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Original Article

Identification of Extracellular Vesicles Derived from Plasma Using MALDI-TOF MS: Influence of Operating Conditions

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Abstract

Introduction: Extracellular vesicles (EVs) are biomolecular messengers secreted by all types of cells. It passes messages through molecular cargoes such as proteins, nucleic acids, and lipids from the parent cell to the target cell. EVs derived from the plasma are used for minimally invasive biopsy and as a possible biomarker to monitor the progression and severity of the disease. In this study, the matrix-assisted laser desorption and ionization-time of flight mass spectrometry approach was used to characterize and study the protein fingerprint region of plasma-derived extracellular vesicles.

Materials and Methods: At first, 3 ml of blood sample was collected from five boys of 5-10 years in sodium citrate tubes, and the samples were centrifuged within an hour to extract plasma. The total exosome isolation (TEI) method was used to obtain high-yield plasma-derived EVs, and the EVs were stored at -80 °C for further analysis. The isolated intact EVs were mixed with an optimal concentration of sinapinic acid matrix (20 mg/ml) in a 1:1 ratio for fingerprint analysis using matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS). We found that the following operational conditions yielded good and high-resolution spectra: broad mass range (2000- 20000 m/z), detector gain (30x), and laser shot (50, 300, and 1000) with 100% laser intensity. The obtained MALDI TOF spectral peaks of plasma EVs (pEVs) matched the reported biomarkers.

Results: Based on the analyses, we improved the crucial experimental conditions and identified five distinct peaks at m/z = 3315, 6630, 9421, 8875, and 8917. C4A, C3, and apolipoproteins A-II, C-I, C-II, and C-III were identified by comparing MALDI-TOF MS data with existing reports.

Conclusions: MALDI-TOF MS-based EV analysis can support the development of protein biomarker screening tools for early diagnosis.

Keywords: Extracellular Vesicles, Matrix-Assisted Laser Desorption-Ionization Mass Spectrometry, Peptide Mapping, Characterization

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Introduction

All types of cells actively release extracellular vesicles (EVs), which are of various shapes, sizes, and biological abilities.[1,2] Depending upon where it is secreted, the lipid composition and the mechanism of EVs vary. EVs are linked to numerous biological processes necessary for intercellular chemical communication.[3] The distinctive qualities of EVs include molecular information delivery to reprogram the receiving cell,[4] evoking a minimal immunological response from the recipient cells.[5] EVs carry proteins, lipids, and nucleic acids from the parental cells as a part of their cargo to preserve the normal physiological condition. They are proven to display a patient's disease physiology. Numerous cancers, such as breast, pancreatic, and lung cancers, can be diagnosed using membrane epidermal growth factor receptors (EGFRs) as markers.[6-8] EVs from mesenchymal stem cells move through blood vessels to the site of tumor cells and recruit active cargoes to induce apoptosis. They are frequently present in biological fluids and have been identified as a source of biological markers and for liquid biopsy[9] for detecting, monitoring, and comprehending diseases such as prostate cancer,[10] lung cancer,[11,12] and neurodegenerative disorders.[13,14]

The most common techniques to characterize the EVs include TEM, SEM, DLS, zeta sizer, nanoparticle tracking analysis, western blotting, ELISA, flow cytometry, nanoLC-MS/MS, and sequencing,[15-21] In the last decade, untargeted strategy analyses have frequently used liquid chromatography -mass spectrometry. Researchers have discovered new biomarkers

and gained a deeper mechanistic understanding of the diseases.[22,23] These techniques can reveal EV characteristics and biological information, but they are complicated to use, require extensive setup, and have high equipment requirements, which restrict their potential for clinical use. Alpha 1-antitrypsin and histone H2B1K proteins identified in urine have been used for the diagnosis and prognosis of urothelial carcinoma.[24] Platelet factor 4 is a plasma-derived EV biomarker for liver diseases.[25] MALDI-TOF MS has gained increasing interest in the characterization of EVs derived from urine and plasma due to their high-throughput, low sample consumption, easy sample preparation, label-free direct analysis, easy operation, and low-cost per sample.[26,27] MALDI-TOF MS, a soft ionization technique, helps detect the EV constituents directly without lysing the samples.[11,28] The sturdy lipid layer of EVs is deformed and becomes soft due to acetonitrile and strong trifluoroacetic acid in the matrix.[29]

EV fingerprints result from reading electric signals into the mass spectrum of EV-associated ion peaks. With this method, extracellular vesicles can be rapidly and thoroughly analyzed. For the representative case, if a 384-spot MALDI target plate is used, it can measure 384 samples in 2 h. In a short period, we can analyze 384 samples parallelly and rapidly using minimal sample volume to study the fingerprint region of EVs.

This study describes the optimum parameters for obtaining MALDI-TOF data that could be used for clinical purposes. Although the previous literature used MALDI-TOF to analyze the exosome proteins, a comprehensive analysis of the operating conditions and their influence on the data output is not reported. In this study, we tested important operational parameters, such as the choice of matrix, pre-analytical variables, and the preparation of the sample. This study will be very useful for large-scale analysis of clinical samples and automation.

Materials and Methods

Study Subjects

Five healthy pediatric children from the age group of 5-10 participated in the study, which was approved by the ethics committee of Sri Ramachandra Memorial (SRM) medical college hospital and research center (1886/IEC/2020) and was carried out according to the guidelines of Helsinki.[30] Each participant signed informed consent to participate in the study. Peripheral whole venous blood was collected in commercially available vacutainer tubes containing sodium citrate (BD Vacutainer, BD, USA) in the morning and processed within an hour after sampling.

