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Thiago Verano-Braga *Editor*

Mass Spectrometry-Based Approaches for Treating Human Diseases and Diagnostics

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This series of volumes focuses on concepts, techniques and recent advances in the field of proteomics, interactomics, metabolomics and systems biology. Recent advances in various 'omics' technologies enable quantitative monitoring of myriad various biological molecules in a high-throughput manner, and allow determination of their variation between different biological states on a genomic scale. Now that the sequencing of various genomes, from prokaryotes to humans, has provided the list and linear sequence of proteins and RNA that build living organisms, defining the complete set of interactions that sustain life constitutes one of the key challenges of the postgenomic era. This series is intended to cover experimental approaches for defining protein-protein, protein-RNA, protein-DNA and protein-lipid interactions; as well as theoretical approaches dealing with data analysis, integration and modeling and ethical issues.

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Mass Spectrometry-Based Approaches for Treating Human Diseases and Diagnostics

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Preface

Omics, a multidisciplinary field encompassing genomics, transcriptomics, proteomics, metabolomics, lipidomics, and more, has witnessed remarkable growth in recent decades. This expansion owes much to the advancements in mass spectrometry (MS) instruments, innovative sample preparation techniques, and enhanced bioinformatics tools. Today, omic approaches are integral to the study of human diseases, driving transformative changes in the field of Molecular Medicine.

This book delves into the dynamic landscape of molecular medicine, where mass spectrometry-based omic technologies, such as proteomics and metabolomics, are playing pivotal roles. These methodologies enable comprehensive molecular-level characterizations, paving the way for the discovery of novel biomarkers and therapeutic targets. The ultimate aspiration is personalized medicine, and omics offers valuable insights in this pursuit.

Within these pages, readers will encounter a wealth of information on the latest technological advancements in omics. The book navigates through the application of omics to gain deeper insights into human diseases, encompassing biomarker discovery, validation, and mechanistic understandings that can inform innovative treatments.

The initial chapters are dedicated to cutting-edge methods for isolating and characterizing extracellular vesicles (EVs) and introduce the innovative bioinformatic tool "Glycosort," which enhances the post-processing of large-scale intact glycopeptide analyses. Subsequently, the book presents a series of review articles showcasing how omics approaches are illuminating various facets of human diseases, including those caused by viruses (SARS-CoV-2 and Zika Virus), psychiatric disorders, neurodegenerative diseases – including their impact in the intricate process of memory formation – and conditions affecting the cardiovascular, renal, and endocrine systems.

This book holds particular relevance for students, scientists, and healthcare professionals eager to explore the molecular intricacies of human diseases. It provides a valuable resource for those interested in identifying potential new diagnostic biomarkers and gaining a deeper understanding of disease progression.

Belo Horizonte, MG, Brazil — Thiago Verano-Braga

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1.1 Introduction

1.1.1 Extracellular Vesicle Biosynthesis and Biological Functions in Diseases

Extracellular vesicles (EVs) are characterized as nanoparticles enclosed by a lipid bilayer membrane secreted by the cells [1, 2]. The EVs can be classified into three main types, according to the origin and size of two main types of EVs are distinguished based on their biogenesis, known as exosomes and ectosomes [3]. More specifically; exosomes, microvesicles, and apoptotic bodies [3–5]. The exosomes

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are particles with 50–150 nm diameter, with endosomal biogenesis in the multivesicular bodies (MVBs) and amphisomes. The exosome's biosynthesis occurs by inward budding endosomal vesicles driven by the endosomal sorting complex required for transport (ESCRT) machinery and fusion of these multivesicular bodies or amphisomes with the plasma membrane, resulting in the release of intraluminal vesicles (exosomes) [3, 6–8]. Microvesicles are medium-sized EVs (200–800 nm) that originated from cell membrane budding and are less abundant than small EVs [3, 9]. Microvesicles are released from many cells, such as neurons, platelets, red blood cells, astrocytes, and endothelial cells, in both healthy and pathological states [10, 11]. Apoptotic bodies are the largest EVs, comprising >1 μm released during the apoptotic process [12]. The heterogeneity of the EVs is related to the type, function, and state of the origin cell such as different mechanisms of cell death [3]. The EVs carry acid nucleic (DNA, mRNA, miRNA) proteins [13, 14], transcription factors, growth factors, signaling molecules, and lipids from one cell to another cell [15–19]. EVs are involved in several biological functions beyond the transport of molecules between cells, such as cell-cell communication and activation of target cells through EV surface ligands [20]. Moreover, EVs are involved in angiogenesis [21], inflammation [22], coagulation [23], antigen presentation in immune response [24, 25], and fetal-maternal communication [26]. In diseases, EVs also participate in several biological processes. For example, in glioblastoma, cells secret mRNA, miRNA, and angiogenic proteins encapsulated in exosomes to target microvascular endothelial cells inducing angiogenesis [27]. EVs also participate in oncogenesis, cancer progression, and metastasis [28]. Isolated EVs have been described as potential prognostic and diagnostic tools in prostatic cancer [29, 30], ovarian cancer [31], lung adenocarcinoma [32], colon cancer [33], bladder cancer [11], breast cancer [34], and melanoma [35]. Moreover, EVs released from the brain were involved in Alzheimer's disease mechanisms [36].

EVs are important in the host-pathogen interaction, being secreted both from the host and the pathogen. For example, parasites can deliver EVs that modulate the host's immune response. Moreover, macrophages infected by *Toxoplasma gondii* release EVs, which are pro-inflammatory [37]. *Leishmania*-delivered exosomes are involved in the modulation of macrophage/dendritic cells' response to infection.

