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Isolation of Exosome Nanoparticles from Human Cerebrospinal Fluid for Proteomic Analysis

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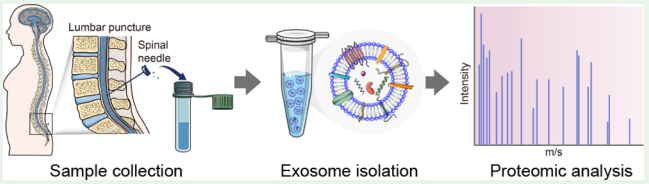
ABSTRACT: Exosomes are nanoscale (30−150 nm) biological vesicles that are actively released from living cells and circulating into all body fluids. Recently, exosomes in cerebrospinal fluid (CSF) have been recognized as promising biomarkers for central nervous system (CNS) neoplasms. In this study, we report a label-free method that can be used to rapidly isolate exosomes from CSF for proteomic analysis. Compared to ultracentrifugation and polyethylene glycol-based precipitation, our method isolates exosomes from 2 mL of CSF within 10 min, which is 18 times and 72 times shorter, respectively; the yield was increased by 4.47 times and 2.09 times and the purity was increased by 4.54 times and 9.76 times, respectively. The proteomic analysis further revealed that the exosomes isolated by our method identified more exosome-related proteins, which may reflect the physiological status of diseases for exosome-based diagnosis. Therefore, the effective isolation of pure exosomes from CSF samples for protein analysis will benefit the downstream analysis and clinical translation of exosomes, thus promoting the early diagnosis of CNS neoplasms.



KEYWORDS: *cerebrospinal fluid, exosomes, purification and enrichment, proteomics, diagnostics*

INTRODUCTION

Exosomes, which are nanometer-sized (30−150 nm) vesicles released from living cells into the extracellular space, play a vital role in different physiological and pathological processes.[1,2] Exosomes are found in many bodily fluids, such as blood,[3] urine,[4] saliva,[5] and cerebrospinal fluid (CSF).[6] The exosomal cargo carries disease-specific molecular biomarkers and can reflect the real-time physiological status of the disease.[7−10]

As the ultrafiltrate of plasma produced by specialized ependymal cells,[11−13] CSF provides mechanical and immune protection to the central nervous system (CNS) neoplasms.[14] Because it is difficult to collect living tissues from the brain, CSF is the primary source for liquid biopsy when diagnosing CNS-related diseases, including the brain tumor and Alzheimer's disease (AD). CSF directly connects with the brain tissue through the blood−brain barrier, thus CSF exosomes could accurately reflects the pathological changes in the brain, spinal cord, and meninges.[11]

However, Albumin and immunoglobulin, the two most abundant proteins in CSF, may reduce the identification of low-abundance proteins and cause significant information loss in exosome-based diagnostics.[15] Therefore, an additional purification step is usually required before the analysis of CSF. The current mainstream exosome isolation methods, such as ultracentrifugation (UC)[16] and polyethylene glycol-based precipitation method (PEG method),[17] often require long processing times and large sample volumes. At the same time, these often fail to deliver high-quality exosomes with regard to purity and yield.[18] Therefore, next-generation methods are necessary to obtain high-purity exosomes and establish a reliable protocol for brain disease diagnostics based on CSF exosomes.

In this study, we applied our early developed label-free exosome isolation method (exosome detection via the ultrafast-isolation system (EXODUS)[19]) for the fast isolation of pure exosomes from CSF. The system consists of an automatic workstation and a microfluidic device with a pair of nanoporous membranes (with pore size of 20 nm and a diameter of 13 mm) for clog-free ultrafiltration based on the principle of negative pressure oscillation.[19] The sensitivity and efficiency of EXODUS for isolation and characterization of CSF exosomes were characterized by comparing with UC and PEG methods.

RESULTS AND DISCUSSION

Workflow for the Isolation and Analysis of CSF Exosomes. Figure 1 shows a schematic diagram of exosome isolation from the CSF sample and proteomic analysis of exosomal proteins. After collecting the CSF sample by lumbar puncture (Figure 1a), the EXODUS device[19] (Figure 1b) was

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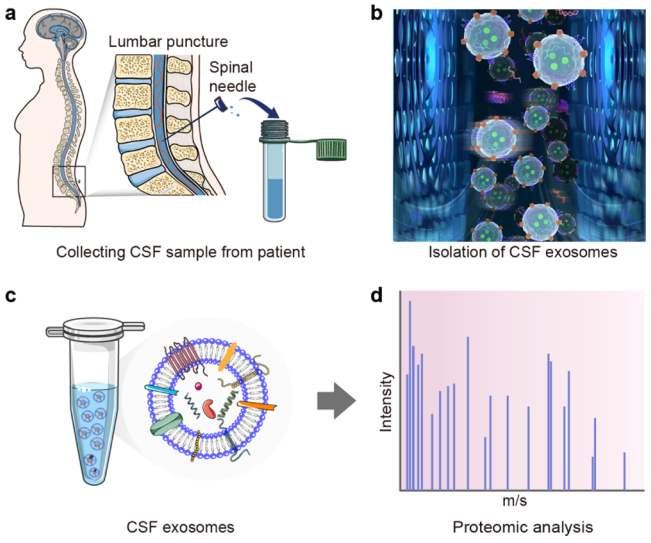

Figure 1. Schematic of exosome isolation and downstream analysis of exosomal proteins from CSF. (a) Collection of CSF by lumbar puncture. (b) Schematic of EXODUS showing the working principle of the double-filter based ultrafiltration with the negative pressure oscillation. Collection of purified exosomes (c) in a tube for (d) proteomic analysis.

used to separate exosomes from the CSF sample (Figure 1c) in an automatic, rapid, high-purity, and high-yield manner. Then, the collected exosomes were analyzed by downstream proteomics (Figure 1d).