Extracellular Vesicle Isolation from Plasma Samples

We used the Total Exosome Isolation (TEI) kit (Invitrogen, USA) for exosome isolation. The protocol for isolation is described elsewhere.[31,32] The clarified plasma volumes ranging from 0.1 to 1 ml were processed with proteinase K to remove bulk plasma proteins other than extracellular vesicle proteins. The EV pellet was then resuspended in 1X PBS. The EV samples were stored at -80 °C for further downstream analysis.

Morphology of Plasma-derived Intact EVs by High-resolution Transmission Electron Microscopy (HR-TEM)

Plasma EVs were imaged using a Jeol TEM – 2100 plus (Tokyo, Japan). The imaging was performed with high-resolution TEM with a magnification scale of 20 nm to 1 micron at 220KV applied voltage. The samples were laid on a copper grid coated with carbon (200 mesh). Sample preparation was carried out without negative staining or fixation. The sample-coated grid was incubated in a closed container at 37 °C overnight.

DLS and Zeta Potential Analysis of Plasma EVs

Dynamic light scattering was used to measure the particle size, and zeta potential helped study the colloidal stability of plasma extracellular vesicles obtained from normal individuals. A litesizer 500 Nano Particle Analyzer (Anton-Paar, Graz, Austria) was used to evaluate the particle size of plasma EVs through dynamic light scattering. This analyzer can detect particle diameters ranging from 0.3 nm to 10 µm with a minimal concentration of 0.1 mg/ml. Plasma extracellular vesicle suspension was tested to ensure particle stability and uniform dispersion in the suspension. One µl of EV samples was diluted in 1 ml of 1XPBS (1:1000 ratio) and vortexed gently for particle size distribution analysis. The magnitudes were read three times each, and the average spectra were used for comparison.

The Selection of the Matrix

The choice of the matrix and its concentration are necessary to acquire good quality spectral results from plasma-derived extracellular vesicles in MALDI-TOF MS analysis. Commonly used matrices for EV characterization in MALDI-TOF MS are sinapinic acid (SA), 2,5 dihydroxybenzoic acid (DHB), and α-cyano-4-hydroxycinnamic acid (HCCA). Two concentrations of 5 and 20 mg/ml were tested.

MALDI-TOF MS Analysis for EV Protein Profiling

Equal volumes of EVs and the matrix were dropped on the MALDI target plate, and 1 µl of each sample was spotted in triplicate and measured. After that, the target plate was subjected to a Bruker UltrafleXtreme II MALDI-TOF MS/MS instrument with a facilitated nitrogen laser (337 nm) for measurement. Mass spectrometry fingerprints of plasma EVs were generated within few minutes under pulsed laser irradiation. The accelerating voltage for all experiments was 20 kV, and the measurement mode was positive linear. The following criteria were established: Ion source 1: 19.99 kV;

ion source 2: 18.78 kV; lens: 6 kV; mass range m/z 2000-20,000; positive linear mode; 100% laser intensity; 45% offset and 30% range; 2000.0 Hz laser frequency; 20 detector gain; suppress up to 450 Da;250 ns pulsed ion extraction: 300 laser shots through random walk movement for each spot. Protein mixtures (PSI and PSII) provided by Bruker were used to calibrate the instrument externally. The spectra were then loaded into the Flex analysis 3.4 programs (Bruker Daltonics, Bremen, Germany), where they were baseline subtracted and smoothened.

Results and Discussion

High-resolution Transmission Electron Microscopy

The total exosome isolation method helps to extract a good yield of extracellular vesicles than ultracentrifugation, which can be advantageous when working with minimal clinical samples. It is a simple method that requires minimal equipment making it easy to perform in a clinical setting whereas the PROSPR and ultracentrifugation are more complex and time-consuming procedures that can take several days to complete. TEI results in less contamination from non-EV proteins compared to the ultracentrifugation method. The EVs isolated using the TEI method are more compatible than other methods with downstream applications such as western blot, ELISA, and mass spectrometry.

HR-TEM images confirmed the morphology and size of plasma EVs, and the micrographs were processed in ImageJ analysis software to understand particle size distribution. The simple air-drying method for sample preparation yielded high-quality HR-TEM micrographs at 2000x magnification at 220 KV. The micrographs show evenly distributed circular vesicles, and the average size of plasma-derived EVs is ± 150 nm[33] (Figure 1a). ImageJ software further uses the micrographs by calibrating their scale, converting them to an 8-bit grayscale format and changing the threshold values for binarization and particle tagging, as shown in (Figure 1b). The number of EV particles in 1 µl was ˜ 10400 and 1 ml of a sample contains ˜ 10×10^6 counted manually.

Table 1. ImageJ Analysis of the Distribution of EV Particles Obtained Using the TEI Method

S.No	TEM grid mesh size	EV Isolation Method	Number of Particles in one square	Number of Particles in 1µl of the sample	Total number of EV-like particles in 1 ml of sample
1.	200	TEI	52	10400	10×10^6

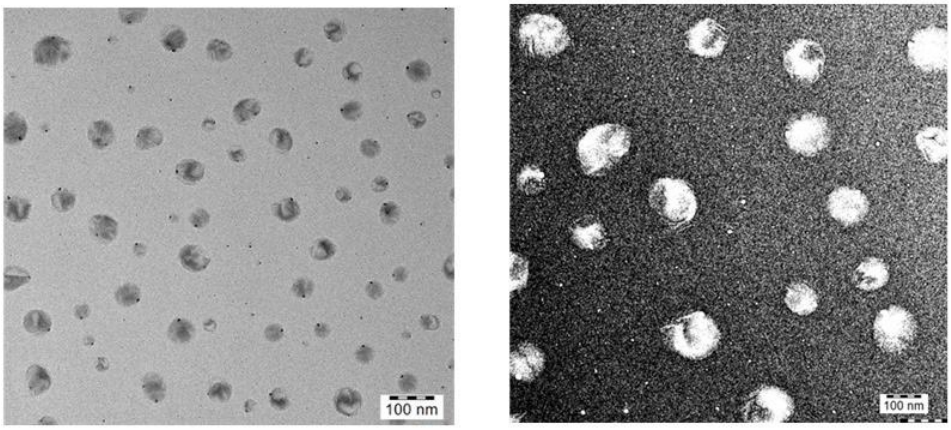

Figure 1. (a) High Resolution-Transmission electron micrographs with a resolution of 100 nm shows the morphology and size distribution of Plasma EVs obtained by TEI method; **(b)** Image J analysis of HRTEM micrographs to study the size distribution of plasma EV particles.

