

An isolation system to collect high quality and purify extracellular vesicles from serum

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Abstract

Background: Extracellular vesicles (EVs) are membrane encapsulated nanoparticles that function as carriers and play a role in intercellular communication. There are a large number of EVs in the blood and serve as an indicator of pathophysiological conditions. Studies on the basics and application of EVs are hampered by the limitations of current protocols to isolate EVs from blood. However, current isolation methods are difficult to achieve a balance between yield and purity.

Results: Firstly, we use Sepharose-4B to build a self-made size exclusion chromatography (SEC) column and perform separation and identification. Then we use the SEC column to systematically compare the efficiency with the most common EV isolation methods: ultracentrifugation (UC) and total exosomes isolation commercial kit (TEI). The EVs isolated through different methods were characterized the yield and size of EVs, analyzed their protein profiles, the morphology and purity were observed under the transmission electron microscope. To further improve the quality and purity, we combined SEC and UC methods and established a two-steps method to isolated EVs from serum.

Conclusion: Our study presents the combination of size-exclusion chromatography and ultracentrifugation as a feasible and time-saving method to isolate high quality and purity extracellular vesicles from serum.

Background

Extracellular vesicles (EVs) are membrane encapsulated nanoparticles that are released by all cell types into extracellular space and serving as multicomponent intercellular signaling devices in a diverse range of physiological and pathophysiological processes(1, 2). EVs contain diverse cellular molecules such as nucleic acids (DNA, RNA, miRNA) and proteins, and the different states of cells will directly affect the changes in the contents of EVs. EVs are also found in all biological fluids and rendered them attractive as minimally invasive liquid biopsies(3-11). Moreover, the amount of EVs in blood plasma or serum has been estimated between 10^7 and 10^{12} EV/ml(12, 13). Therefore, EVs presented in the blood have attracted the most interest as novel biomarkers for cancer, kidney, cardiovascular and neurodegenerative diseases(14-19).

But a mass of evidence shows that there are a large number of contaminants in the blood including non-vesicles protein complexes and lipoproteins particles like HDL, LDL, and VLDL. And the total number of these contaminant particles is at least 10^5 fold higher than EV(20). Due to the similar size, lipoproteins and protein aggregates are co-isolated with EVs(21, 22). This may result in contamination in downstream experiments and affect the experimental results. Despite the existence of multiple reports which compare EVs isolation methods, there is currently no agreement on an optimal technique to yield high-purity EVs from blood(16, 21, 23-26).

With the application of different technical methodologies, separation methods based on different principles have been proposed. Taking into account the convenient operation of each method, the easy

availability of materials, and the dependence on special equipment, the current separation of blood-derived EVs mainly focuses on the following three methods: ultracentrifugation (UC), polymer-based precipitation kits, and size-exclusion chromatography (SEC)(26-28). UC separates EVs based on different gradients of centrifugal force and has always been the standard method for EVs separation. However, UC still has certain deficiencies in the separation yield and residual other non-vesicular protein complexes and apolipoprotein particles(16, 21, 29). The polymer-based precipitation method can quickly and conveniently precipitate EVs to obtain the maximum separation yield. However, for the complex components and physical and chemical properties of blood, the non-selective precipitation feature of this method has caused much non-EVs serum or plasma protein impurities to be precipitated. Therefore, its separation purity has always been criticized(21, 30). SEC has attracted much attention and application for the separation of EVs from blood. SEC does not require centrifugation and the addition of reagents and can quickly obtain high-quality EVs(21, 22). However, since based on the principle of size, many apolipoprotein particles, cholesterol, or protein complexes similar in size to EVs are difficult to completely remove by the SEC method. Therefore, the purity of EVs obtained by the SEC is compromised(23, 31, 32). Due to the heterogeneity and overlapping characteristics with other constituents of biofluids, the separation of EVs with high purity and quality still has certain challenges.

Since it is difficult to balance the separation quality and purity by only one method, the strategy of combining different separation methods has been proposed and proved to be effective in improving separation efficiency and quality. Studies have reported that the combination of UC and the precipitation kit is compared with the precipitation kit alone, and the results show that the combined method significantly improves the separation efficiency(33). Similarly, using a combination of UC and SEC methods also showed better separation results (34, 35). Not only limited to the combination of the two methods, but a recent study also proposed a three-step protocol to achieve high yield and purity of EVs separation by sequentially using PEG precipitation, iohexol density gradients, and SEC(36). More and more studies have shown that the combination of different methods can achieve better separation results. However, for clinical and translational application aspects, avoiding the use of complex equipment and tedious operating procedures, and using the combination of existing, easily accessible, and easy-to-operate methods to achieve efficient separation of extracellular vesicles is currently urgently needed.