Comparison of Exosome-Isolation Performance between EXODUS and Conventional Methods (UC and PEG). The performance of EXODUS was compared with UC and PEG method in varied metrics by isolating exosomes from aliquots of CSF samples (2 mL) with the same final volume of 200 μL for subsequent analysis. ZetaView system, Qubit, dynamic light scattering (DLS), western blotting (WB), transmission electron microscopy (TEM), and Bioanalyzer were used to characterize the isolated exosomes. As shown in Figure 2a and Supporting Information Table S1, the isolation time for EXODUS (within 10 min) was much shorter (all $p < 0.001$) than the PEG method (12.5 h) and UC (3 h). The PEG method required an additional incubation time (more than 12 h).

Protein and particle amounts are commonly used to evaluate the quality of exosomes.[18] To determine the size distribution, freshly isolated exosomes were analyzed using the ZetaView system. Exosomes isolated using UC and EXODUS were less than 140 nm in size, while exosomes isolated by the PEG method showed a broader size distribution with a shift toward a bigger size (154.83 ± 6.66 nm), which may be due to the fact that polymer-based precipitation methods simultaneously separate non-vesicular contaminants (including lipoproteins). Moreover, regarding the yield of particles, EXODUS isolated 2.3-fold and 2.9-fold more particles compared to the PEG method and UC, respectively (Figure 2b, Supporting Information Table S1).

The total amount of exosome proteins was determined using a Qubit protein assay kit (Q33212, Invitrogen, California, USA). PEG-precipitated exosomes had the most abundant proteins ($p < 0.001$), followed by the EXODUS and UC (Supporting Information Figure S2a and Table S1). We then calculated the particle to protein ratio (ratio of particle number to protein amount) of the exosomes purified by three methods, which have been used as an indication marker of protein contaminants and exosome purity.[18] EXODUS had shown the lowest ($p < 0.001$) protein contamination and the highest exosome purity among the three methods. The lower purity of the UC method could result from lower yields of exosomes, while a lower purity of the PEG method may be due to the heavy contamination of non-exosome proteins (Supporting Information Figure S2b and Table S1). A recent study has shown that shorter multiple cycles of UC provide a higher exosome purity from biological fluids compared to most other methods or one long cycle of UC.[20] Therefore, we compared the EXODUS results with the multiple-cycle method. The results showed that the yield and protein content of exosomes were greatly reduced, and the relative purity of exosomes was lower when using multiple cycles of UC compared to EXODUS (Supporting Information Figure S1 and Table S2).

The particle and protein amounts were insufficient to fully analyze the yield and purity of exosomes since ZetaView system cannot differentiate exosomes from molecules of similar size (*e.g.*, small lipoproteins). To address this issue, we used western blotting and silver staining to analyze exosomal proteinss, including Mac-2BP, flotillin 1, CD81, and CD9, as well as non-exosomal proteins such as albumin (ALB) and immunoglobulin (IgG). The two sample-loading methods, equal-mass gel-loading (2 μg of total proteins) and equal-volume gel-loading (30 μL of each sample) were used. The western blotting results (Figure 2c,d (left), Supporting Information Figure S2d–g) and the relative gray value (Supporting Information Figure 2h,i) showed that exosomes enriched by EXODUS exhibited higher ($p < 0.05$) signal intensities of exosome-specific proteins and lower signal intensities of non-exosomal proteins compared to the UC and PEG methods. Besides, silver staining demonstrated that EXODUS removed the most abundant proteins compared to the PEG method and UC (Figure 2c,d, right, and Supporting Information Figure S2d–g, right). The PEG method unsurprisingly showed the highest protein yield due to its isolation mechanism. In addition, the residual PEG in the sample caused the deviation of protein molecular weight and band dispersion.

To evaluate particle stability and integrity, the zeta potential of isolated exosomes was measured using DLS. Typically, a higher magnitude of zeta potential indicates higher repulsion between the particles in suspension, suggesting higher dispersion stability. Supporting Information Figure S2c shows that the negative charges of exosomes isolated by UC and EXODUS were very close. In contrast, exosomes from the PEG method showed the least charge reflecting the worst particle dispersion. We then characterized exosome morphology using negative staining TEM. TEM images revealed that the vesicles separated by UC and EXODUS had a similar size and morphology, while the PEG-isolated exosomes were aggregated and non-uniform (Figure 2e). Statistical analysis of the particle size distribution in the TEM image of exosomes separated by EXODUS showed that the mean size was 56.46 ± 4.88 nm, the median size was 50.35 nm, and the diameter was less than 140 nm (Supporting Information Figure S3).

Furthermore, we also compared the separation effect of EXODUS with the size-exclusion chromatography (SEC) method (Figure 2f–h, Supporting Information Figure S4, and Table S3). There was no significant difference in the size distribution of exosomes separated by EXODUS and SEC