DLS and Zeta Potential Analysis

Dynamic light scattering analysis shows that the heterogeneous size of plasma EVs ranges from 100 nm to 350 nm in 1X PBS at pH 7.2 dispersion medium. The average plasma EV size is 150 nm, as shown in Figure 2a. The TEI method obtained EVs with excellent stability and widespread distribution. The physicochemical properties of EVs, such as particle size and surface charge, determine the EV's interaction with biological systems.[34]

Plasma EVs isolated by TEI had electrokinetic potential magnitude ranging from -125 mV to +76 mV, resulting in polydispersity and good stability of EV particles with significantly less agglomeration (Figure 2b).

Criteria Concerning the Analysis of EV Proteins by MALDI-TOF MS

Sample and Matrix Preparation

The pre-analytical variables that influence the extraction of plasma EVs are collection and handling of blood samples, repeated freeze-thaw cycles, sample quality, non-extracellular vesicle components, extraction, and storage conditions. Each variable was optimized and validated for extraction of EVs

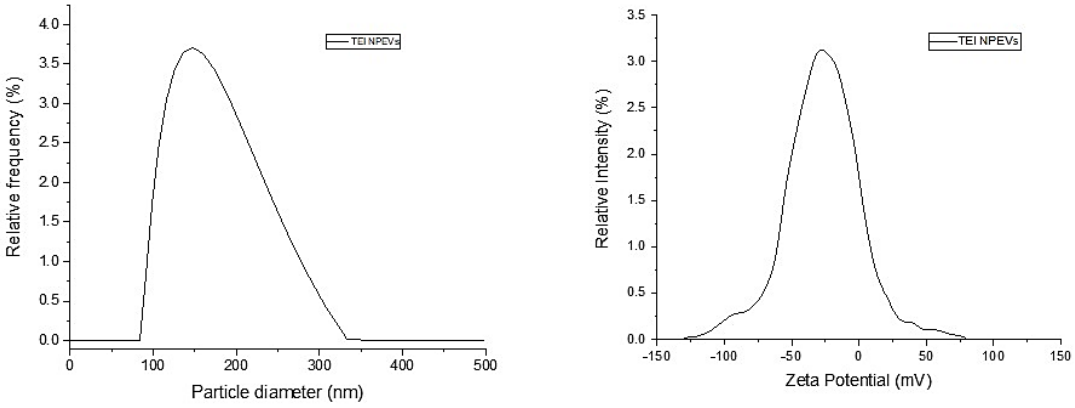

Figure 2. (a) Dynamic light scattering analysis shows that the heterogeneous size of plasma EVs ranges from 100 nm to 350 nm; **(b)** Zeta potential analysis represents the electrokinetic potential magnitude ranging from -125 mV to +76 mV, resulting in polydispersity and good stability of Plasma EVs.

from plasma.[35]

The high quality and yield of EV samples are the main requirements to sensitize MALDI-TOF MS analysis. The polymer-based precipitation method provides highly abundant and good-quality extracellular vesicles from plasma. As mentioned earlier, 1 µl of the sample containing ˜10,400 EV particles is required for MALDI-TOF MS investigation. For the analysis of EV samples, acetonitrile and water at equal ratio (50:50) were recommended as appropriate solvents, and they produced favorable MALDI results. 0.1% of TFA improves the ion signals from the EV sample. The matrices made in organic solvents are acetonitrile and trifluoroacetic acid for best EV protein profiling (5 mg/ml or 20 mg/ml of each matrix in 50% acetonitrile and 0.1% TFA in water).

Selection of the Suitable Matrix

One µl of the EV sample was spotted on the MALDI target plate, allowed to air dry at room temperature (a few minutes) and overlaid with one µl of the matrix, resulting in a consistent coating of tiny, granular matrix crystals for examination.[36] Three matrices SA, HCCA, and DHB at concentrations of 5 mg/ml and 20 mg/ml co-crystallized with plasma EV samples on a ground steel MTP 384 target plate (Bruker Daltonics, Bremen, Germany) were used for comparison of samples (Figure 3).

The EV fingerprint signature was amplified and repeated by covering the dried sample with matrices. Prolonged drying time, sample concentration, and diameter of matrices improved the signal quality by adding the total number of laser shots taken in a particular spot.

In contrast to DHB, which creates needle-shaped crystals or no crystals on the PEVs, SA and HCCA efficiently generate granule-like crystals with the PEV sample, as seen in the picture at 5 mg/ml and 20 mg/ml matrix concentrations. Recorded signals for HCCA and DHB both had low resolution and signal-to-noise ratios. Contrarily, SA produces significant results in granule-shaped crystal formation, coverage, and signal quality than other matrices, as shown in (Figure 3&4).

SA matrix solution at 20 mg/ml generates the most ion signals with the best resolution compared to a 5 mg/ml SA matrix solution[11,37] as shown in Figure 5. As a result, SA acts mainly as a matrix for plasma EV applications, and the higher concentration of the matrix generates high quality coverage[11,37] and mass spectrum.

