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Isolation of small extracellular vesicles from a drop of plasma via EXODUS and their fingerprint proteomics profiling by MALDI-TOF MS

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ABSTRACT

Small extracellular vesicles (sEVs) in blood have emerged as the most promising biomarkers for clinical diagnostics and prognostics. However, isolation and identification of intact sEVs from blood are the major obstacles for basic research and clinical translations. Here, we report rapid isolation and sensitive detection of plasma sEVs by an integrative platform of sEV detection via the ultrafast-isolation system (EXODUS) and the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). We can achieve label-free isolation of sEVs with relatively high recovery and purity by the EXODUS purification method from 20 μL of plasma, which was compared with polyethylene glycol-based precipitation and ultracentrifugation methods. We have profiled the fingerprints of the intact sEVs isolated from the different volumes of plasma using MALDI-TOF MS within 1 h. Further, we have evaluated the reproducibility and identified the metabolomic biomarkers of plasma sEVs via LC-ESI-MS/MS. We believe the combination of rapid EXODUS isolation and MALDI-TOF MS detection may serve as a clinical translation method for fast and high-throughput biomarker detection and screening.

1. Introduction

Small extracellular vesicles (sEVs), secreted by almost all living cells (Choi and Yong, 2015), are lipid-bilayer-enclosed vesicles with diameters ranging from 30 to 150 nm (O'Driscoll 2015; Raposo and Stoorvogel, 2013; Tkach and Théry, 2016). sEVs contain proteins, nucleic acids, metabolites, and lipids (Schuld et al., 2014; Shao et al., 2012) playing diverse roles in intercellular communication and a variety of important physiological and pathological processes in intra- and intercellular environments (Hannafon and Ding, 2013; Mathivanan et al., 2010; Simons and Raposo, 2009; Yoon et al., 2014). sEVs are present in most body fluids, such as blood, urine, saliva, cerebrospinal fluid, and ascites, which are considered as potential biomarkers and targeted therapeutic agents for many diseases, including cancer, neurological disorders, diabetes, and renal diseases (Buzas et al., 2014; Melo et al., 2015; Mitchell et al., 2009; EL Andaloussi et al., 2013). As the most promising and reliable biomarker provider, blood also contains various secretions, which are from almost all human cells. However, the isolation of high yield and purity sEVs from blood becomes relevant difficult for basic research and clinical applications (Melo et al., 2015).

Despite sEVs as potential biomarkers having recently attracted substantial interest, the basic study and clinical translation of plasma sEVs are limited because of their laborious and low-efficiency isolation process and characterization approaches (Witwer et al., 2013). Ultracentrifugation (UC) is the most commonly used method for sEV isolation; however, it is time-consuming (>3 h), low-yielding, and may alter sEV morphology and function (Coumans et al., 2017; Taylor and Shah, 2015). To further improve the purification of sEVs after UC, the sucrose

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gradient is often used, resulting in an even longer processing time (>16 h) (Coumans et al., 2017; Greening et al., 2015). Furthermore, sEV purification methods, that use precipitation reagents, such as polyethylene glycol (PEG), are discarded because of polymer contamination, making it inappropriate for downstream protein-based analysis (Kooijmans et al., 2016; Taylor and Shah, 2015). It has been reported that ultrafiltration has adequate sEV purity results. However, the filter has a limited lifetime, and extra cleaning is needed. It causes protein aggregation, contamination, and excessive shear which can reduce sEV yield and integrity (Li et al., 2017). The microfluidic technology-based isolation method has advantages in precision patterning of nanopores and the capability of integrated sensors; however, it has the limitation of handling large biological sample volumes such as urine and blood (Jeong et al., 2016; Wunsch et al., 2016). A method of rapid, feasible, and efficient isolation of plasma sEVs is therefore needed for research and clinical applications.

At present, western blotting of specific sEV protein markers is one of the most widely used methods for sEV identification. However, it has low sensitivity and is laborious, time-consuming, and is not available for absolute quantification, limiting its clinical application. In the last decade, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been demonstrated as one of the best analytical tools for detection and precise analysis of biomolecules such as peptides and proteins (Bizzini and Greub, 2010). Its high sensitivity and throughput enable the simultaneous detection of hundreds of molecules over a broad mass range. MALDI-TOF MS was used for the rapid identification and classification of micro-organisms by recording their characteristic protein profiles (Stübiger et al., 2018; Zhu et al., 2019). The mechanism of MALDI facilitates sEV input in their intact form, which significantly reduces the processing time by avoiding the sample preparation step (Zhu et al., 2019).