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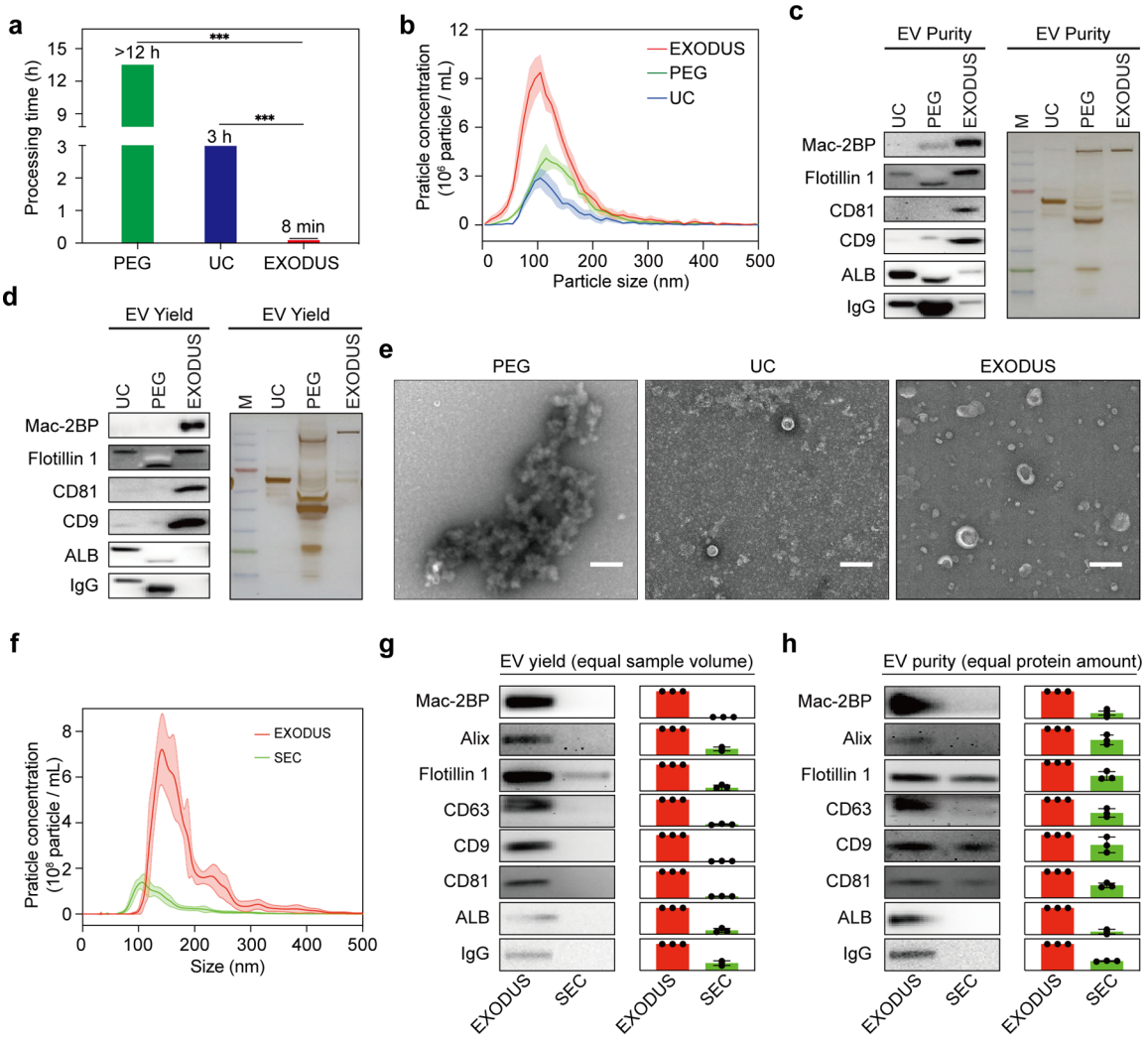

Figure 2. Comparison of the EXODUS with UC and PEG method for exosome isolation. (a) The processing time of EXODUS (5−10 min), UC (3 h), and PEG method (>12 h). (b) The NTA profiles of exosomes isolated from CSF. (c,d) Western blotting analysis of exosomal proteins (Mac-2BP, flotillin 1, CD9, and CD81), non-exosomal proteins (ALB and IgG). (c) Equal-mass gel-loading (2 μg) and (d) equal-volume gel-loading (30 μL) to evaluate the purity and yield of exosomes, respectively. (e) TEM images of exosomes isolated by PEG method, UC, and EXODUS. Scale bar = 200 nm. (f) NTA profiles of exosomes isolated from CSF using EXODUS and SEC. (g) Equal-volume gel-loading (30 μL) analysis of exosomal proteins by western blotting and the relative gray value for evaluating the yield of exosomes. (h) Equal-mass gel-loading (2 μg) analysis of exosomal proteins by western blotting and the relative gray value characterization for evaluating the purity of exosomes. The experiments were repeated three times.

method (Figures 3a and 2f, and Supporting Information Table S3). The concentration of particles and proteins and yield of exosomes separated by EXODUS were significantly higher than those by SEC (Supporting Information Figure S4b,c and Table S3). The yield of exosomes isolated by EXODUS was about 5.5 times higher than that of SEC (Supporting Information Figure S4c). However, the purity (particle number/protein amount) of exosomes isolated by EXODUS was slightly lower than that of SEC (Supporting Information Figure S4c). The western blotting results and the relative gray value (Figure 2g,h and Supporting Information Figures S4d,e) showed that exosomes enriched by EXODUS exhibited higher signal intensities of exosome-specific proteins, as compared to SEC. This is consistent with the results displayed in Supporting Information Figure S4c. To meet downstream analysis, SEC method requires a large amount of starting biological fluid to compensate for the yield. Although the SEC's sample processing time is about 20 min, it still requires a lot of hands-on steps to prepare the separation column, washing and (re)equilibration. In addition, the SEC method needs about 2h concentration steps before and after sample separation, which may result in a decrease in yield.