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Additionally, parasites can use EVs to evade host immunity. For example, *Trypanosoma cruzi* uses host microvesicles to evade host immunity [38–41]. Bacteria are also able to produce and release EVs, which are essential in the pathogenicity, cell-cell signaling between intra- and inter-species, and different organisms via transference of the EV content. In Gram-positive bacteria, the EVs are spherical bilayers with sizes between 20 and 400 nm [42, 43]. In Gram-negative bacteria, particles are described as outer membrane vesicles (OMVs) and display several roles, such as antibiotic resistance, nucleic acid transference, biofilm synthesis, and host-bacteria interaction [44, 45]. Specifically, during infection, host cells can incorporate bacterial OMVs by endocytosis via different mechanisms: clathrin-dependent, caveolin-mediated, lipid raft, and membrane fusion [44]. Some inhibitors can be used to block OMV endocytosis, such as chlorpromazine (blocks clathrin-coated pit formation), dynasore (inhibits dynamin GTP activity in the caveolin-mediated endocytosis), wortmannin (inhibits the phosphatidylinositol kinases in the lipid raft endocytosis), and cytochalasin D (depolymerizes actin in the membrane fusion) [44]. Fungi EV's content can activate the host immune system once recognized by pattern recognition receptors expressed by leukocytes [46]. Among the fungal EV cargo, described as virulence factors are lipids (phospholipids, sterols, glycolipids) [47, 48], proteins [47], polysaccharides [49], nucleic acids [50], and pigments such as melanin [51]. Among the proteins identified in the EV cargo, urease and phosphatase were described in EV-released *C. neoformans*. Urease is characterized in *C. neoformans* as a relevant virulence factor, involved in the host central nervous system invasion [52]. Fungi EVs are involved in adhesion in the brain microvasculature in *C. neoformans* [53] and stimulation of cytokine production by macrophage (IL-10, IL-12, TGF-β) and dendritic cells (IL-10, IL-12, TNF-α) in *C. albicans* [54]. EVs are also important in viral infections. Infected cells produce EVs that mediate the communication between infected and uninfected cells, supporting the virus survival and manipulating the host's immune response [55].

The EVs' molecular repertoire defines their molecular and biological functions. Several reviews have investigated the analytical and computational methods to characterize EVs' transcriptome, proteome, and metabolome in different pathophysiological conditions. Using mass spectrometry-based proteomics, in addition to the isolation and characterization of the proteins, it is possible to identify and characterize the EV proteins. Lischnig and colleagues [56] isolated large and small EVs using differential ultracentrifugation combined with density gradient centrifugation. In this study, more than 6000 proteins were identified in EVs isolated from breast cancer cell lines (MDA-MB-231-luc-D3H1, MDA-MB-231-luc-D3H2LN, and MDA-MB-231-luc-BMD2a), where 818 and 1567 proteins were enriched in small EVs and large EVs, respectively, isolated from all three cell lines. Most of the 1567 enriched proteins in large EVs were related to organelles such as mitochondrion, endoplasmic reticulum, and ribosomes, while the enriched proteins in small EVs were associated with multivesicular bodies, plasma membrane, and Golgi apparatus [56]. These results highlighted that the differences among the EV types are also at the protein content level. The EV proteome alterations are also observed during physical activities [57], where differences in

the EV proteins are released in the plasma over time points. The results showed regulation of 322 proteins among exercise and rest time points, mostly involved with multivesicular body formation, membrane trafficking, lipid rafts, vesicle adhesion, and chaperone [57]. EV proteome from *B. anthracis* [58], *S. aureus* [42], *L. monocytogenes* [59], *P. acnes* [60], and *F. alocis* [61] were analyzed using mass spectrometry-based proteomics to characterize the proteins involved in the EV release. The presence of penicillin-binding proteins (PBPs) and autolysins (transpeptidases related to cell wall homeostasis) as EV cargo suggests that the modification of the cell wall plays a role in the vesicle release. As in eukaryotes, bacterial EVs contain not only nucleic acids but also cytosolic and secreted proteins [44]. Metabolomics is described as other omics science potentially recommended to elucidate the influence of cell metabolism in physical changes [62]. Metabolomics analyses were performed in urinary EVs from prostate cancer patients (PCa) and benign prostate hyperplasia (BPH). A total of 248 metabolites were identified, including amino acids and lipids. In this comparison, 76 differentially regulated metabolites were identified between PCa and BPH [63]. The regulated metabolites were mainly phosphatidylcholines (higher in BPH), sterols (higher in PCa), and fatty acid esters (higher in PCa). Moreover, vitamins were identified as increased and glycerolipids decreased in PCa-released EVs. Interestingly, metabolite regulation changed according to PCa stages 2 and 3. In this context, three ceramides Cer(d18:1/16:0), Cer(d18:1/20:0), and Cer (d18:1/22:0), one glycerophospholipid (30:0), and stearoylcarnitine (18:0) showed different levels between stages 2 and 3. All these findings support the urinary-released EVs as a non-invasive source for metabolite analysis in PCa [63]. Additionally, RNA, another EV cargo, has gained the interest of the study as a potential biomarker. Transcriptomic profiling of plasma from multiple sclerosis patients showed a differential number and cargo of EVs. Using techniques such as differential ultracentrifugation and RNA-sequencing, EVs were isolated from 20 patients and 8 controls to identify differential expression of RNA, specifically linear and circRNAs [64]. The main finding was the identification of 6575 circRNAs in multiple sclerosis compared with healthy controls. Moreover, the study reported differences in the number of circRNAs and linear RNAs in the comparisons between multiple sclerosis and healthy controls or between different stages of the disease: relapsing-remitting vs secondary progressive multiple sclerosis (MS), highlighting the importance of further studies to analyze the function of RNAs in the progression of MS [64]. Transcriptomics analysis of EVs was also applied to infectious diseases. Meningher et al. [65] analyzed 26 schistosomiasis-positive patients compared to 17 healthy controls and detected *Schistosoma* microRNAs in EVs isolated from host sera. All four schistosomal RNAs (bantam, miR-2c-3p, miR-3488, and miR-2a-5p) showed significant differences between the EVs isolated from infected and healthy individuals [65], suggesting the EVs as potential sources of diagnosis in schistosomiasis infection.