To overcome these challenges in achieving the effective isolation of intact sEVs from urine and saliva, we have demonstrated a sEV isolation platform, a label-free method (Chen et al., 2021). In this study, by comparing UC and PEG, we mainly focus on the isolating and purifying of sEVs from a drop of plasma in less than 20 min. We then further integrated EXODUS with MALDI-TOF MS to obtain the high sensitivity of proteomic fingerprints of intact sEVs from 20 μL of human plasma. The implementation of label-free purification of sEVs followed by MALDI-TOF MS characterization and further analysis, such as metabolism, provides a promising approach for speeding up sEV-based basic research and clinical translations.

2. Experimental section

2.1. Plasma samples preparation, handling, and storage

Blood samples used in this study were obtained from healthy donors (Ethics Committee in Clinal Research of the First Affiliated Hospital of Wenzhou Medical University (Issuing Number 2019011)). 10 mL of blood samples were collected in EDTA vacutainer tubes and processed within 2 h. They were then centrifuged at 1500 g for 15 min to remove cells, followed by centrifugation of supernatants at 3000 g for 15 min (Kalra et al., 2013). The resulting supernatants (plasma) were aliquoted into 1.5 mL microcentrifuge tubes and were stored at −80 °C for future use.

2.2. Isolation of plasma sEVs using different methods

EXODUS: Trace plasma samples (20 μL) were diluted in 5 mL with 1X PBS and filtered through a 0.22 μm syringe filter (Millipore). The filtered plasma solutions were then loaded into the EXODUS device (Chen et al., 2021). Protein fragments, lipids, nucleic acids, and other small impurities pass through nanopores under the alternating negative pressing with sEVs (diameter >20 nm) remaining inside the chamber. The 200 μL of the purified sEV 1X PBS solution was transferred to a microcentrifuge tube and stored at 4 °C for MALDI-TOF analysis and −80 °C for metabolomics (Liu et al., 2017). UC: 20 μL of plasma was centrifuged at 120,000 g at 4 °C for 90 min using an ultracentrifuge (Hitachi, CP100NX) to collect sEVs (Sunkara et al., 2019). The sEVs were then resuspended in 1X PBS to reach the 200 μL and saved at 4 °C and −80 °C, respectively. PEG: we used a commercial kit (ExoQuick® ULTRA EV Isolation Kit for Serum and Plasma, System biosciences) to isolate 20 μL of plasma according to protocol. The isolated sEVs solution was kept at the same condition as EXODUS and UC for next-step characterizations.

2.3. Western blotting and SDS-PAGE electrophoresis

Protein concentrations of sEV samples were measured by a Qubit™ 3 Fluorometer (Thermo Fisher Scientific, Waltham, MA). Samples were then denatured for 10 min by heating at 100 °C, after which equal amounts of proteins were separated by 4%–20% SDS-PAGE gels (GenScript, Hong Kong). Next, gels were transferred to 0.22 μm PVDF membranes (GE Healthcare Biosciences, Piscataway, NJ) and blocked with 5% skim milk (dissolved in PBS with 0.01% Tween 20) for 1 h at room temperature on a shaker. Membranes were then incubated overnight at 4 °C with the following primary antibodies: 1:1000 anti-Alix (sc-53540; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 1:1000 anti-Mac-2BP (sc-374541; Santa Cruz Biotechnology, Inc.), 1:1000 anti-TSG101 (NB200-112; Novus Biologicals, Littleton, CO), 1:1000 anti-flottilin1 (sc-74566; Santa Cruz Biotechnology, Inc.), 1:1000 anti-CD 9 (13403S; Cell Signaling Technology, Danvers, MA), and 1:1000 anti-albumin (sc-271605; Santa Cruz Biotechnology, Inc.). Next, membranes were incubated with the appropriate secondary antibodies (7076S; Cell Signaling Technology) for 1 h. Immunoreactive bands were detected via chemiluminescence methods, and densitometry analysis was performed using the Image J software.