Characterization of Exosomal RNAs. Since exosomes are considered an important source for RNA biomarkers, we determined the total RNA amount in exosomes using the Bioanalyzer. We evaluated the yield and size distribution of the exosomal RNAs isolated by the three methods (Supporting Information Figure S5), which exhibited the same size range (<100 nt) corresponding to the small RNA, consistent with the characteristics of exosome-RNA molecules.[21] Long RNA (>200 nt) was not observed due to its small amount in exosomes. With regard to the RNA yield, EXODUS showed the highest intensity of RNA peak compared to the other methods (Supporting Information Figure S5).

Proteomic Profiling of Exosomes. High-throughput mass spectrometry (MS) has been applied for systematically

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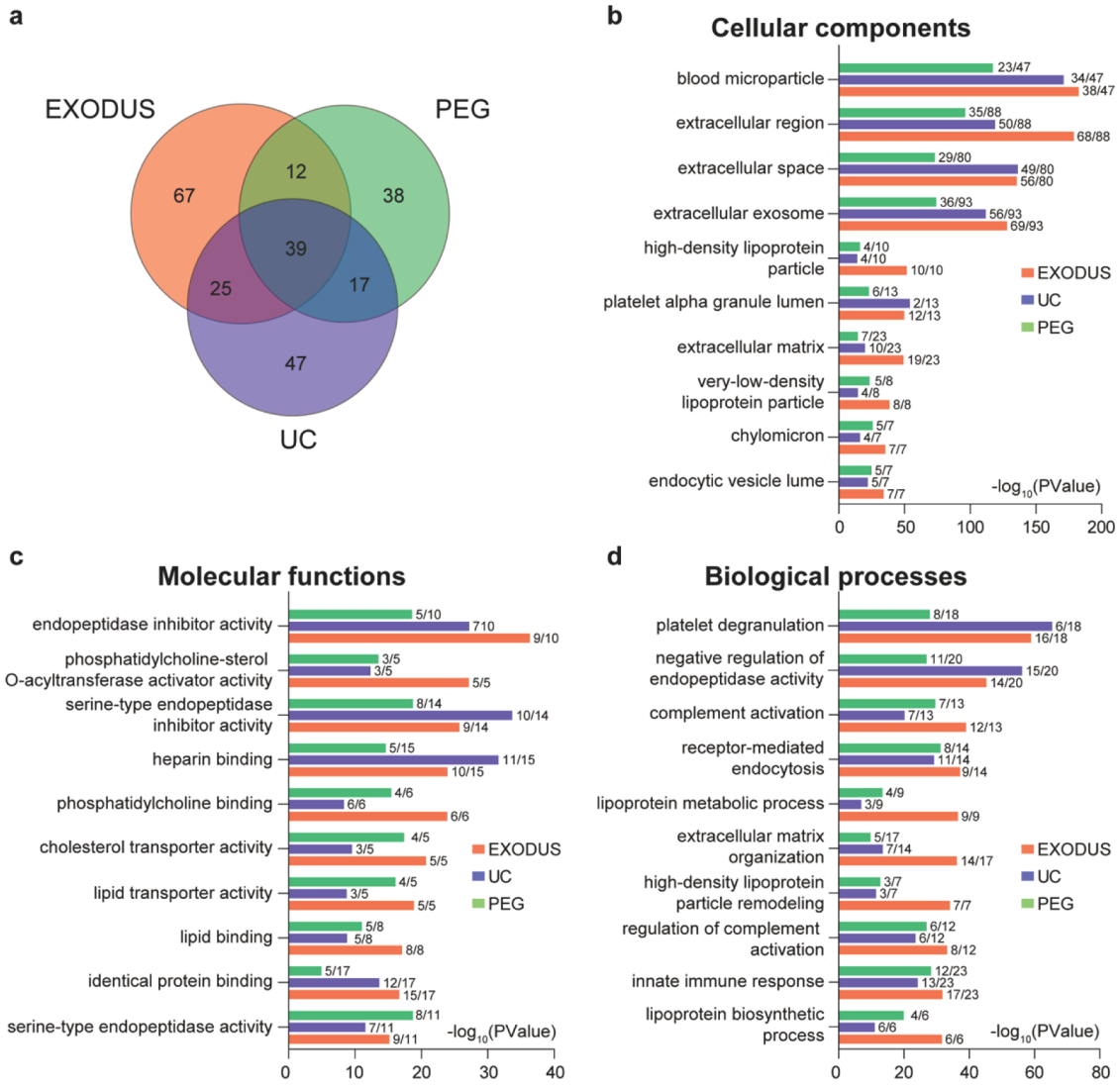

Figure 3. Qualitative and quantitative proteomic analysis of exosomes using LC−MS/MS. (a) Venn diagram of exosomal proteins identified by EXODUS, PEG, and UC methods. (b−d) Iexosomal proteins in CSF-derived exosomes from the three isolation methods: analyzed by GO. For the (b) cellular components, (c) molecular functions, and (d) biological processes of CSF-derived exosomal proteins based on GO analysis.

analyzing thousands of proteins in complex biological samples.[8] We developed a workflow for collecting liquid chromatography−mass spectrometry (LC−MS/MS) data of exosome proteins using a Q-Exactive Orbitrap HF hybrid mass spectrometer combined with an Ultimate 3000 RSLC nano-HPLC system. We then compared protein classification and amounts from proteomic analysis of exosomes isolated by EXODUS, PEG, and UC methods.

The Venn diagram shows the overlap of the identified proteins from the three isolation methods. In total, 245 proteins (Supporting Information Table S4) were identified with 58% (143/245), 52% (129/245), and 44% (107/245) by EXODUS, UC, and PEG method, respectively. Among them, 39 proteins were commonly expressed from exosomes isolated by the three methods (Figure 3a). Fewer proteins were detected by the PEG method, probably due to the presence of high-abundance proteins, which prevented low-abundance proteins from being identified. The number of uniquely identified proteins was 67 for EXODUS, 47 for UC, and 38 for the PEG method.