1.1.2 Omics Science Approaches for Extracellular Vesicle Characterization

Innovative methods for EV proteome characterization have been developed and applied in healthy and diseased conditions [66]. The EVs' proteome of human breast cancer subtype (triple-negative and HER2-positive) cell lines was characterized using ultracentrifugation with sequential centrifugation steps to remove cell debris and large EVs (10,000× *g*, 40 min), followed by two ultracentrifugation (UC) cycles at 120,000× *g* for 2 h each. This study identified a total of 4992 proteins and 2299 differently regulated proteins (FDR 5%) using a mass spectrometry (MS) approach. Most of the proteins identified in this study were reported in the EV databases ExoCarta and Vesiclepedia (>90%), and their biological processes and cellular component are related to cell-cell communication and EVs, respectively [67]. Mass spectrometry-based proteomics was applied to identify disease patterns and potential biomarkers for breast cancer by analyzing EV proteome. For this, Risha et al. (2020) [68] isolated EVs from a metastatic cancer cell line (MDA-MB-231) and a human breast epithelial cell line (MCF-10A) using three methods for isolation: ExoQuick, ultracentrifugation, and the combined ultrafiltration-ultracentrifugation. Among the three isolation methods, ExoQuick showed five proteins, while UC and UF-UC presented 986 and 503 proteins, respectively. Using the UC method, the total proteome of MDA-MB-231 and MCF-10A identified 1107 and 726 proteins, respectively. The gene ontology displayed extracellular exosomes as enriched cellular components. Moreover, 36 kinases were reported among the MDA-MB-231 exosomal proteins, and 3 exosomal membrane proteins (GLUT-1, Glypican-1, and ADAM-10) were hypothesized as a biomarker for breast cancer prognosis [68].

In addition to EV proteome analysis in several types of diseases, post-translational modifications (PTMs) in EVs are also widely studied, focusing on the alteration of PTMs in cancers, pregnancy, and infection [69–71]. EVs can be isolated from several types of sources, such as cell culture and biofluids (bile, blood, urine, breast milk, semen, cerebrospinal fluid, saliva, and amniotic fluid) [72–76]. Due to this, there are many methods for EV isolation: UC, size exclusion chromatography (SEC), UF, density gradient, immunoaffinity, and microfluidics. Each method displays differences in specificity, EV recovery, and purity. Each method requires a minimum sample input volume and shows different time and cost-associated procedures [77]. In some studies, the combination of more than one method is used to enrich the EVs. For example, ultrafiltration (UF) combined with size exclusion chromatography (SEC) enhances the EVs' isolation from cell culture media [78]. The EV isolation techniques and their technical challenges were reviewed by Ramirez et al. [79] and Stam et al. [80]. The gradient UC is another technique to separate aggregates from EVs using a sucrose gradient, where different particles float in different densities (e.g., exosomes floatation 1.08–1.22 g/mL on a sucrose

gradient) [81, 82]. Moreover, the combination of differential ultracentrifugation followed by gradient UC (sucrose) was applied by Alexander et al. [83] to separate the contaminants. Among the isolation methods currently available, UC is the gold standard method [84]. In this technique, the isolation is based on the size of the particle. Larger vesicles are isolated by centrifugal force between 200 and 1000× *g*, while microvesicles and the exosomes form pellets by a centrifugal force of 10,000–20,000× *g* and 100,000× *g*, respectively [85–87]. The main disadvantage of UC is related to the low recovery of the EVs and time consumption, once the UC steps require 1–16 h of equipment use [88, 89]. Recently, Gao et al. [90] presented a novel isolation method for extracellular vesicles with high specificity using metal oxide titanium dioxide (TiO_2) microparticles. In EV isolation, the interaction occurs based on the reversible binding of TiO_2 to the acidic phosphate groups present in the phospholipids in the EV bilayer membrane [90]. TiO_2 microspheres are used to isolate phosphopeptides [91] and sialylated glycopeptides [92]. During the enrichment of these biomolecules, the pH of the loading solution is kept acidic to increase selectivity toward acidic residue-containing peptides. For the EV isolation, the pH of the loading solution is kept neutral [92]. This methodology allows EV isolation from both cell culture supernatants as also biofluids such as plasma, serum, and urine [93, 94]. Moreover, the TiO_2 method showed a similar size profile of EVs compared to UC and smaller EVs compared to SEC fractions. Additionally, the time consumption is reduced in hours compared to UC and SEC methods [93]. The application of TiO_2 was also efficient to analyze EVs isolated from the plasma of diabetic retinopathy (DR) patients to perform metabolomics analysis. The analysis compared diabetes mellitus (DM) and DR patients, stratified into two groups: non-proliferative DR (NPDR, early stage) and proliferative DR (PDR, advanced stage). The results showed a total of 344 metabolites identified using mass spectrometry-based proteomics. Several types of metabolites as amino acids, benzene, and aldehydes were identified. Regulation of metabolites was observed in the comparison between NPDR and DM, where uracil, Ile-Phe, 4-acetamidobutyric acid, and Met-Hyp were upregulated, while lysophosphatidylcholine, sphingomyelins, and free fatty acid were downregulated in NPDR compared to DM. Taken together, these results highlight the relevant functions of EV metabolism during DR and its progression [93]. Considering the EV isolation from complex samples, such as plasma/serum, the TiO_2 enrichment method is described as a simple, rapid, low-cost, and low-volume sample required. Moreover, the technique shows a reduced plasma protein contamination (albumin, IgG) compared to the UC method, due to the high specificity to isolate the EVs. During the EV isolation, the co-purification of the lipoproteins (ApoE, ApoB, ApoC-II, etc.) is also observed due to the similarities between these EVs and lipoproteins, such as the size and density, being a major challenge of the current techniques during the EV isolation from plasma samples [95–98]. Here, we present an adapted method for EV enrichment using TiO_2 microspheres with low plasma volume and a step-by-step protocol, focusing on MALDI and LC-MS/MS proteomics approaches. An overview of the EV enrichment by the TiO_2 method is represented in Fig. 1.1.