2.4. Nanoparticle tracking analysis (NTA)

The concentration and size distribution of sEVs were measured by an NTA system (Nanosight NS300, Malvern Panalytical, UK). Purified sEV samples were vortexed and diluted with 220 nm pre-filtered PBS to obtain the recommended 25 to 100 particles/frame in the NTA system. All measurements were performed under identical settings to ensure consistent results. Each sample was analyzed three times, and mean values were plotted.

2.5. Transmission electron microscope (TEM)

The preparation of sEV samples for TEM analysis was based on a previously published protocol, with minor modifications (Théry et al., 2006). sEV samples were added to carbon-coated copper grids. The carbon-coated surface was kept wet during sample preparation, whereas the uncoated side was kept dry. Isolated sEV samples were mixed with an equal volume of 4% paraformaldehyde for 20 min. Next, 20 μL of the fixed sEV solution was placed onto the grid and incubated for 1 h. Grids were then washed with 100 μL of PBS and blocked with 50 μL of 1% glutaraldehyde for 5 min. Negative samples staining was achieved using 1% uranium acetate for 30 s. The grids were dried with silica gel overnight. Imaging was performed in an FEI Tecnai TEM operated at 200 kV.

2.6. MALDI-TOF MS analysis

sEV samples, isolated as mentioned above, were spotted onto a MALDI target plate (MSP 96 target ground steel; Bruker Daltonics, Bremen, Germany) and vacuum-dried with a CentriVap Benchtop Vacuum Concentrator (Labconco, Kansas City, MO) at room temperature; 0.8 μL of a saturated matrix solution (4-hydroxy-α-cyanocinnamic acid; Bruker Daltonics, Bremen, Germany) in 50% acetonitrile-0.1% trifluoroacetic acid was then added to sample spots. MALDI-TOF/MS was conducted in an autoflex max MALDI-TOF (Bruker Daltonics) benchtop

instrument. Intact sEVs extracted from plasma were spotted three times onto a MALDI target plate and each spot was measured three times, resulting in nine mass spectra for each isolate. Identification of the detected protein peaks was performed by comparing them with a database, using TagIdent (https://web.expasy.org/tagident/) (Swiss Institute of Bioinformatics). The m/z value of the experimental protein cluster was searched against all possible tag permutations in the UniProtKB/Swiss-Prot database of *Homo sapiens* at a mass tolerance of 0.1% to allow for deviations caused by small posttranslational modifications (PTMs) or shifts due to experimental errors.

2.7. Metabolism

Samples were prepared as follows (Puhka et al., 2017): 1 mL of 70% MeOH was added to the sample after freeze-drying, followed by vortexing 30 s, freezing in liquid nitrogen for 5 min, and cooling with ice for 3 min (this procedure was repeated three times). Next, the sample was vortexed for 30 s, exposed to 30 Hz ultrasound for 3 min at 4 °C, vortexed for 30 s, centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant collected. After concentrating the extract at 4 °C, 150 μL of 70% MeOH were added, followed by vortexing for 30 s, centrifuging at 12,000 rpm at 4 °C for 10 min, and collecting the supernatant for testing. UPLC Conditions: sample extracts were analyzed, using an LC-ESI-MS/MS system (UPLC, Shim-pack UFLC SHIMADZU CBM A system, https://www.shimadzu.com/; MS, QTRAP® 6500+ System, https://sciex.com/). The analytical conditions were as follows: ACQUITY UPLC HSS T3 C18 column (2.1 × 100 mm, 1.8 μm particle size); column temperature, 40 °C; flow rate, 0.4 mL/min; injection volume, 2 μL; solvent system, water (0.04% acetic acid): acetonitrile (0.04% acetic acid); gradient program, 95:5 V/V at 0 min, 5:95 V/V at 11.0 min, 5:95 V/V at 12.0 min, 95:5 V/V at 12.1 min, 95:5 V/V at 14.0 min. ESI-Q TRAP-MS/MS: LIT and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (QTRAP), QTRAP® 6500+ LC-MS/MS System, equipped with an ESI Turbo Ion-Spray interface, operating in positive and negative ion mode and controlled by Analyst 1.6.3 software (Sciex). The ESI source operation parameters were as follows: source temperature 500 °C, ion spray (IS) at 5,500 V (positive) and −4,500 V (negative), ion source gas I (GSI), gas II (GSII), and curtain gas (CUR) were set at 55, 60, and 25.0 psi, respectively; the collision gas (CAD) was high. Instrument tuning and mass calibration were performed with 10 and 100 μmol/L polypropylene glycol solutions in QQQ and LIT modes, respectively. A specific set of MRM transitions were monitored for each period according to the metabolites eluted within this period.