MALDI-TOF MS Parameter Optimization for Plasma-derived Extracellular Vesicles

The ground steel MTP 384 target plate (Bruker Daltonics, Bremen, Germany) was loaded into the UltrafleXtreme II MALDI-TOF MS/MS instrument (Bruker Daltonics, Bremen, Germany) for MALDI-TOF MS measurement. We optimized the instrument parameters and set them as follows: broad mass range (2000- 20000 m/z), detector gain (30x), 100% laser intensity and laser shot (50, 300, and 1000), 45% offset, and 30% range; 2000.0 Hz laser frequency; 20 detector gain; suppress up to 450 Da; and 250 ns pulsed ion extraction; through random walk movement to analyze the EVs from biological fluids in positive linear mode with 20 kV accelerating voltage. Instrument parameters such as pulse delay, profiling mode, and accelerating voltage was optimized to achieve good resolution. Optimal settings for a broad mass range (2000-20000 m/z) require a lower accelerating voltage (18-20 KV) and a higher delay time (200-800 ns). The laser shots that aggregated a single spectrum varied between 30-750 when used in the EV profiling mode. The protonated ions flow linearly and are recorded by the detector.

MALDI-TOF MS Analysis of Intact Extracellular Vesicles Derived from Plasma

The MALDI-TOF mass spectrum of plasma-derived EVs is highly reliable, indicating stability acquired at the mass range of 2000-20,000 and the summing of 300 shots at a particular spot. The spectra were baseline subtracted and spectral smoothened in Flex analysis 3.4 software (Bruker

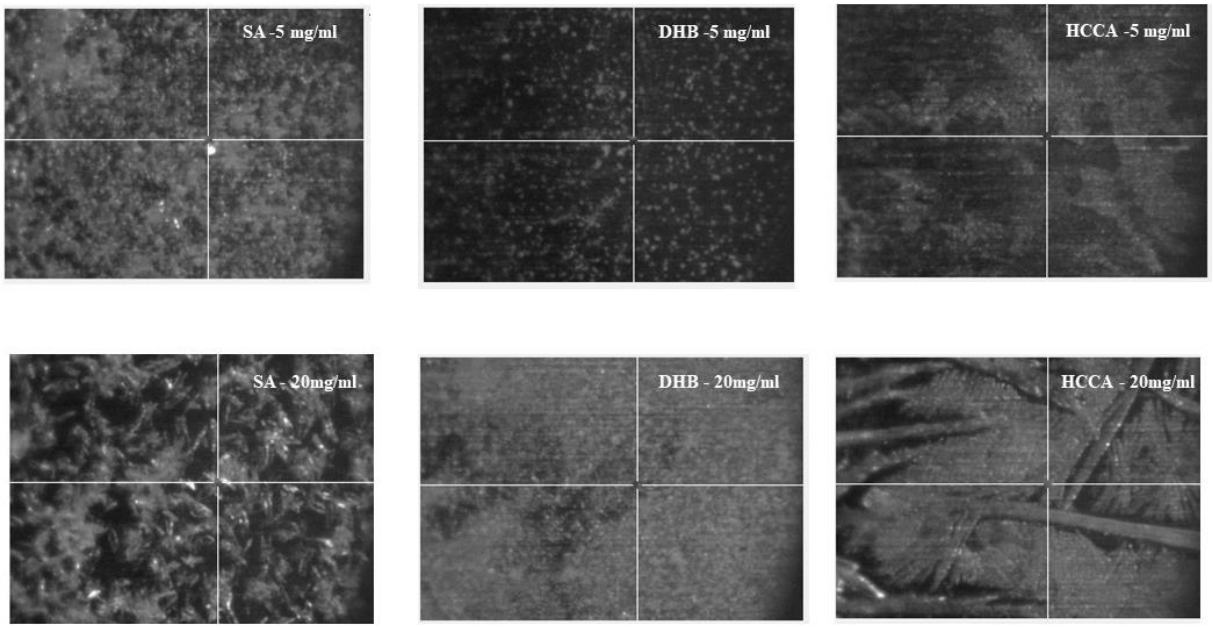

Figure 3. Co-crystallization of plasma EVs with the Matrix Concentration (5 mg/ml) and (20 mg/ml) of Sinapinic Acid (SA), Dihydroxy Benzoic Acid (DHB) and alpha-Cyano-4- hydroxycinnamic Acid (HCCA).

