identification.[127]

B. OMICs profiling

OMICs profiling aims to identify, characterize, and quantify biological components involved in the construction and function of exosomes, such as proteins, lipids, and metabolites.[128] Exosome surface proteins contain information about their origin, which is useful for classifying exosomes recovered from biofluids.[129] Proteome profiling may be carried out by extracting and sequentially analyzing proteins using MALDI-MS for top-down protein identification or fingerprint analysis. Lipidomic composition analysis by MALDI-MS may not be as useful as proteomics because lipid detection by MALDI-MS is highly selective for the specific matrix.[48] A lipidomic profiling study on plasma exosomes found that only sphingomyelin, phosphoinositol, and ceramide species could be identified with high confidence when employing the 9-aminoacridine matrix in negative mode.[81] Furthermore, there were no significant differences in lipid composition between plasma and its exosomes, indicating the classification of exosomes based on lipid composition may not be feasible.[81]

C. Statistical analysis

Software tools Proteome Discoverer™ (Thermo) for proteomics[130] and the LIPID MAPS® for lipidomics[131] now allow for direct analysis of acquired mass spectra.[127,128] However, obtaining numerous datasets may make it difficult to determine the relevant features in the spectrum. One straightforward method compares features with the largest quantitative changes, although significant differences between two classes (e.g., control and sample) may not represent mechanistic differences.[132] In this case, statistical techniques may be beneficial. There are two methods in statistical analysis: univariate and multivariate methods. Analyses of variance (ANOVAs), *t*-tests, and *p*-values are examples of univariate analysis, in which scores are used to rank input characteristics.[132] *P*-value is often used in exosome research to measure the level of significance of a feature.[133] For multivariate analysis, PCA, PLS-DA and clustering methods are frequently used in exosome studies.[43,67] Multivariate techniques are more resistant to error than univariate methods, which makes them more accurate for feature selection.[132] Metaboanalyst 5.0[134], SPSS, and MATLAB are examples of statistical analysis software packages.[131]

V. Conclusion

MALDI-MS is a powerful tool for exosome research, much like LC-MS/MS. The insufficient amount of exosome research publications employing MALDI-MS requires attention as this method is well-suited for clinical use. While the approach has drawbacks, such as difficulty detecting a wide variety of exosome lipid species due to matrix selectivity, the knowledge gained will be valuable for future research. We outlined three commonly utilized exosome research strategies when using MALDI-MS: fingerprint analysis, OMICs profiling, and statistical

analysis, all of which can be dependent and independent of one another. Fingerprint analysis may be the most promising method in terms of the clinical applicability of MALDI-MS for exosome research. The focus of MALDI-MS sample preparation should be the identification of a standard method for preparing exosomes while avoiding common issues, such as hot spot formation. Furthermore, we propose developing a MALDI-MS mass spectral fingerprint database for exosomes, similar to SpectraBank, for microbiological characterization. All mass spectra of exosomes would be gathered from a respected origin, ensuring that all references are consistent and valid. We recommend additional exosome studies be performed on MALDI-MS to fully realize the potential of this technique and maximize its usefulness.

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