2.8. Statistical analysis

Data represent the mean ± SD of triplicate determinations from three independent experiments. Figures were modified with Adobe Illustrator (Adobe Systems Incorporated, San Jose, CA) and GraphPad Prism 7.0 (GraphPad Software, San Diego, CA).

3. Results and discussion

3.1. Rapid isolation of plasma sEVs for MALDI-TOF MS analysis

The workflow for isolation and quantitation of sEVs in plasma is shown in Fig. 1 and Fig. S1. First, the blood is collected and separated by the centrifugation method (Fig. S1A). Fig. S1B shows the scheme of sEV isolation, which provides a new strategy of ultrafiltration by the induced vibrational oscillation of nanoporous membranes that form a fluidic cavity. It allows highly efficient isolation of sEVs from plasma dilutions. In brief, after cell-free plasma was obtained from human whole blood by centrifugation, sEVs were isolated as previously reported (Chen et al., 2021). Due to its novel design with negative pressure oscillation, plasma sEVs are rapidly and non-invasively enriched with high purity and recovery (Fig. S1C). By loading purified samples and matrix onto a MALDI target plate (Fig. S1D), downstream analysis is then performed for sEV-protein profiling in a MALDI-TOF MS benchtop instrument with high sensitivity and efficiency (Figs. S1E and F).

3.2. Comparison of sEVs isolated by different methods from human plasma

We compared the EXODUS method with UC and PEG-based precipitation about the size and purity of sEVs isolated from 20 μL plasma. The particle size distribution was characterized by NTA. sEVs isolated by EXODUS and PEG had a unimodal peak centered at 65 nm and 95 nm respectively (Fig. 2A and C), whereas sEVs purified by UC had diverse peaks, ranging from 81 nm to 253 nm (Fig. 2B). EXODUS and PEG ($\sim 10^{10}$ particles/mL) also isolated sEVs from 20 μL plasma >50-fold higher than that with UC ($\sim 10^{8}$ particles/mL) (Fig. 2D). The total time of isolating sEVs from 20 μL plasma with different methods was shown in Fig. 2E, and EXODUS needs 18 min (including sample preparation time), which was much shorter than UC and PEG. Taken together, the purity of sEVs isolated with EXODUS was higher than that with UC and PEG (Fig. 2F). The TEM image in Fig. 2G confirmed the presence of intact sEVs with the hallmark cup-shaped structure, demonstrating that high-purity sEVs were successfully enriched from human plasma. Next,