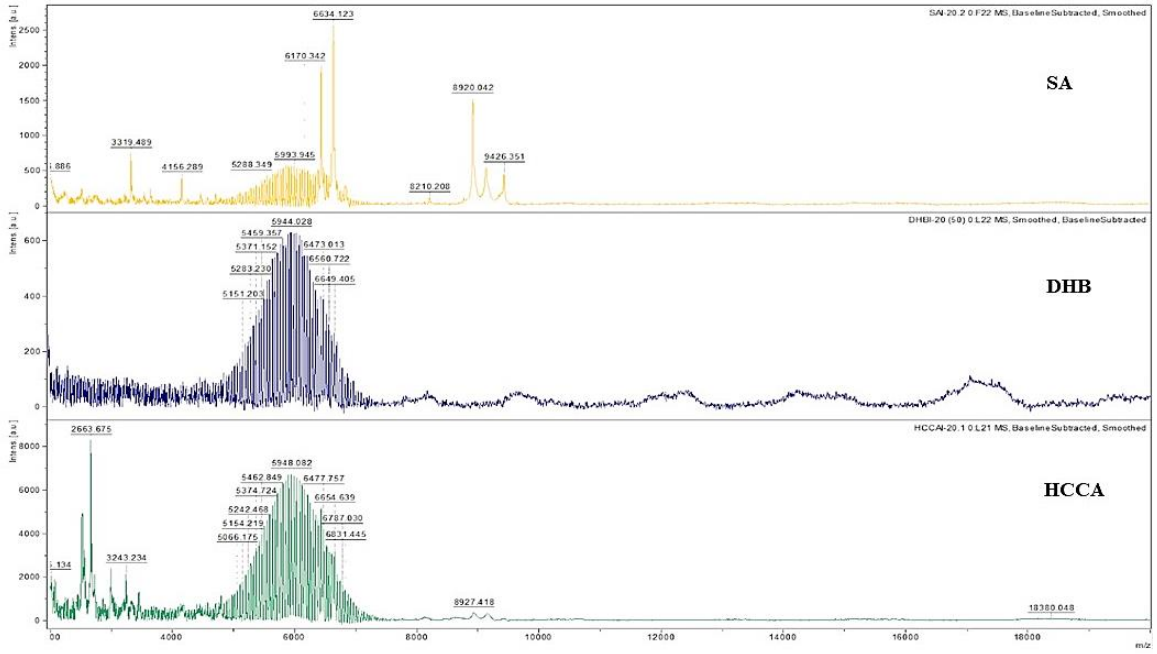

Figure 4. The intact plasma EVs at the matrix concentration (5 mg/ml) of Sinapinic Acid (SA), Dihydroxy Benzoic Acid (DHB), and α-Cyano-4-hydroxycinnamic Acid (HCCA) were analyzed by MALDI-TOF where the SA provided better spectrum.

Table 2. Advantages and Limitations of Sinapinic Acid, α-cyano-4-hydroxycinnamic Acid and 2,5-dihydroxy Benzoic Acid

Matrices	Advantages	Limitations
Sinapinic Acid (SA)	Good repeatability & covers a wide mass range (>10kDa)	Ability to produce small crystals but not suitable for glycoproteins
α-cyano-4-hydroxycinnamic acid (CHCA)	Maximum sensitivity and resolution for peptide detection.	only employed for polypeptide analysis
2,5-dihydroxy benzoic acid (2,5-DHB)	Improve the ionization of the sample and the high sensitivity and detection of lower mass ions.	significant crystallization capacity, strong polarity, and poor homogeneity

Daltonics, Bremen, Germany). In MALDI-TOF MS spectra, the fingerprint region of intact EVs at optimized parameters shows distinct peaks with m/z = 3315, 6630, 6432, 7807, 9130, 9421, 8875, and 8917. Proteomic analysis was carried out on plasma-derived EVs isolated from healthy individuals to determine those peaks' molecular and biological roles. The

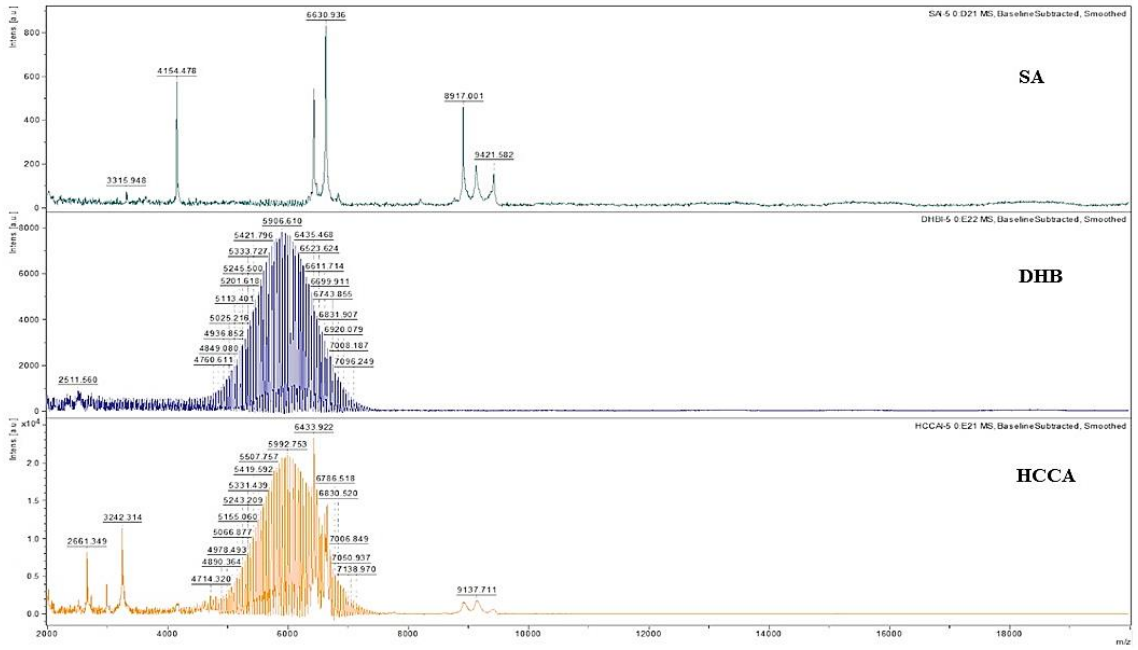

Figure 5. The intact Plasma EVs at the matrix concentration (20 mg/ml) of Sinapinic Acid (SA), Dihydroxy Benzoic Acid (DHB), and α-Cyano-4-hydroxycinnamic Acid (HCCA) were analyzed by MALDI-TOF MS.

biological functions of the four protein biomarkers are displayed in Table 2 with respective mass.

These peaks represent apolipoproteins A-II, C-I, C-II, C-III, C4A (complement component 4A), and C3 (complement component 3).[38,39] The UniProt database shows the cellular compartment, and biological and molecular functions of the above proteins match the role of intact plasma EVs.