We then performed gene ontology (GO) analysis of the unique proteins from exosomes isolated by the three methods. The number of exosome-associated proteins was 33, 18, and 9 for EXODUS, UC, and PEG method, respectively. A higher number of proteins associated with exosomes from EXODUS indicated the most effectiveness in removing high-abundance protein contaminates as well as obtaining the highest isolation yield of exosomes.

Besides, GO annotation analysis in DAVID Bioinformatics Resources 6.7 (https://david.ncifcrf.gov/) was used to categorize the proteins enriched from three isolation methods as cellular components (Figure 3b), molecular functions (Figure 3c), and biological processes (Figure 3d). The top 10 statistically significant items are listed in sequence. Each category represents the enriched functions of and specific proteins with the data expressed as the $\log_{10}p$ value. The higher the p-value is, the richer the function of the relative term. Regarding cellular components, these terms indicate that blood microparticles, extracellular region, extracellular space, and extracellular exosomes are the most enriched parts of constitutive exosomes, which are similar to the vesicles and

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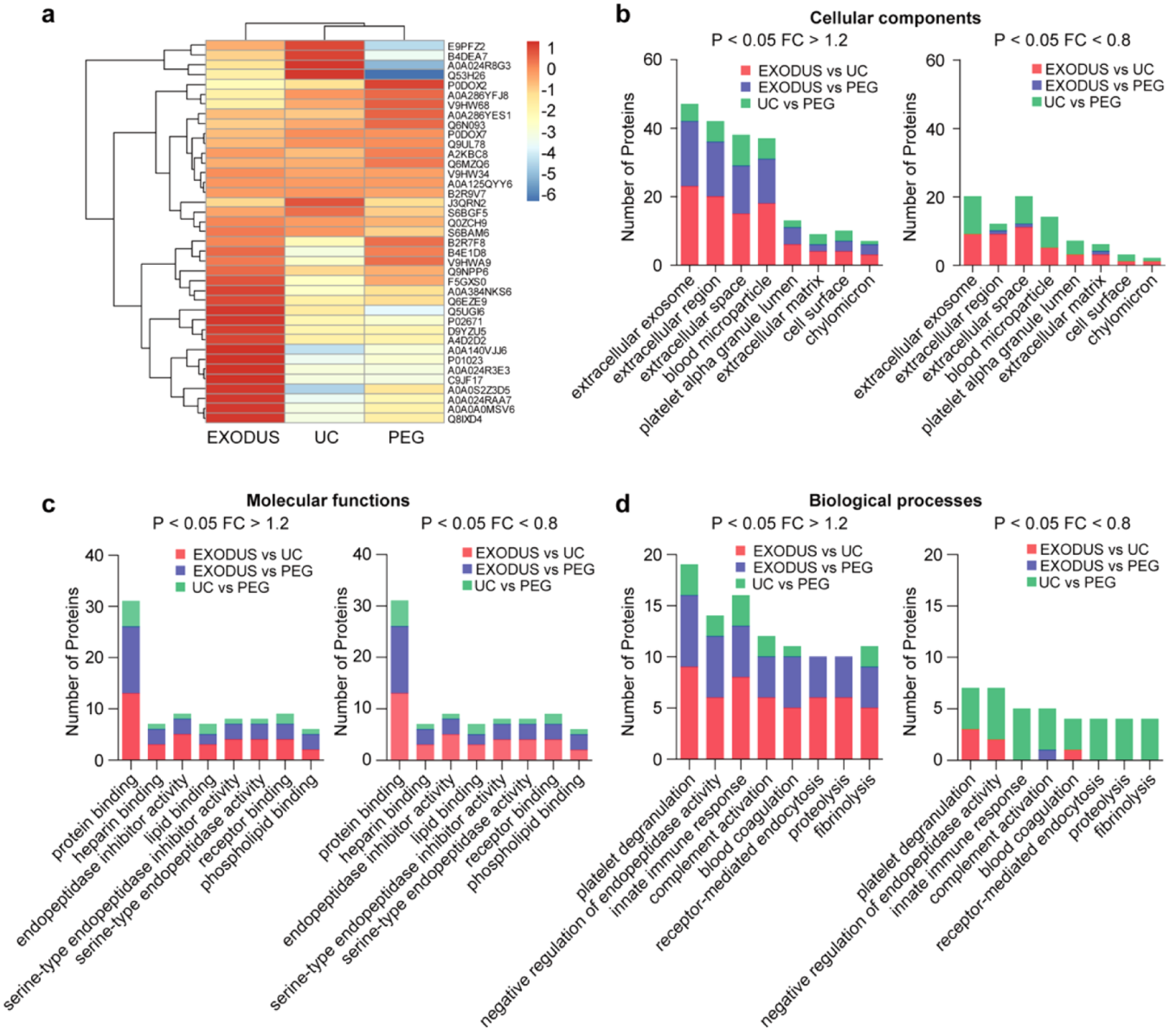

Figure 4. Quantitative proteomic analysis of the enriched exosomal proteins. (a) The heat map of the enrichment levels of significantly different proteins in different methods. Higher enrichment and lower enrichment are represented by red and blue colors, respectively. The Differentiation and classification of enriched proteins for (b) cellular components, (c) molecular functions, and (d) biological processes using GO annotation analysis.

extracellular properties of these exosome proteins. The highly enriched items, including blood microparticles, high-density lipoprotein particles, and very low-density lipoprotein particles, confirm that CSF is derived from the plasma.[13] In the extracellular exosome (Figure 3b), the exosome proteins isolated by the EXODUS (69) contained the most species, accounting for 74% of the total proteins detected (93), followed by UC (56) and then the PEG method (36). The results indicated that EXODUS showed the best performance for CSF-exosome isolation.