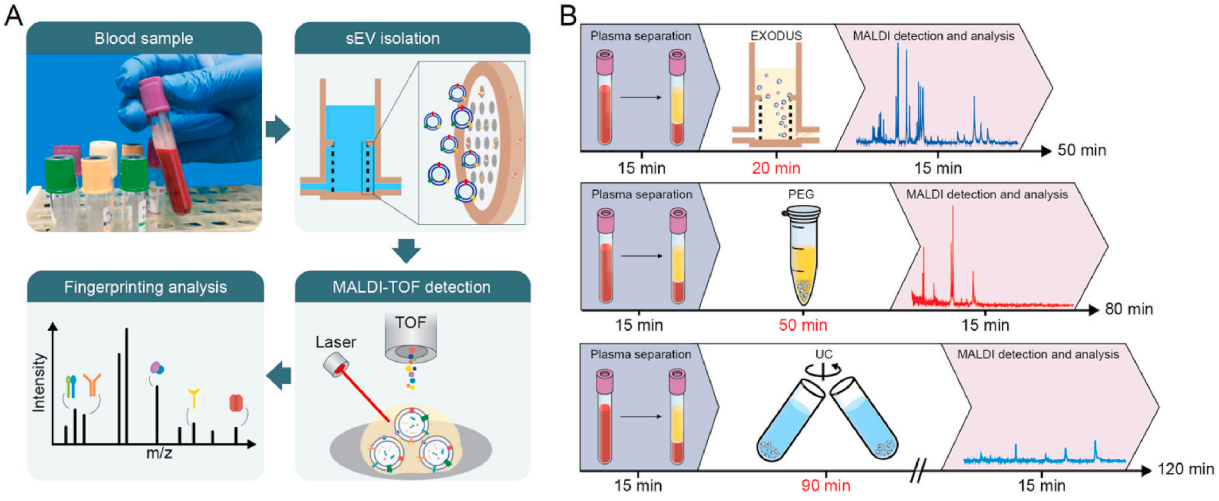

Fig. 1. Rapid isolation and MALDI-TOF detection of sEVs: (A) Blood separation, purification of plasma sEVs by EXODUS, MALDI-TOF of detection, and fingerprinting analysis of sEVs. (B) Comparison of intact sEV-based MALDI-TOF analysis among EXODUS, PEG, and UC.