The apolipoprotein family consists of apo A-II, C-I, C-II, C-III, and C-IV. All lipoproteins, except LDL, relate to apolipoproteins C-I and C-II, but LDL also contains apo C-III. The apolipoprotein A-II involved in predicting coronary artery disease, and its protective effects on atherosclerosis have been studied.[40] Apo C-I lowers the lipoprotein and hepatic lipase rate, which indicates gastric and renal cell carcinoma[41] and pancreatic cancer.[42] Apo C-II involves transporting free fatty acids as energy sources to all energy-dependent tissues. Apo C-III regulates the triglyceride level and reduces the risk of cardiovascular problems, atherosclerosis, and lipidemia.[43] Complement factors C3 and C4A associated with components of serum extracellular vesicles have been reported.[44] The levels of these complement proteins indicate COVID-19 severity[45] and autoimmune diseases such as systemic lupus erythematosus.[46] The proteins mentioned above are packed inside the extracellular vesicles and are involved in important biological functions such as vesicle-mediated transport, signaling, lipid metabolic processes, and immune response. Bloodborne EVs can transmit signals to distant targets such as the CNS, and crossing the blood-brain barrier (BBB) is the best example of vesicle-mediated transport.[47] The uptake of the molecular cargo from plasma EVs may alter recipient cells' genetic makeup and biological functions.[47,48] EVs immunomodulate and defend against causative agents in inflammatory diseases and cancer. Neutrophil-derived EVs promote inflammation in blood-carrying vessels reported in atherosclerosis.[49] EV channels pathogenic or protective biological signals in an autocrine or paracrine manner. Existing studies show that EVs involved in the overexpression of the PI3K signal are directly linked to many cancer types.[50] Adipocyte- and hepatocyte-derived circulating EVs are metabolic markers that track lipid and glucose metabolism. EV levels are directly proportional to lipid and glucose metabolism parameters.[51,52] Estes's group studied the variations in lipid metabolism and abnormal levels of EVs that influence Alzheimer's and Parkinson's disease progression.

MALDI-TOF MS Analysis of Proteinase K and 1X RIPA Lysis Buffer-treated Plasma Extracellular Vesicles

Salts, lipids, and high molecular weight proteins are all present in the complex biological fluid known as plasma, which can affect the MS profiling of EVs produced from plasma. Proteinase K (PK) at a concentration of 20 mg/ml degrades bulk plasma protein like albumin, which co-precipitates along with EV extraction from plasma.[53] The MALDI mass spectrum of PK-treated EVs at the optimized parameters: sinapinic Acid, 20 mg/ml at three different shots of 50, 300, and 1000 showed more protein peaks compared to the intact plasma EV profile pattern in Figure 6. The high molecular mass protein peptides such as 10030, 13537, and 18355 might be from the EV surface proteins (CD63, CD81, and CD9). The MALDI mass spectra are good at 50 and 1000 shots, but 300 hits show more peaks than the other two.

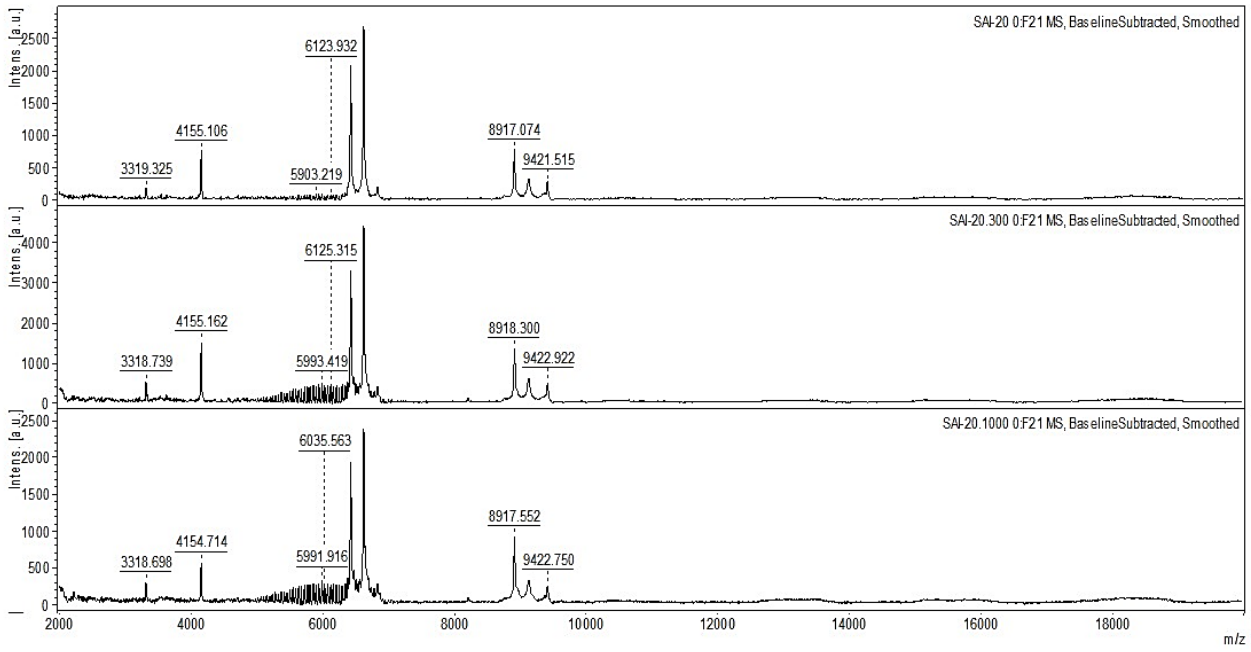

Figure 6. The intact Plasma EVs at the matrix concentration (20 mg/ml) of Sinapinic Acid at 50, 300, 1000 laser shots. Among the three sets of conditions 300 shots provided well-defined peaks with high intensity.