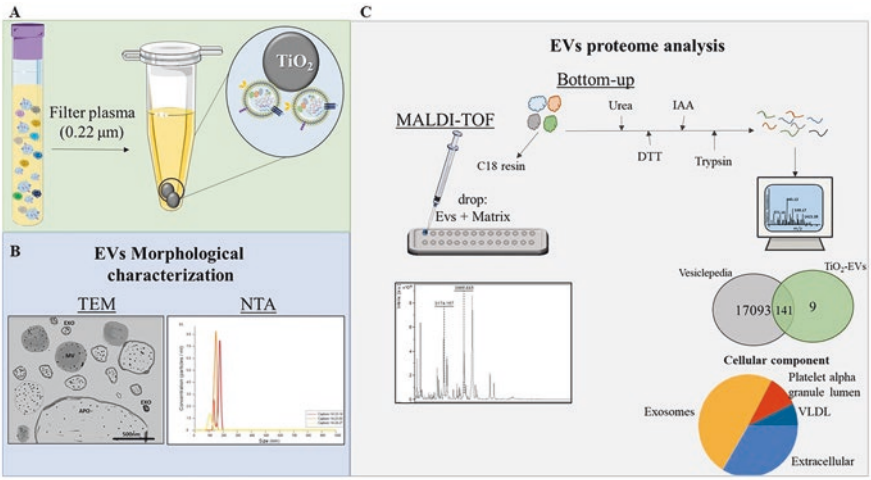

Fig. 1.1 Schematic representation of EV isolation method using TiO_2 microspheres. (**a**) 100 µl of plasma was filtered using a 0.22-µm syringe filter with GHP Membrane (PALL Life Sciences, USA) directly into the 5 mg TiO_2 bead microtube, incubating for 5 minutes at 4 °C, max speed at thermomixer. Three washing steps with filtered PBS are required to remove the non-EV molecules. The elution of EVs is performed with 10% ammonium hydroxide, pH 11.3. (**b**) The morphological characterization of EVs can be performed using transmission electron microscopy (TEM) and nanotracking analysis (NTA). Moreover, the EV proteome analysis is performed by mass spectrometry-based proteomics, MALDI-TOF, and LC-MS/MS

1.2 Material and Methods

1.2.1 Plasma Samples

Peripheral blood was collected at delivery into vacuum tubes containing heparin. Plasma was isolated by centrifugation at 1200× *g* for 10 min at 4 °C and maintained at −80 °C until use. Human plasma samples from healthy controls were supported by ethical clearance provided by the Ethic Committee in Research Involving Human Beings from the Biomedical Sciences Institute of the São Paulo University under the Certificate of Presentation to the Ethic Appreciation (CAAE) numbers 03930812.8.0000.5467 and 32707720.0.0000.5467, according to the Resolution No. 466/12 from the Brazilian National Health Committee.

1.2.2 Enrichment of Plasma EVs Using TiO_2 Microspheres

Add 5 mg of TiO_2 beads (Titansphere TiO, 10 µm, GL Sciences) into the low binding microtube. Thaw plasma samples on ice. Then, mix them by vortexing followed by spinning down before filtering 100 µl of plasma using a 0.2-µm syringe filter

with GHP Membrane (PALL Life Sciences, USA) directly into the TiO_2 bead microtube. Plasma was mixed with TiO_2 beads by vortexing roughly for 40 s and incubated in a thermomixer system at 2000 RPM for 5 min at 4 °C. The beads were spun down for 1 min using a bench centrifuge, and remove the supernatant, once the EVs are adsorbed by TiO_2 beads. TiO_2 beads were washed three times briefly with 200 µl of filtered phosphate-buffered saline (PBS) solution, mixed by vortexing, and incubated in a thermomixer system at 2000 RPM for 5 min at 4 °C. The beads were spun down to remove the maximum volume of PBS solution before the EV elution step to avoid alterations in the elution buffer's final concentration. The EV elution was performed by adding 50 µl of fresh elution buffer, which consists of 10% ammonium hydroxide (pH 11.3), and incubation in a thermomixer system at 2000 RPM for 15 min at 4 °C. Then, centrifuge for 10,000× *g* for 10 min at 4 °C to pellet the TiO_2 beads, and collect the EVs in the supernatant. The following steps can vary according to the next analysis (MALDI-TOF MS, LC-MS/MS, etc.).