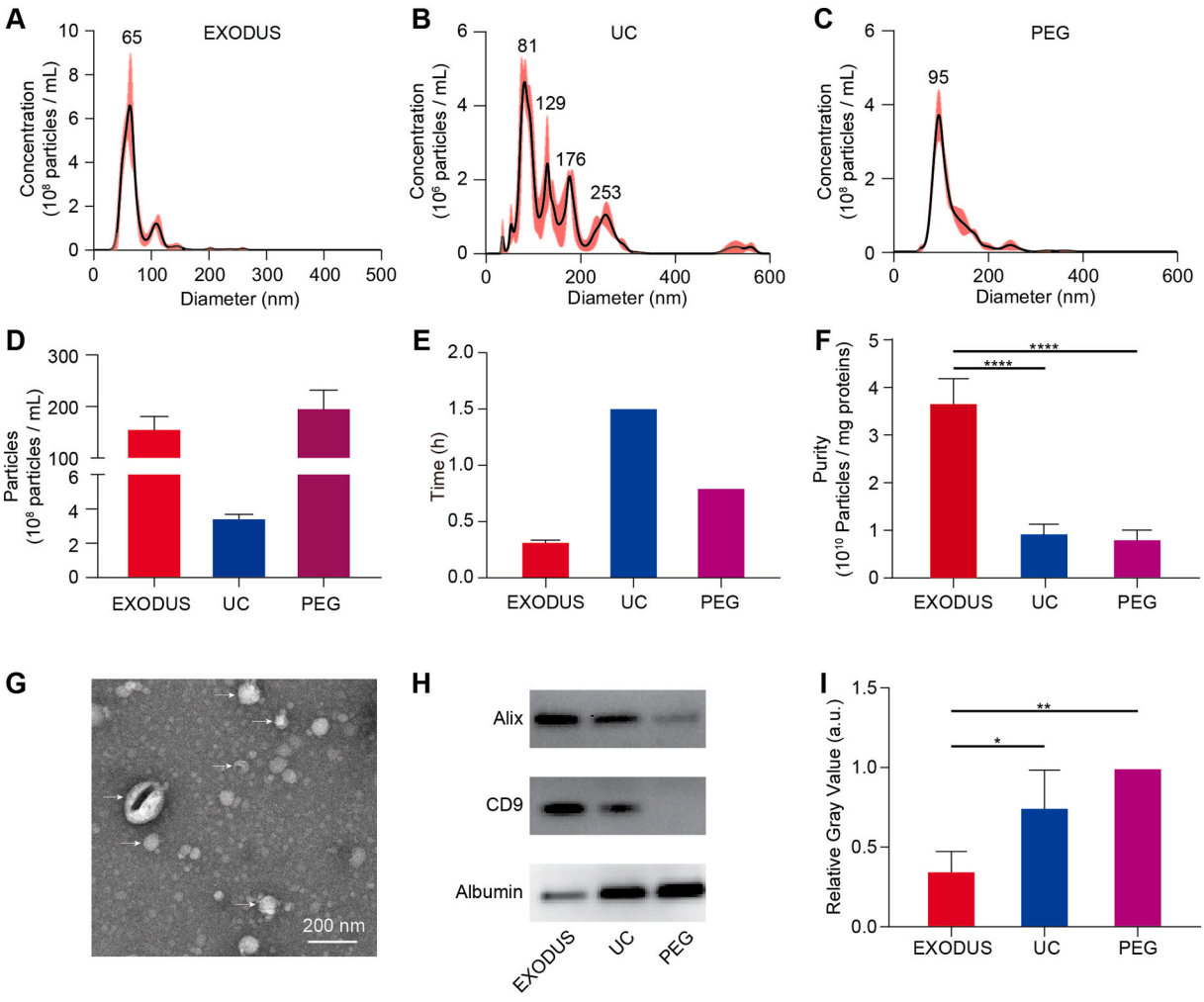

Fig. 2. Rapid isolation of high purity sEVs from human plasma. The size distribution of sEVs isolated using (A) EXODUS, (B) UC, and (C) PEG. The particle numbers (D), isolation time (E), and purity (F) of sEVs isolated by EXODUS, UC, and PEG. (G) TEM image of sEVs isolated by EXODUS. (H) Western blotting results of sEV-specific proteins and non-specific proteins. (I) The relative gray value of albumin in sEVs isolated by EXODUS, UC, and PEG.

we used western blotting for verification at the protein level. As shown in Fig. 2H and I, the sEV-specific protein (Alix and CD9) bands as the yield were more evident in EXODUS-isolated sEVs, and the non-specific protein (Albumin) band as purity was less than that in PEG- and UC-isolated sEVs, demonstrating that EXODUS was better than PEG and UC. And the recovery efficiency of EXODUS is about 90% (Fig. S4). In summary, EXODUS is a high-speed and highly sensitive approach for sEVs isolation from 20 μL plasma.

3.3. MALDI-TOF MS analysis of intact sEVs

Proteomics profiling of EXODUS-isolated sEVs was carried out by MALDI-TOF MS. To evaluate the sensitivity, sEV samples isolated from incremental volumes of plasma with EXODUS technology were analyzed with their mass spectra as shown in Fig. 3A. Overall, peak intensities gradually decreased as the plasma loading volume was reduced from 30 to 5 μL (Fig. 3A). At the same time, particle numbers also gradually decreased in a linear manner (Fig. 3B). However, only a few peaks were observed using 5 μL of plasma. This result indicates that the mass spectrum can detect a signal only when the number of particles reached a certain level. The signal-to-noise (S/N) ratios for each plasma loading volume were plotted in Fig. 3C, in which S/N ratios significantly decreased from 25 to 2 as the loading volume decreased from 15 to 10 μL. We can also observe that when the plasma loading volume increased from 20 μL to 30 μL, the number of particles still increased linearly, but peaks S/N ratios showed a slower increase rate in this range (Fig. 3B and C). We further analyzed the number of spectral peaks detected at different plasma loading volumes. As shown in Fig. 3D, the number of peaks did not have a significant increase when the plasma loading volume was higher than 15 μL, indicating that the informative mass spectrum was achieved with 1.5×10^{10} particles, and 20 μL of plasma loading volume was sufficient for downstream analysis. We isolated and analyzed the same sample three times independently (Fig. S5A). The results of mass spectrometry showed acceptable reproducibility (Fig. S5B), indicating that EXODUS has the potential to be a stable method for the isolation of sEVs from plasma.

To identify the proteins of the most abundant peaks, the TagIdent was used to search the peak at the m/z value with a mass tolerance of 0.1%. The potential candidates are shown in Supplement Table 1. In this protein list, VMO1, RNAS2, SAP3, and BTG2 have been reported to be present in sEVs according to the database (Gonzales et al., 2009; Gonzalez-Begne et al., 2009; Nguyen et al., 2019; Principe et al., 2013; Sabapatha et al., 2006). Among them, SAP3 was related to conversion to advanced age-related macular degeneration (AMD) and could be used as a plasma biomarker that reflects the systemic changes in protein expression in patients with AMD (Lynch et al., 2020). The risk judgment on AMD may become the application target of our method. Recently, Syntenin-1 has been reported as a novel exosomal biomarker (Kugeratski et al., 2021). However, there is no similar discovery in our research results, which may be related to the different proteomics analysis