Figure 3c shows the GO enrichment analysis of molecular functions, which include the endopeptidase inhibitor activity, serine-type endopeptidase inhibitor activity, and heparin-binding. These abundant molecular functions are closely related to the corresponding biological processes, such as receptor-mediated endocytosis, which indicate the molecular functional interaction of each protein and their participation in these meaningful biological processes. Besides, other processes may also be associated with the exosomes' specific functions, such as complement binding and immune response. For biological processes (Figure 3d), the exosomal proteins are mainly involved in platelet degranulation, complement activation, receptor-mediated endocytosis, and innate immune response, which indicate that CSF exosomes may also contribute to the immune system.

Differentially enriched exosomal proteins (see Supporting Information Table S5) were clustered by the three isolation methods and visualized with heat maps (Figure 4a). The cellular components, molecular functions, and biological processes were classified for the different enriched proteins. Compared to UC and PEG method, most EXODUS proteins were associated with extracellular exosomes, extracellular region, extracellular space, and other cellular components, in which extracellular exosome-related proteins were significantly

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enriched in the exosomes separated by EXODUS (Figure 4b). This suggests that exosomes isolated by EXODUS carrying more exosome-associated biomarkers, supporting that the EXODUS isolation method has greater potential for exosome-based clinical applications. Molecular functional analysis showed that exosomes isolated uisng the EXODUS method had a higher enrichment of proteins related to protein binding, receptor binding, and endopeptidase inhibitor activity than those of UC and PEG method (Figure 4c), indicating that the EXODUS has less influence on the function of exosome molecules during the isolation process. With regard to biological processes, we observed that EXODUS had higher enrichment of proteins related to innate immune response, receptor-mediated endocytosis, and complement activation than achieved by UC and PEG method (Figure 4d), thus suggesting that the molecular function was closely related to the corresponding biological processes (*e.g.*, receptor binding and inverted immune response), and that exosomes, which carry the proteins for molecular functional interactions, participate in these meaningful biological processes. In summary, exosomes isolated by the EXODUS not only carry more exosome-associated proteins but also can promote biological processes such as immune response and complement activation.

In summary, compared to the UC and PEG method, exosomes separated by EXODUS showed a higher purity. Still, a certain number of high-abundant proteins were detected from all tested isolation methods, which may mask the signals of low-abundant proteins. Therefore, to achieve a better detection of proteins with low-abundance, the more sensitive MS detection strategy might be needed such as the tandem mass tag (TMT) method or 4-dimensional MS detection method for the future investigations.

MATERIALS AND METHODS

Collection and Storage of CSF Sample. All CSF samples used in this experiment were provided by Tongji Hospital of Huazhong University of Science and Technology and were approved by the ethics committee. A dedicated clinical expert approved each patient before collection, and the written consent was obtained from each patient.

The CSF was collected from five patients treated for hydrocephalus.[22] As part of routine clinical management, a CSF drain was inserted peri-operatively, after which the CSF was collected. Approximately, 50−100 mL of lumbar puncture catheter drainage fluid was collected from each patient. Before the experiment, we mixed the collected different human CSF samples to ensure that the method comparison would not interfere with the differences between samples from different patients. The CSF samples were collected using a waist-piercing pipe, centrifuged at 500*g* for 10 min at 4 °C, to remove any intact cells. The supernatant was collected and stored at -80 °C until further use.

Exosome Isolation using EXODUS. The EXODUS method for isolating exosomes has been recently developed in our laboratory.[19] In brief, the CSF sample was thawed on ice, after which 2 mL of CSF sample was centrifuged at 2500*g* (Rotor S-4-72, Centrifuge 5804 R, Eppendorf AG, Hamburg, Germany) for 10 min to remove the cell debris. Next, the sample was filtered using a 0.22 μm membrane filter (FPE-204-013, JET BIOFIL, Guangzhou, China) to eliminate microvesicles and apoptotic bodies. Subsequently, a 10 mL centrifugation tube containing 2 mL CSF sample (1 mL per time) was then placed into the sample position in the EXODUS, after which the sample was added to the EXODUS device [anodic aluminum oxide (AAO) membrane: pore diameters, 20 nm; diameter, 13 mm] automatically by a needle for exosome isolation. All parameters, including the negative pressure, conversion time, single injection volume, and repeating cycles, were set to -30 kPa, 10 s, 1 mL, and 2 times, respectively, for exosome isolation. Before use, the device was autoclaved at 121 °C for 15 min to avoid contamination. Before processing CSF samples, the chip membrane was conditioned by flushing with 1 mL PBS 2 times. After the separation procedure was over, the EXODUS device automatically popped out, and the chip was removed from the workstation to obtain the exosomal suspension (about 200 μL).

Exosome Isolation using UC. The exosome isolation by UC was performed according to the previously described method.[16] In brief, the CSF sample was thawed on ice, after which 2 mL of CSF sample was centrifuged at 2500*g* for 10 min to remove the cell debris. The sample from the last step was then filtered using a 0.22 μm membrane filter to eliminate microvesicles and apoptotic bodies. The supernatant was then centrifuged using an ultracentrifuge (P40ST, Ultracentrifuge CP100NX, himac-science, Tokyo, Japan) under the conditions of 120,000*g*, 4 °C for 2 h. A pipette was used to carefully remove the supernatant, and the exosome pellet was resuspended in 200 μL of PBS and stored at −80 °C until further use.