Although combination methods can improve the isolation effect of EVs, the yield of EVs decreased with the increase of steps. How to simplify the isolation method and increase the yield is a major problem to the basis and application of EVs at present(28). How to effectively isolate EVs from small samples is also needed at present. In this study, we refer to the method of Boing et al.(37) to build an SEC column with Sepharose-4B. Then we systematically compared recovery efficiency and purity with SEC, UC, and TEI. We further used an approach combining SEC and enrichment using UC or TEI respectively to optimize separation purity and recovery efficiency.

Results

SEC column efficiently separate EVs from serum protein

The Schematic diagram shows the procedures of the SEC isolation method and downstream characteristics (Fig.1a). To determine the fractions of EV particles and verify that the SEC method can effectively isolate EVs from serum, we collected 24 eluent fractions, detected the particle concentration and protein concentration of each fraction by Nanoparticle Tracking Analysis (NTA) and BCA protein assay. The distribution of proteins and EV particles in all 24 fractions shows that EV particles can be well separated from serum proteins by SEC (Fig. 1b). As seen from Fig. 1b, EV particles are concentrated in 8-13 fractions and the protein concentration is relatively low. From the 14th fraction, the concentration of EV particles is reduced, and the protein concentration is significantly increased. To directly visualize the efficacy of SEC to separate vesicles from serum proteins, all collected fractions were loaded for gel electrophoresis. Proteins became detectable from 11 fractions, but the bulk of the protein elutes from fraction 15 onwards, which is consistent with the protein distribution curve in Fig. 1b (Fig. 1c). Furthermore, we identified the markers of EV and lipoprotein contamination by Western Blot in 8-13 fractions and 14-24 fractions respectively. The EV markers CD9 and CD81 are highly expressed in 8-13 fractions, but low or undetected in 14-24 fractions. Conversely, apoA1, a lipoprotein associated with both HDL and chylomicrons, was highly expressed in 14-24 fractions (Fig. 1d). Transmission electron microscopy (TEM) was used to observe the collected EV fraction. As seen from Fig. 1e, it can be observed that fractions 9-11 contained structures of EV, with a characteristic cup-shaped appearance (the black arrow indicated). Besides, homogeneously smaller solid spherical structures could be discerned, which are characteristic of lipoprotein particles (the white arrow indicated). The protein highly enriched fraction 20 was also observed (Fig. 1e). However, the image of fraction 20 shows a blurry and thick protein layer and it is difficult to find the existence of vesicles. Based on the above results, it can be concluded that the self-made SEC column can well separate EVs from complex serum protein, and EVs enriched in the 8-13 fractions with good morphology.

SEC well balance the yield and purity compared with UC and TEI methods

To analyze the yield and purity of different methods, we compare SEC with the commonly used ultracentrifugation and Total Exosomes Isolation (TEI) kit (Invitrogen by Thermo Fisher Scientific, USA). To systematically compare recovery efficiency, an equal initial volume of platelet-free serum was used for each isolation method and rendered a final volume of 100 μ l (see Fig. 2a for a schematic representation). The EVs pellets collected by UC were invisible by the naked eye and yielded only 0.05 ± 0.02 mg protein per ml serum, TEI-induced EVs pellets were visible and yielded as much as 2.6 ± 0.4 mg protein per ml serum. The yield of EVs protein obtained by SEC was 1.2 ± 0.01 mg protein per ml serum which is between UC and TEI (Fig. 2b). UC, SEC and TEI yield 4.4×10^{10} , 1.3×10^{12} , and 2.4×10^{12} particles per ml serum, respectively, as determined by NTA (Fig. 2c). Moreover, we compare the three methods with the ratio of particle to protein, and the results show that SEC has outstanding recovery efficiency (Fig. 3d). According to measured size by NTA, those particles may be in the range of 70-120 nm and may represent EV. SEC-EVs have the smallest particle size of 85 nm, and the particle sizes of UC-EVs and TEI-EVs were similar, at 112 and 117 nm, respectively (Fig. 2e). The relative protein yield of these three methods was