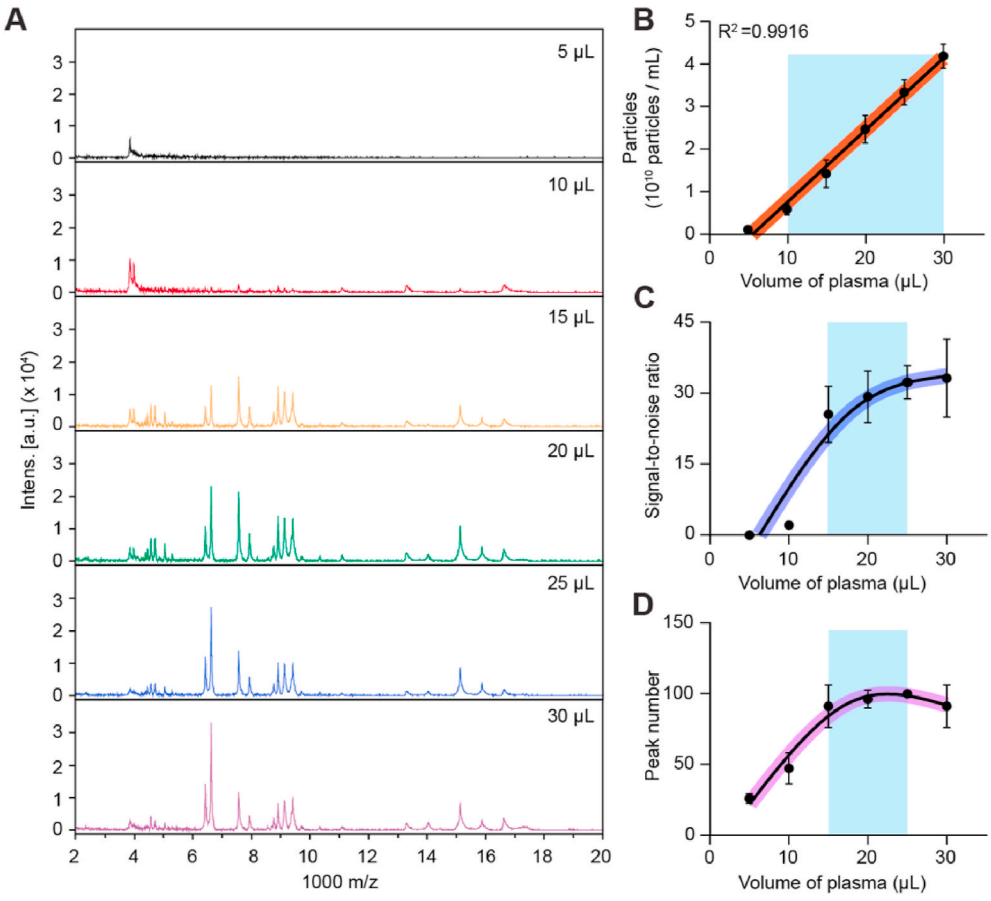

Fig. 3. Profile fingerprint of sEVs isolated from the different volumes of plasma using MALDI-TOF. (A) MALDI-TOF mass spectrum of enriched sEVs from different volumes (5μL–30μL) of human plasma over a mass range of m/z 2,000–20,000. (B) Particle number of enriched sEVs measured by NTA. (C) Comparison of signal-to-noise ratio from a different volume of human plasma. (D) The number of peaks detected by MALDI-TOF.

methods used.

Zhu et al. reported the use of MALDI to detect exosomes, which were isolated by the PEG precipitation method (Zhu et al., 2019). The origin of the sEVs was then identified from normal or tumor cells. We compared our mass spectrum results with those isolated by UC and PEG precipitation. As shown in Fig. S6, abundant peaks were observed in EXODUS and PEG methods, but more peaks were detected from sEVs isolated by the EXODUS purification method (100 peaks at S/N $\geq$ 3) than those isolated by PEG precipitation (25 peaks). After concentrating the sample to a certain concentration (~10^{10} particles/mL), we can also obtain many peaks from sEVs isolated by the UC with MALDI, but the signal intensity of the peak is lower than that of EXODUS and PEG (Fig. S6). Thakur et al. reported the use of label-free sensitive localized surface plasmon resonance (LSPR) and atomic force microscopy (AFM) biosensors to detect levels of MCT1 and CD147 in serum-derived exosomes from the mouse model of glioma (Thakur et al., 2020). Compared with its detection of single-molecule interactions, our method has the potential to detect multiple molecules at the same time, but cannot select the molecules to be detected.