1.2.3 EV Morphological Characterization by Transmission Electron Microscopy and Nanoparticle Tracking Analysis (NTA)

Below are reported detailed procedures for the morphological and molecular characterization of the isolated EVs. The procedures can be adapted based on the equipment and techniques used.

The 10% ammonium water needs to be exchanged for a proper solution preparing the Amicon® Ultra Centrifugation Filters 30 kDa cut-off (Merck Millipore, Billerica, MA, USA) by adding 200 µl of filtered PBS, and centrifuge 10,000× *g* for 1 min at 4 °C. Then, the eluted EV sample was added to the Amicon filter, and ammonium hydroxide was diluted 10x by adding PBS to complete the volume. The ammonium hydroxide was removed by centrifugation at 10,000× *g* for 5 min at 4 °C. The recovery of EVs was performed by inverting the Amicon filter into a new tube, and centrifuge 10,000× *g* for 5 min at 4 °C. The recovered EVs were placed into a new LoBind tube, and store the samples at 4 °C until further analysis.

1.2.3.1 For NTA Analysis

The EV samples were ten times diluted by filtered sterile PBS and injected in the sample chamber with sterile insulin syringes (BD, New Jersey, USA) until the liquid reached the tip of the nozzle. All measurements are performed at room temperature, with samples being kept on ice between analyses. The software used for capturing and analyzing the data is NTA 3.4. For the NTA analysis, a 500-µL volume of the isolated EV sample was manually injected into the laser chamber of the NanoSight NS300 instrument. The injection was carefully carried out to prevent any

introduction of air bubbles or sample loss. Readings were taken in triplicate for each sample, with each reading lasting 30 s at a frame rate of 25 frames per second. This allowed for the tracking and measurement of EVs in real time, capturing their Brownian motion. NTA was set up to three measurements for each sample for 30 s per recording, with manual shutter and gain adjustments, and kept for all samples. A value between 2.0 and 3.0 for screen gain and 5.0 and 6.0 camera level was selected for the analysis and kept for the following samples. The supplied data is used to measure the number of particles, the average and median size of vesicles obtained, and mode, D10, D50, and D90, comparing the profiles between the groups.

1.2.3.2 For TEM Analysis

The EVs were fixed with 0.2% paraformaldehyde (w/v) for at least 18 h. Then, 10 μL of samples was incubated for 7 min in a 300-mesh grid with carbon-coated Formvar film ((Electron Microscopy Sciences, Hatfield, USA). The grid was incubated on 10 μL of 1.75% uranyl acetate (w/v) for 7 min. The samples were dried at room temperature for 2 h and imaged with TEM at 100 kV (CM-10, Philips, Eindhoven, the Netherlands).

1.2.4 EV Plasma Proteome Identification Using MALDI-TOF MS and LC-MS/MS

1.2.4.1 For the MALDI-TOF MS Approach

- The recovered EVs were lysed by adding 50 μL of 50% acetonitrile in 1% trifluoroacetic acid's final concentration. Reserve 10% of the total volume for protein quantification (Qubit/Nanodrop), and dry down the lysed EV vacuum centrifuge.
- Resuspend the dried sample in 1% trifluoroacetic acid, and pass it through polymeric resin disks C18.
- Prepare the matrix solution (α-cyano-4-hydroxycinnamic acid [HCCA]) by dissolving in acetonitrile/water 50:50 vol/vol containing 2.5% trifluoroacetic acid at 10 mg/ml, mixed with the sample, directly spotted in duplicate onto a stainless steel MALDI target plate (Bruker Daltonics), and analyzed in a MALDI-TOF Autoflex speed smart beam mass spectrometer (Bruker Daltonics) using FlexControl software (version 3.3.108.0; Bruker Daltonics).
- MALDI-TOF Autoflex Setup: Spectra recording in the positive linear mode (laser frequency, 500 Hz; extraction delay time, 390 ns; ion source 1 voltage, 19.5 kV; ion source 2 voltage, 18.4 kV; lens voltage, 8.5 kV; mass range, 2400–20,000 Da). Spectra acquisition: automatic run mode to avoid subjective interference with the data acquisition. For each sample, 5000 shots, in 500-shot steps, were summed. All spectra calibration: using Protein Calibration Standard

I (Insulin [M + H]+ = 5734.52, Cytochrome C [M+ 2H]2+ = 6181.05, Myoglobin [M+ 2H]2+ = 8476.66, Ubiquitin I [M + H]+ = 8565.76, Cytochrome C [M + H]+ = 12,360.97, Myoglobin [M + H]+ = 16,952.31) (Bruker Daltonics). EV spectra are log-transformed using the square root method, followed by spectra smoothing using the Savitzky-Golay method and a half window size of 10. A base correction is required using the TopHat method. The intensity should be normalized using the total ion current method and peak identification with a half-window size of 10 and signal-to-noise ratios of 5 and 10. Raw data were imported to MALDIquantForeign [99] package and processed using the MALDIquant package [99] for TiO_2-enriched analyses.