Exosome Isolation using PEG Method. The PEG-based exosome precipitation method (EXOCG50A-1, System Biosciences, California, USA) was performed for exosome isolation according to the manufacturer's instructions. Before exosome isolation, the CSF samples were centrifuged at 2500*g* and filtered using a 0.22 μm membrane filter. To start the precipitation, 2 mL of the filtrate from the last step was gently mixed with 0.64 mL of ExoQuick solution. Thereafter, the mixture was incubated at 4 °C for at least 12 h, and the ExoQuick solution was then spun down by centrifugation at 1500*g* (Rotor FA-45-24-11, Centrifuge 5424 R, Eppendorf AG, Hamburg, Germany) for 30 min. The supernatant was discarded, and the exosome pellet was resuspended in 200 μL of PBS and stored at −80 °C until further use.

Nanoparticle Tracking Analysis. The exosome concentration and particle distribution were determined using ZetaView (PMX110-s, Particle Metrix, Meerbusch, Germany). All samples were diluted in PBS to a final volume of 1 mL. For each measurement, two cycles were performed by scanning 11 cell positions for each and capturing 60 frames per position under the following settings: focus: autofocus; camera sensitivity for all samples: 70; shutter: 70; scattering intensity: 4.0; temperature: 25 °C. After capture, the videos were analyzed using in-build ZetaView software PMX110 V3.0 with specific analysis parameters: max area: 1000, min area: 5, min brightness: 20. Hardware: embedded laser: 40 mW at 488 nm, camera: CMOS.

Western Blotting Analysis. Exosomes were lysed with a loading buffer (P0015L, Beyotime Biotechnology, Shanghai, China) and boiled at 100 °C for 10 min. 2 μg or 35 μL of total protein of each sample was separated in a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) blotting membrane (10600023, GE Healthcare Life Science, Freiburg, Germany). Membranes were blocked in 1× PBS-T (0.5% Tween-20) with 5% non-fat dry milk for 1 h at room temperature and then incubated with the following primary antibodies: mouse anti-flotillin 1 (1:1000) (610820, BD Biosciences, New York, USA), anti-Mac-2BP (1:1000) (sc-374541, Santa Cruz Biotechnology, Texas, USA), anti-CD81 (1:500) (SC-166029, Santa Cruz Biotechnology, Texas, USA), anti-CD9 (1:500) (sc-13118, Santa Cruz Biotechnology, Texas, USA), anti-ALB (1:1000) (ab151742, Abcam, Cambridge, UK), and anti-IgG (1:1000) (ab109489, Abcam, Cambridge, UK). After the washing step, the blots were incubated with an HRP-conjugated anti-mouse IgG secondary antibody (1:3000) (7076S, Cell Signaling Technology, Massachusetts, USA) at 4 °C for 1 h. The signals were measured using a Feike class super-sensitive ECL luminous liquid (Pei Qing Science & Technology, Shanghai, China). Chemiluminescence was detected using the JS-M8 luminescence image analyzer (JS-M8, Pei Qing Science & Technology, Shanghai, China).

Zeta Potential Measurement. The purified samples were diluted in PBS for zeta potential analysis, which was performed by DLS using a Zetasizer Nano ZS90 (ZS90, Malvern Panalytical, Malvern, UK). Before the measurement, the samples were loaded

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onto the instrument at a 90° angle to the light source. All experiments were performed at a constant temperature of 25 °C and analyzed with Dispersion Technology software.

Transmission Electron Microscopy. For TEM, 20 μL of CSF exosome samples was placed on 200 mesh carbon-coated copper grids (BZ11022a, Beijing Zhongjingkeyi Technology Co., Ltd., Beijing, China) and incubated for 30 min at room temperature. The excess samples were wiped with a filter paper and left to dry for an hour and then negatively stained with filtered aqueous 2% uranyl acetate for 30 s. The negatively stained excess dye was then blotted dry with a filter paper and placed in a vacuum drying oven. Samples were then examined in an FEI Tecnai transmission electron microscope at an accelerating voltage of 100 kV.

RNA Extraction and Analysis. CSF exosomes were processed for RNA extraction using the RNeasy Mini Kit (74104, QIAGEN, Frankfurt, Germany) according to the manufacturer's protocol. QiAzol Lysis Reagent buffer (700 μL) and chloroform (140 μL) were added to each sample. The samples were mixed well for 30 s and then incubated for 5 min at room temperature. The phase separation was carried out by centrifugation at 12,000*g* at 4 °C for 15 min. The upper aqueous phase was collected and 1.5 volumes of absolute ethanol were added. Subsequently, 700 μL of RWT buffer and 500 μL of RPE buffer were sequentially added, and RNA was extracted by centrifugation. Finally, the RNA sample was eluted with 30−50 μL of RNase-free water. Total RNA was analyzed using the Qubit RNA HS Assay Kit (Q32855, Invitrogen, Thermo Fisher Scientific, Massachusetts, USA), and RNA quality was assessed using Qsep1 (Portable Single-Channel CGE System, Qsep1, Bioptic, Taiwan, China) to confirm the yield and the size distribution.

MS Sample Preparation and Tryptic Digestion. Purified exosomes were solubilized in the lysis solution containing 8 M urea, 30 mM *N*-2-hydroxyethyl piperazine-*N*′-2-ethane sulfonic acid (HEPES), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM ethylenediaminetetraacetic acid (EDTA), and the total protein concentration was determined using the BCA assay. 20 μg of protein volume was taken for each sample, and dithiothreitol was added to a final concentration of 10 mM in a 56 °C water bath for 1 h. After removal, iodoacetamide (IAM, Promega) was added to a final concentration of 55 mM and was left in the dark room for 1 h. After reductive alkylation, the sample was added to a 10 kDa ultrafiltration tube and centrifuged at 14,000*g* at 4 °C for 40 min, after which the waste liquid was discarded. Next, 200 μL of 25 mM ammonium bicarbonate was added and centrifuged at 14,000*g* at 4 °C for 40 min, after which the waste solution was discarded. The above steps were repeated twice. Next, 0.9 μg of trypsin was added in a 37 °C water bath for 24 h (trypsin/protein = 1:22). The digestion fluid was collected, lyophilized, and the peptide was reconstituted with 40 μL of 0.1% formic acid (FA) per tube.