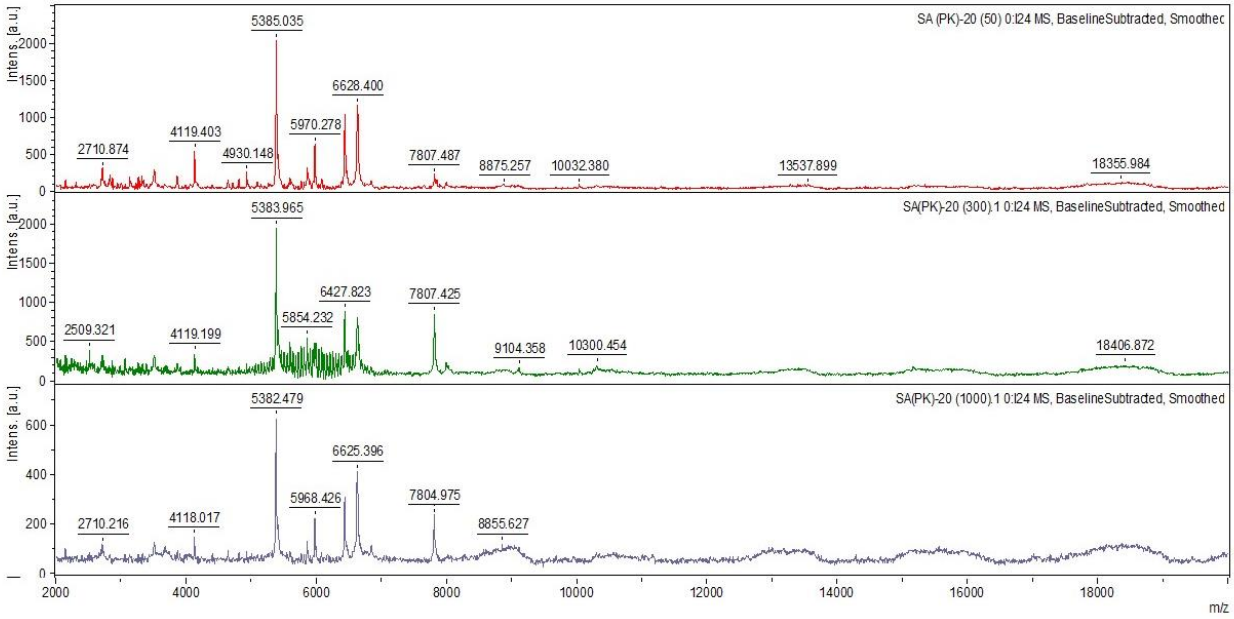

Figure 7. The intact Plasma EVs treated with Proteinase K at the matrix concentration (20 mg/ml) of Sinapinic Acid at 50, 300, 1000 shots. Proteinase K treatment improved the resolution in all the experimental conditions.

The outer membrane of EVs is 5 nm thick, and a sturdy layer protects the molecular contents of EVs derived from the source. The bilipid layer of EVs was distorted using 1X RIPA lysis buffer to release the molecular content.[54] Figure 7 illustrates the peak pattern, which is generally similar to that of proteinase K-treated plasma EVs, but with some particularly intense peaks at three different shots, including 4123, 5390, 6432, and 6630. On the other hand, the peaks 2710, 8855, and 13,537 have not been reflected in the 1X RIPA-lysed EV mass spectrum.

Comparing the plasma-borne EV proteome profile after the treatment of Proteinase K and 1X RIPA lysis buffer, more peaks were found in the mass spectra of Proteinase K-treated EVs than in the EVs treated with 1X RIPA lysis buffer. The proteome pattern was better at 50 and 300 shots at 1000 shots for PK-treated EVs. At 300 shots, the peaks are intense and reproduce the pattern. The mass peaks of PK-treated EVs include 2710, 4119, 5385, 5970, 6628, and 7807. In the

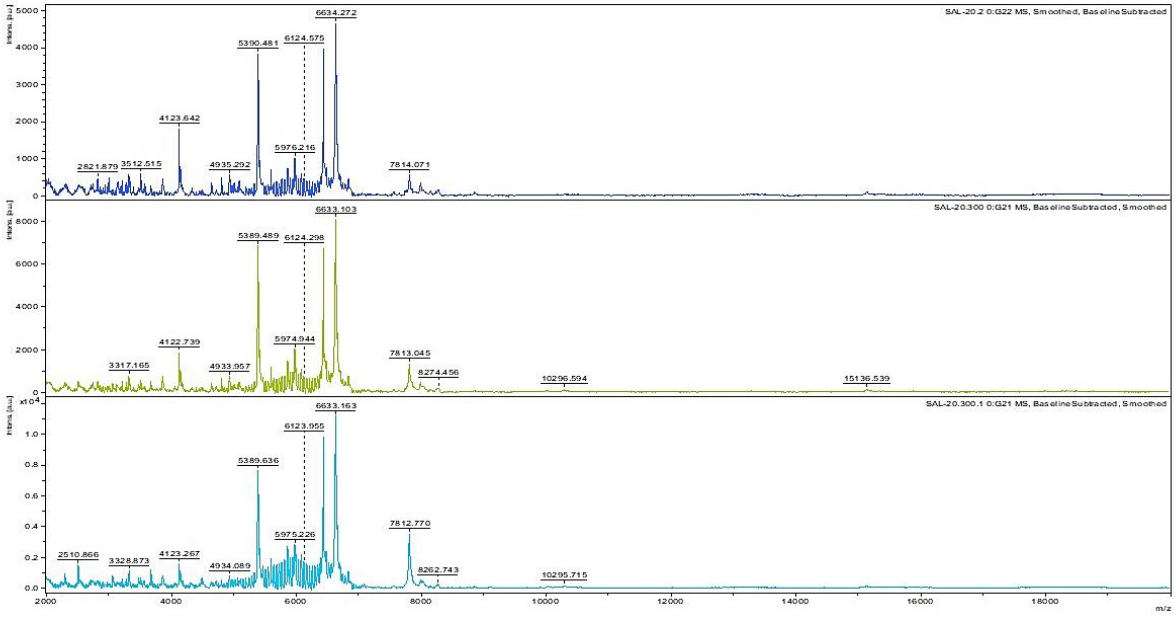

Figure 8. Plasma-derived EVs lysed with 1X RIPA lysis buffer and analyzed at the matrix concentration (20 mg/ml) of Sinapic Acid at 50, 300, 1000 shots. The RIPA treatment increased the m/z range and proteins with higher molecular weight could be observed.