1.2.4.2 For LC-MS/MS Analysis

- Before adding the EVs into the 30 kDa Amicon filter, wash the membrane with 200 µl of filtered PBS, and centrifuge 10,000× *g* for 1 min at 4 °C.
- Add the eluted EV sample to a 30 kDa Amicon filter, and complete the volume with PBS to dilute ten times the ammonium hydroxide. Centrifuge 10,000× *g* for 5 min at 4 °C.
- Recover the concentrated sample by inverting the Amicon filter into a new tube, and centrifuge 10,000× *g* for 5 min at 4 °C.
- Collect the recovered EVs into a new LoBind tube, and add 4 M urea with a cocktail protease inhibitor for sonication for 1 h in the ice bath to lysis the EV membrane.
- Protein quantification was performed before adding 10 mM dithiothreitol (DTT) final concentration in 50 mM ammonium bicarbonate to the samples and incubating at 30 °C for 45 min.
- Add 40 mM iodoacetamide in 50 mM ammonium bicarbonate (final concentration), incubate for 30 min at room temperature, and be protected from the light.
- To quench the iodoacetamide alkylation, add 5 mM DTT final concentration, and incubate for 15 min at 30 °C.
- Before adding the trypsin (1:50, w/w), dilute ten times the urea with 50 mM ammonium bicarbonate, and confirm that the pH is between 7 and 8.
- Add trypsin and incubate for 16 h, at 30 °C, and 400 RPM on a thermoshaker. After this time, add 1% trifluoroacetic acid final concentration to stop the trypsin reaction. Check the pH (should be below 3).
- Centrifuge the samples for 10 min at 10.000× *g*, recover the supernatant, and proceed with the desalting protocol.
- For desalting protocol, insert two disks C18 microcolumn placed to a P200 pipette tip.
- Add 100 µl of activation solution (acetonitrile (PA) in the C18 resin, followed by 100 µl of 0.1% trifluoroacetic acid (TFA).
- Load peptides in the C18 column, and wash them three times with 0.1% TFA.

- Elute the peptides with 50% acetonitrile/0.1% trifluoroacetic acid followed by 70% acetonitrile/0.1% trifluoroacetic acid.
- Dry the samples in a vacuum concentrator, and store them in a −20 °C freezer until LC-MS/MS analysis.

1.2.5 Bioinformatics Analysis of Mass Spectrometry-Based Proteomics (LC-MS/MS)

- Resuspend the samples in 0.1% formic acid, and load on ReproSil-Pur C18 AQ (Dr. Maisch, Ammerbuch-Entringen, Germany) in-house packed trap column (2 cm × 100 μm inner diameter 5 μm) by reverse-phase chromatography, operated on an EASY-nLC II system (Thermo Scientific) and flow 300 nL/minute. Mobile phase water/0.1% FA as solvent A and solvent B (95% ACN/0.1% FA). Gradient 5–28% of solvent B for 25 min, 28–40% of B for 3 min, and 40–95% of B for 2 min. The EASY-nLC II is coupled into an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific) and operates in positive mode ion. The mass spectrometer acquired a full MS scan at 120,000 full-width half maximum (FWHM) resolution with a 350–1550 Da mass range. The top 20 most intense peptide ions should be selected from MS for high energy collision-induced dissociation (HCD) fragmentation (collision energy: 30.0 V).
- The MS output files are imported to protein identification into MaxQuant software [100]. In MaxQuant, for protein identification, the database search engine Andromeda was used against UniProt *Homo sapiens* (20,361 entries released). The following parameters can be used: carbamidomethylation of cysteine (57.021464 Da) as a fixed modification, oxidation of methionine (15.994915 Da), and N-terminal acetylation protein (42.010565 Da) as variable modifications. Full trypsin with a maximum of two missed cleavages as enzyme specificity. The minimum peptide length can be set to seven amino acids. For label-free quantification, use the "match between runs" feature in MaxQuant, which can identify the transfer between the samples based on the retention time and accurate mass, with a 0.7-min match time window and 20-min alignment time window.

1.3 Results of the EV Morphological Characterization and Proteome Identification

The results shown below were obtained from a plasma sample. The size distribution of the isolated EVs was obtained by NTA analysis. This technique is based on the Brownian motion using Stokes-Einstein equation to determine the nanoparticle size and concentration [101]. TiO_2-enriched EVs showed a size means and mode size of

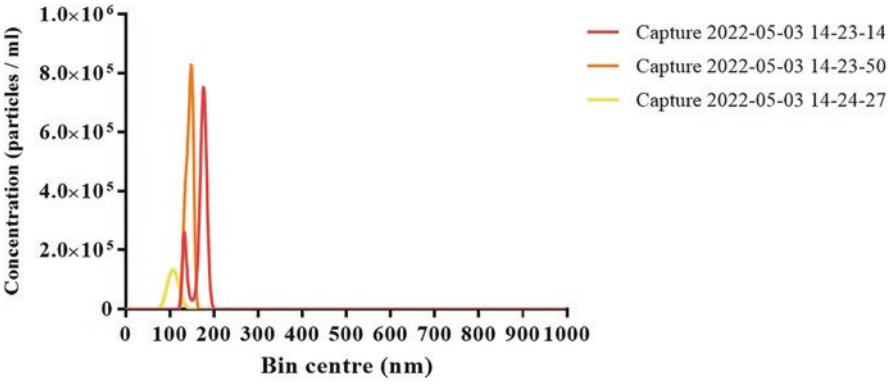

Fig. 1.2 TiO_2-enriched EV characterization based on the size and concentrations measured by NTA. 500 µL from the isolated EV sample was manually injected into the laser chamber. Triplicate readings were taken for each sample, with each reading lasting 30 s at a frame rate of 25 frames per second. This allowed for real-time tracking and measurement of EVs by capturing their Brownian motion