also confirmed by SDS-PAGE followed by staining with Bio-Safe Coomassie G-250 (Fig. 2f). As seen from Fig.2f, the content of EV protein isolated from 1ml platelet-free serum by UC is very low, and the band is not distinct. So, to effectively compare, we tried to use 3 times the original volume of serum to isolate the same final volume of EVs by UC (Fig. 2e). EV proteins yielded by UC and TEI have distinct bands, but the electrophoretic bands are uneven due to the residual contaminants such as carbohydrates in the SEC collected component (Fig. 2e). Also, the EV markers CD9, CD63, and CD81 were highly expressed in SEC-EVs. The expression of EV markers isolated by UC from the initial serum volume of 1ml was very low or even unexpressed. The expression level was obvious after being isolated from 3 times the initial volume of serum, but the expression level in CD63 was still very low. The EVs yielded by TEI normally expressed CD9 and CD63, but CD81 was not detected (Fig. 2g). ApoA1 was also more efficiently collected by TEI than UC and SEC (Fig. 2g). All these three methods yielded EVs contained structures reminiscent of EV, with a characteristic cup-shaped appearance (the black arrow indicated) (Fig. 2h). However, the image of UC-EVs has a relatively clean background under electron microscopy, and the apolipoprotein is rare. The image of SEC-EVs has many vesicles similar in shape and size to EV may be apolipoprotein particles (the white arrow indicated). The image of TEI-EVs has the most complex background, and there are a large number of solid particles (the white arrow indicated), consistent with the biochemical data. (Fig. 2h). Overall, TEI harvests a high yield of protein and particles. But based on TEM and biochemical results, TEI is difficult to remove apolipoproteins, so the separation purity of TEI is unsatisfactory. UC method has better separation purity, but low protein and particle yield is its shortage. By contrast, SEC is outstanding in all aspects and balance both isolation purity and yield.

SEC+UC combined method achieves the optimization of quality and purity

In the previous comparison, we found that the SEC method well balanced the purity and yield. But SEC still has some shortcomings that need to be improved. As seen from the SDS-PAGE results in Fig. 2e that SEC is difficult to completely remove carbohydrates and other impurities in the serum. But UC and TEI can solve the shortcoming of SEC. Therefore, we consider combining these three methods. First, SEC is used to separate vesicles from protein components, and then UC or TEI is used to enrich the EVs separated by SEC to solve optimize separation efficiency and purity (see Fig. 3a for a schematic representation).

From the results, compared with using SEC alone, the amount of EVs protein obtained by SEC+UC and SEC+TEI was significantly reduced, which were 0.1 ± 0.07 and 0.19 ± 0.06 mg protein per ml serum, respectively. The results of particle concentration detection by NTA showed that SEC+UC obtained 6.1×10^8 and SEC+TEI obtained 9.2×10^9 particles per ml serum, both of which were lower than SEC alone (Fig. 3b,3c). We can see from the result of SDS-PAGE electrophoresis that the combined method solves the problems of carbohydrate residues that exist when SEC is alone, and the bands are distinct (Fig. 3f). For the particle size distribution results, SEC+TEI and SEC alone have the same particle diameter of 85nm, while the particle diameter obtained by the SEC+UC method is 132nm (Fig. 3e). Since the amount of EV protein obtained from 1 ml of platelet-free serum through the SEC+UC method is relatively low. We also expanded the serum sample volume by 3 times for the experiment. The results of the western blot showed that SEC+UC and SEC+TEI both expressed EV markers CD9, CD63, and CD81. Unfortunately, the

combined method of SEC+TEI did not remove the apolipoprotein particles. The SEC+UC method has better performance (Fig. 3g). Similarly, we observe the isolated EVs by TEM. Surprisingly, although the results of protein and particle content show that SEC+UC isolates the least yield of EVs from 1ml serum, abundant vesicle aggregation was observed under the electron microscope and they all showed a characteristic cup-shaped appearance of EV. And compared with SCE alone, the background of SEC+UC is clean, and the number of vesicles under the same scale is also large. It may be due to the centrifugal force of ultracentrifugation that caused vesicles to aggregate. This also explains the result of NTA's detection of particle size (Fig. 3d) that the SEC+UC method has a relatively high particle diameter (Fig. 3h). For the ratio of particle to protein, the separation efficiency of the SEC+UC method is higher than that of SEC alone. The ratio of the SEC+TEI method is significantly high, however, based on the analysis of WB and TEM results, this method cannot remove the apolipoproteins, which leads to a high ratio of particle to protein. This also shows that this ratio is not a good indicator of purity (Fig. 3d).