Table 3. List of Proteins Obtained from the Fingerprint Region of MALDI-TOF Spectra of the Plasma Extracellular Vesicles

Feature Peak m/z	Protein Name	UNIPROT Accession number	Cellular compartment	Molecular function	Biological function
3315	C4A-Complement Component 4A	P0C0L4	Endoplasmic reticulum, Plasma membrane and extra cellular space	Hydrolase activity, molecular function regulator activity and catalytic activity	Vesicle-mediated transport, inflammatory and defense response.
6630 9421	Apolipoprotein C-III	P02656	Extracellular space and early endosome	Lipid binding, catalytic activity, hydrolase activity	Lipid metabolic processes, vesicle mediated transport and signaling
8875	C3-Complement component 3	P01024	Extracellular space	Hydrolase and catalytic activity	Immune and defense response
8917	Apolipoprotein C-II	P02655	Early endosome and extracellular space	Lipid binding, hydrolase activity and molecular function regulator activity	Lipid metabolic process and vesicle-mediated transport
7807	Apolipoprotein A-II	P02652	Extracellular region, extracellular space, endosome and cytosol	Transporter activity, lipid binding, transferase activity,	Immune response, lipid metabolic processes, Inflammatory response, protein modification process, anatomical structure development, Membrane organization, protein-containing complex assembly

lysis buffer treatment, the mass spectra of plasma EVs were good at 300 shots and 1000 shots. At 300 shots, the mass spectra of lysis buffer-treated EVs covered high molecular mass peptides such as 10296 and 12136 with fewer artifacts as shown in Figure 8. 1X RIPA lysis buffer-treated EVs show poor reproducible proteome patterns in mass spectrometry analysis.[54] The salt and detergents in the 1X RIPA lysis buffer cause artifacts in mass spectra.[55]

Limitations of the Study

However, EVs and lipoproteins are abundant in biofluids, presenting detection challenges. Furthermore, the half-life, variability of turn-over, and heterogeneity of vesicle sources in the body are all factors that are poorly understood, especially in clinical populations. Although MALDI-TOF MS profiling of plasma-derived EVs can study the protein fingerprinting of EVs collected by the polymer-based precipitation method, an inadequate amount of protein to do MS/MS analysis. The technology restriction is that EV fingerprint recognition is only possible if the spectrum database contains peptide mass fingerprints from various cell types. It is preferred to develop a MALDI TOF MS/MS-based protein database for EV proteins from biological fluids and software for MALDI-TOF analysis in the future.

Conclusion

Extracellular vesicles are attractive components in the clinical area of diagnosis and therapeutics. Studying and characterizing the fingerprint regions of EVs derived from biofluids help understand the molecular composition and

biological function. In this study, only a minimal volume of human blood is required for the EV fingerprint detection test from blood collection to mass spectral generation, which takes less than 3 h due to the advent of commercial kits for quick exosome precipitation and the use of MS for quick extracellular vesicle analysis. The benefits of profiling plasma extracellular vesicles in MALDI-TOF MS are analysis of EV composition without labeling, robust detection, and high-scalable analysis. The accuracy of the mass of the protein measurement depends on several factors. Many proteins are sufficiently massive that resolution is not achieved from additional species, such as salts or matrix adducts. Under these conditions, such species cause peak broadening, resulting in a slight shift in the measured mass to higher values. The matrix aids in the ionization of analyte molecules and helps isolate analyte molecules in MALDI-TOF MS analysis by absorbing laser energy. The specific and predominant peaks represent protein masses in Table 2 that have a molecular and biological function. Therefore, choosing the suitable matrix is crucial for MALDI-TOF MS analysis. The plasma-extracellular vesicles obtained from the TEI method show high proteome recovery with 20 mg of sinapinic acid matrix in MALDI-TOF MS analysis.

In summary, we showed that MALDI-TOF MS fingerprinting of EVs has excellent potential in clinical applications. The detection and analysis of EVs will study fingerprint regions and be used as a liquid biopsy diagnostic test. The optimized parameters of MALDI-TOF MS can be further applied to identify biomarkers for diseases by fingerprinting EVs derived from other biological fluids.

Authors' Contributions

AR performed the experiments and data analysis and wrote the original draft. AR performed the experiments. RNS, VM., and JM helped with data processing. AR and VV collected the clinical samples. RNS revised the original draft of the manuscript. JM modified the manuscript. RNS and VM supervised all aspects of the work. All authors were involved in the design of the study.

Ethical Approval

This study was approved by the ethics committee of SRM medical college hospital and research center (1886/IEC/2020) and was carried out according to the guidelines of Helsinki. Each participant signed informed consent to participate in the study.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

Acknowledgment

We acknowledge the staff members of SRM Medical College Hospital and Research Center for their assistance in blood sample collection from normal samples. We would also like to thank the SRM-DBT platform and Contemporary Research Services and Skill Development in Advanced Life Sciences Technologies for providing the MALDI-TOF MS instrument facility. We also thank the children and their parents who participated in the study.

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