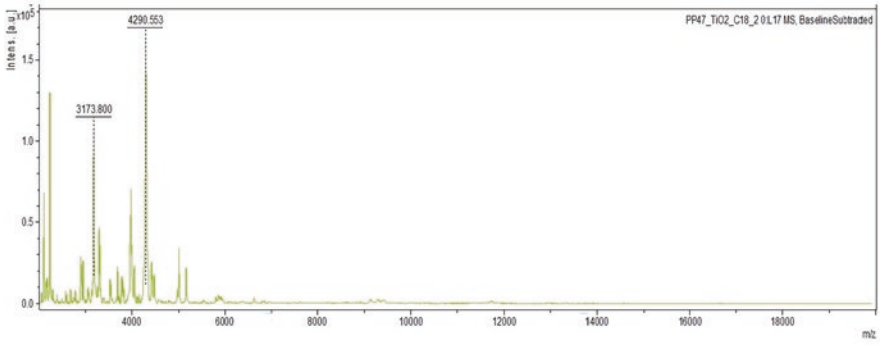

Fig. 1.3 MALDI-TOF MS of the TiO_2-enriched EVs

140.2 nm (±17.5 nm) and 144.4 nm (±20.2 nm), respectively. Moreover, the EV concentration obtained was 1.28e+07 particles/ml (Fig. 1.2).

EV proteome identification was performed using MALDI-TOF MS and LC-MS/MS proteomics approaches. Figure 1.3 shows the TiO_2-enriched EVs' MALDI spectra. EV content showed more peaks established in the region 2000–6000 m/z with the most intense peaks localized around 2000, 3000, and 4000 m/z regions.

Complementary to MALDI-TOF MS proteomics, bottom-up proteomics using LC-MS/MS was performed to identify the proteins in the TiO_2-enriched EVs. A total of 172 proteins were identified in TiO_2-enriched EVs (Supplementary Table 1.1). Based on the gene name, a comparison between the identified proteins and the Vesiclepedia database was performed, showing an overlap of 90.7% of the EV proteins reported in the Vesiclepedia (Fig. 1.4a, Table 1.1). Among the reported EV proteins in Vesiclepedia are Protein S100-A8, Protein S100-A9, Transgelin-2,

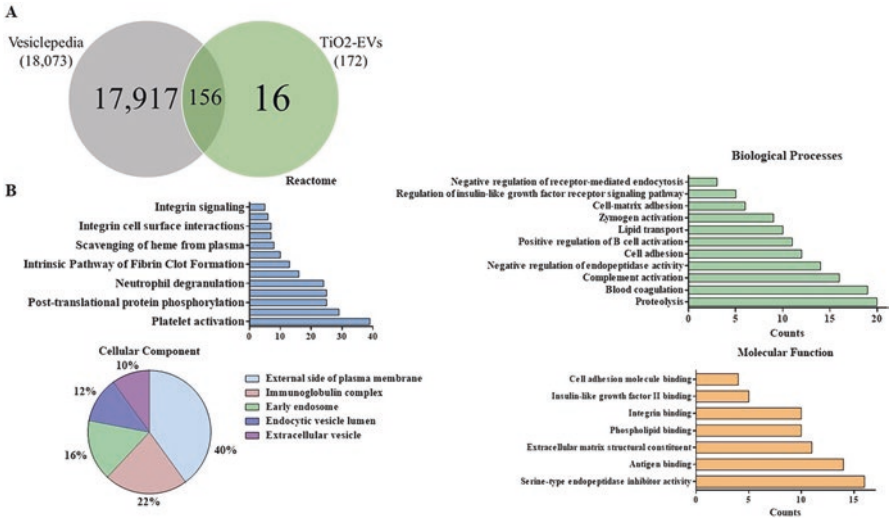

Fig. 1.4 (**a**) Venn diagram of identified proteins in TiO_2-enriched EVs reported in Vesiclepedia. (**b**) Gene ontology of the EV proteins reported in Vesiclepedia

Caspase-7, Talin-1, disintegrin and metalloproteinase domain-containing protein 12 (ADAM-12), destrin, tetranectin, Ras suppressor protein 1, Coronin-1C, transmembrane protease serine 6, and Cystatin-C.

Regarding the gene ontology, the TiO_2-EV proteins reported in Vesiclepedia showed Reactome pathways related to platelet activation, regulation of insulin-like growth factor and uptake by IGFBPs, regulation of complement cascade, and neutrophil degranulation. The biological processes associated with the EV proteins were proteolysis, blood coagulation, complement activation, negative regulation of endopeptidase activity, and cell adhesion. Additionally, molecular functions of EV protein were serine-type endopeptidase inhibitor activity, antigen binding, extracellular matrix structural constituent, phospholipid binding, and integrin binding. Regarding the cellular components, the external side of the plasma membrane, early endosome, extracellular vesicle, and endocytic vesicle lumen were the most enriched of the EV proteins (Fig. 1.4b).