3.4. Metabolism of plasma sEVs

The reproducibility of sEV isolation methods is critical for downstream analysis. To validate this, metabolomic studies of sEVs isolated from 20 μL of plasma were performed via LC-ESI-MS/MS. Through the correlation analysis between samples, a biological repetition between samples in the group was observed. As shown in Fig. S7A, the repeatability of EXODUS and PEG was better than that of UC. In addition, the similarity between EXODUS and UC was higher than that of PEG and UC. The metabolic profiles of sEVs were isolated by different methods as shown in Fig. S7B. Among them, for sEVs isolated by EXODUS, we detected more than 200 metabolites with their content distribution shown in Fig. 4A. The number of various metabolites was shown in Fig. S8. In the characterization, lipids accounted for the highest proportion of total metabolites. sEVs exert significant effects on lipid metabolism, including synthesis, transport, and degradation. Recent findings have confirmed that sEVs act as a biological vehicle and directly transfer lipids such as cholesterol, fatty acids, and eicosanoids (Wang et al., 2020). It is suggested that the plasma sEVs extracted by the EXODUS method have the potential to study the role of exosomal lipid metabolites in diseases. Fig. 4B shows the top 17 metabolites identified from the purified sEVs, in which we performed two independent isolation and analysis, using the aliquots from one plasma sample for comparison. No significant variation was observed between the two replicate determinations, showing fair reproducibility and stability of sEV isolation from the plasma for downstream analysis.

4. Conclusion

In summary, we have validated the rapid and highly sensitive approach for investigating the fingerprints of plasma sEVs, using EXODUS for efficient sEV isolation from 20 μL of plasma samples, and followed with sensitive MALDI-based proteomic profiling. The total processing time of sEV isolation was achieved within 20 min, much faster than ultracentrifugation and other commercial kits. Since a method that can obtain sufficient and reliable information from a small volume of samples is demanded in life sciences and clinical applications, our efficient integrative platform via EXODUS purification technology

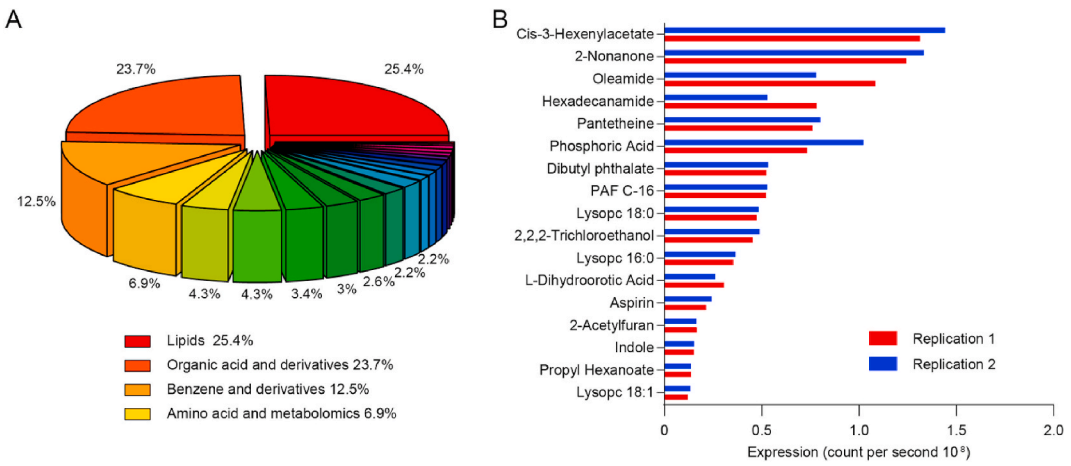

Fig. 4. Metabolism study of plasma sEVs showing high reproducibility via EXODUS. (A) The content of metabolites in sEVs isolated by EXODUS. (B) Expression of top 17 metabolisms of sEVs in two replications using aliquots from one sample.

and MALDI-TOF MS allows us to obtain high isolation recovery of intact sEVs and achieve high detection sensitivity. Our method will provide an efficient solution for acquiring valuable sEV information from a small amount of plasma.

CRediT authorship contribution statement

Wen Ye: processed the whole experiment, prepared the manuscript and Figures. **Reguang Pan:** processed the whole experiment, prepared the manuscript and Figures. **Ke-Qing Shi:** collected and analyzed the clinical samples. **Hui-Ping Li:** collected and analyzed the clinical samples. **Luke P. Lee:** conceived the project and designed the experiments, edited the manuscript, Supervision. **Fei Liu:** conceived the project and designed the experiments, edited the manuscript, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

W.Y. and R.P. contributed equally to this work.

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Appendix A. Supplementary data

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