LC−MS/MS Analysis and Database Search. LC−MS/MS analysis was conducted on an UltiMate 3000 nano-LC system (Thermo Fisher Scientific, Dionex, California, USA) coupled with a Q-Exactive mass spectrometer (Thermo Fisher Scientific Waltham, MA, USA) for 65 min. Next, the peptides were desalted on C18 cartridges (75 μm × 10 cm, 5 μm, 300 Å, Agela Technologies, Delaware, USA), concentrated by vacuum centrifugation, and reconstituted in 40 μL of 0.1% (vol/vol) FA. The gradient comprised an increase from 5 to 30% solvent B (0.1% FA in 98% ACN) over 40 min, 30 to 60% in 5 min, and climbing to 80% in 3 min, then holding at 80% for the last 7 min, all at a constant flow rate of 400 nL/min on a Dionex Ultimate 3000 nano-LC system.

The data-dependent acquisition was performed with an MS scan mass window set in a positive-ion mode and in a data-dependent manner with full MS scan from 350 to 2000 *m*/*z*; the MS/MS scan resolution was 17,500. In addition, the capillary temperature was 320 °C, ion-source voltage was 1800 V, and fragmentation mode was higher collision energy dissociation (HCD).

After the MS was scanned, the original MS file was obtained. Each sample was tested three times (a total of three samples and nine test results). The three test results of different samples were combined and searched. The mass spectra were searched against the UniProt-taxonomy-9606 database restricted to *Homo sapiens* (20,194 sequences) using Mascot Version (1.5.2.8, Matrix Science Ltd., London, UK). Trypsin was specified as the cleavage enzyme with allowances set for up to one missed cleavage. The mass tolerance for precursor ions was set to 15 ppm in the First search and 20 mmu in the Main search. FDR was adjusted to <1%. *T*-test analysis was used to evaluate the significance of the differences.

CONCLUSIONS

CSF-derived exosome nanoparticles are considered as valuable biomarkers for the early molecular diagnosis of brain tumors and neurological diseases. The lack of efficient methods for isolating exosomes from CSF with a high purity remains a significant challenge, which limits the exosome analysis and its application for early-stage disease diagnosis and clinical translation. EXODUS is an efficient and rapid approach for isolating exosomes from human CSF samples compared to the conventional isolation methods, such as UC and PEG method. The main advantages of EXODUS compared to UC and PEG method for the isolation of CSF exosomes are the following: first, it takes less than 10 min for EXODUS to isolate exosomes from 2 mL of CSF samples, which is significantly faster as compared to UC (3 h) and PEG method (12.5 h). Second, EXODUS provides 2.09-fold and 4.47-fold higher particle yield compared to the PEG method and UC, respectively. Considering that EXODUS achieves higher exosome purity than UC and PEG methods, the actual exosome yield should be higher than the values measured by NTA. Thus, to acquire the same yield of exosomes for the PEG method and UC, it is necessary to increase the original sample volume, which is not always possible when analyzing samples such as CSF. Third, EXODUS may carry more exosome-associated biomarkers, indicating that EXODUS has higher potential in exosome-based clinical applications.

In summary, we successfully used the EXODUS method for high-speed isolation of exosome nanoparticles from CSF samples with high purity and high recovery. As a robust and high-efficient exosome-isolation approach, EXODUS may contribute to the basic research of CSF exosomes and speed up the clinical translation of exosomes for the molecular diagnosis of CNS neoplasms.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsanm.0c02622.

Comparison of EXODUS exosome-isolation performance between multiple cycles of UC; comparison of EXODUS exosome-isolation performance between UC and PEG method; statistical bar chart of the TEM image (EXODUS); comparison of EXODUS exosome-isolation performance with SEC; analysis of exosome RNA isolated by PEG method, UC, and EXODUS; comparison of exosome-isolation performances between EXODUS and conventional methods (UC and PEG method); comparison of exosome-isolation performances between EXODUS and multiple cycles of UC; comparison of exosome-isolation performance between EXODUS and SEC; distribution of 245 proteins detected by LC−MS in the three isolation methods; and differentially enriched proteins (PDF)

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M.L. and L.H. contributed equally. All the authors have made a contribution to the manuscript. All the authors have approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AD, Alzheimer's disease
CNS, central nervous system
CSF, cerebrospinal fluid
EXODUS, exosome detection via the ultrafast-isolation system
PEG, polyethylene glycol
UC, ultracentrifugation
AAO, anodic aluminum oxide
MS, mass spectrometry
NTA, nanoparticle-tracking analysis
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel
PVDF, polyvinylidene fluoride
TEM, transmission electron microscopy
DLS, dynamic languages symposium
HCCA, 4-hydroxy-α-cyanocinnamic acid
HEPES, *N*-2-hydroxyethyl piperazine-*N*′-2-ethane sulfonic acid
PMSF, phenylmethylsulfonyl fluoride
EDTA, ethylenediaminetetraacetic acid
IAM, iodoacetamide
FA, formic acid
LC−MS/MS, liquid chromatography−mass spectrometry
HCD, higher collision energy dissociation
TMT, tandem mass tag

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