1.4 Conclusion

The application of the TiO_2 beads for EV enrichment of complex samples, such as plasma, is the most rapid and effective approach using a low volume of samples compared to the UC methods. Short time consumption enables the preparation of a high number of samples, allowing the application in the medical diagnosis. In this method, TiO_2-enriched EVs showed a high concentration (1.28e+07 particles/ml) in NTA analysis, where the particle size was between 140 and 144 nm. Moreover, the

Table 1.1 EV proteins reported in Vesiclepedia

Albumin	Complement C1q subcomponent subunit C	Ceruloplasmin	Hyaluronan-binding protein 2	Inter-alpha-trypsin inhibitor heavy chain H2	Ribonuclease pancreatic
Alpha-1B-glycoprotein	Complement C1r subcomponent	Carboxypeptidase N catalytic chain	Hemoglobin subunit alpha	Inter-alpha-trypsin inhibitor heavy chain H4	Ras suppressor protein 1
Alpha-2-macroglobulin	Complement C1s subcomponent	C-reactive protein	Haptoglobin	Intelectin-1	Protein S100-A8
Actin, cytoplasmic 2	Complement C3	Cysteine and glycine-rich protein 1	Haptoglobin-related protein	Plasma kallikrein	Protein S100-A9
Disintegrin and metalloproteinase domain-containing protein 12	C4b-binding protein alpha chain	Cystatin-C	Hemopexin	Kininogen-1	Serum amyloid A-4 protein
Afamin	C4b-binding protein beta chain	Neutrophil defensin 3	Histidine-rich glycoprotein	Apolipoprotein(a)	Alpha-1-antitrypsin
Angiotensinogen	Complement component C6	Destrin	Insulin-like growth factor II	Leucine-rich repeat and fibronectin type III domain-containing protein 1	Alpha-1-antichymotrypsin
Alpha-2-HS-glycoprotein	Complement component C8 alpha chain	Coagulation factor X	Insulin-like growth factor-binding protein 1	Lysozyme C	Antithrombin-III
Protein AMBP	Complement component C8 beta chain	Coagulation factor XI	Insulin-like growth factor-binding protein 2	Microtubule-associated protein RP/EB family member 2	Heparin cofactor 2
Serum amyloid P-component	Complement component C8 gamma chain	Coagulation factor XII	Insulin-like growth factor-binding protein 3	Alpha-1-acid glycoprotein 1	Pigment epithelium-derived factor

Apolipoprotein A-I	Complement component C9	Prothrombin	Insulin-like growth factor-binding protein 4	Alpha-1-acid glycoprotein 2	Alpha-2-antiplasmin
Apolipoprotein A-II	Adenylyl cyclase-associated protein 1	Coagulation factor VII	Insulin-like growth factor-binding protein 5	Procollagen C-endopeptidase enhancer 1	Plasma protease C1 inhibitor
Apolipoprotein A-IV	Caspase-7	Coagulation factor IX	Immunoglobulin heavy constant alpha 1	Protein disulfide-isomerase A3	Sex hormone-binding globulin
Apolipoprotein B-100	CD5 antigen-like	Fibulin-1	Immunoglobulin heavy constant alpha 2	Plasminogen	Antileukoproteinase
Apolipoprotein C-I	Complement factor B	Ficolin-1	Immunoglobulin heavy constant gamma 1	Serum paraoxonase/ arylesterase 1	Secreted phosphoprotein 24
Apolipoprotein C-II	Complement factor D	Fermitin family homolog 3	Immunoglobulin heavy constant gamma 2	Platelet basic protein	TATA box-binding protein-associated factor RNA polymerase I subunit A
Apolipoprotein C-III	Complement factor H	Fetuin-B	Immunoglobulin heavy constant gamma 3	Peptidyl-prolyl cis-trans isomerase A	Transgelin-2
Apolipoprotein D	Complement factor H-related protein 1	Fibrinogen alpha chain	Immunoglobulin heavy constant gamma 4	Peptidyl-prolyl cis-trans isomerase B	Serotransferrin

(continued)

Table 1.1 (continued)

Albumin	Complement C1q subcomponent subunit C	Ceruloplasmin	Hyaluronan-binding protein 2	Inter-alpha-trypsin inhibitor heavy chain H2	Ribonuclease pancreatic
Apolipoprotein E	Complement factor H-related protein 2	Fibrinogen beta chain	Immunoglobulin heavy constant mu	Proteoglycan 4	Transforming growth factor-beta-induced protein ig-h3
Beta-2-glycoprotein 1	Complement factor H-related protein 5	Fibrinogen gamma chain	Immunoglobulin heavy variable 3–7	Vitamin K-dependent protein C	Thrombospondin-1
Apolipoprotein L1	Cofilin-1	Fibroleukin	Immunoglobulin kappa constant	Vitamin K-dependent protein S	Thrombospondin-4
Zinc-alpha-2-glycoprotein	Properdin	Four and a half LIM domains protein 1	Immunoglobulin kappa variable 1–5	Putative pregnancy-specific beta-1-glycoprotein 7	Intelectin-1
Beta-2-microglobulin	Tetranectin	Filamin-A	Immunoglobulin kappa variable 3–20	Prostaglandin-H2 D-isomerase	Transmembrane protease serine 6
Bridging integrator 2	Clusterin	Fibronectin	Immunoglobulin lambda constant 1	Pregnancy zone protein	Thymosin beta-4
Complement C1q subcomponent subunit A	Collagen alpha-1(XVIII) chain	Vitamin D-binding protein	Immunoglobulin lambda constant 3	Retinoic acid receptor responder protein 2	Transthyretin

high specificity of the interaction between EVs and TiO_2 beads allowed the identification of EV proteins by LC-MS/MS, which their ions are usually suppressed by the most abundant plasma proteins (IgGs, albumin, etc.) and lipoproteins, chylomicrons, VLDL, LDL, and HDL. In conclusion, the combination of the TiO_2 enrichment method applied for EV isolation and high-throughput mass spectrometry techniques allowed the EV characterization and proteome profiling from EVs of a complex and viscous biofluid in an easy and fast way.

Competing Interests The authors declare no competing interests.

Supplementary Material The supplementary material can be downloaded from 10.6084/m9.figshare.24194895.

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