In summary, our results show that the SEC+UC combined method can separate EVs with high quality and purity from serum. The combination of the two methods can optimize the quality and purity of EVs, which is much better than using one method alone.

Discussion

How to effectively isolate extracellular vesicles and simplify the isolation process has always been a problem faced by the current research and application of EVs. In this report, SEC, UC, and TEI were compared for purifying EVs from blood serum. Tab. 1 summarizes and evaluates the results of all the above methods. Our comparison results show that SEC and TEI can obtain high-yield EVs, but the purity is inferior to UC. However, due to the limitations of NTA and BCA, EVs cannot be distinguished from other apolipoprotein particles, and the profile of measured particle number and protein concentration cannot be an accurate evaluation indicator for the purity and yield of EVs. By Western Blot analysis of apolipoprotein marker ApoA, it was excess in the TEI-EVs. Through TEM observation, it was also found that non-vesicle impurity particles also existed in SEC-EVs. Our results in Fig. 2e also showed that SEC-EVs contained some soluble impurities, which was consistent with the results of the published article by Baranyai et al(38). And these soluble factors are more in SEC-EVs than UC-EVs and TEI-EVs. This also further shows the advantages of the UC method over SEC for the separation of blood EVs. Due to its simplicity and commercial availability, the precipitation kit is quite popular. However, this technique is generally not recommended on account of the low purity achieved.

May by the reason of the complex physical and chemical properties of blood make the isolation of EVs more difficult. Some newer techniques such as field flow fractionation and microfluidics have been reported to achieve isolation of EVs and appear promising, but require expensive, specialized equipment, and are relatively low output limited the use. Some other studies have suggested that the combined use of multiple different methods can effectively improve the separation purity of EVs. However, it is not difficult to consider that as the combined operation steps of the method increase, it will inevitably lead to

a decrease in the amount of EVs obtained. Therefore, how to simplify the isolation operation steps and effectively improve the recovery efficiency is a problem that needs to be solved at present.

The main purpose of this research is to combine the most commonly used methods to maximize separation quality and purity. In our comparison results, SEC has the best separation efficiency and balances yield and purity. However, it is necessary to solve the problems of the apolipoprotein particles and soluble impurities. Interestingly, we found that UC can solve the shortcomings of SEC. Therefore, the method of combining SEC and UC achieves the best separation quality and purity. Our results show that the combination method SEC+UC will inevitably cause a decrease in the amount of protein and particles, but the ratio of particle to protein shows that SEC+UC has a better recovery efficiency than the method using SEC alone. However, we also found in the comparison that there are certain problems with the comparison method of the particle to protein ratio. We found that although the particle-to-protein ratio of SEC+TEI is much higher than that of SEC+UC and SEC, it can be seen from the results of Western Blot and TEM that there are still apolipoprotein particles in EVs obtained by SEC+TEI. This shows that the ratio of particle to protein is not a good indicator of the purity of the obtained EVs. This view is consistent with the results of the research done by Kaloyan et al(16). Additionally, our study has the advantage of using equal serum volumes from the same animal for each method biological replicate while maintaining the same final volume, which allowed us to systematically compare contaminating factors in the same experiment. Intriguingly, EVs separated from 1ml of serum by the SEC+UC method have a low total number of particles measured by NTA, but the number of vesicles observed by TEM under the same multiple fields of view is more than any other method. This also confirmed that, as previously reported by others, NTA can only detect the size of particles but cannot distinguish lipoprotein particles and protein aggregates that have a similar size to EVs. The presence of lipoproteins particles and protein aggregates will cause the particle concentration detected by NTA to be inaccurate. Therefore, NTA and protein amount are not desirable parameters for evaluating the amount of EV, there is no obvious correlation between particle and protein.

Conclusion

We have successfully demonstrated that high quality and purity serum derived EVs can be separated by two consecutive simple steps: SEC and UC. Comparing the ratio of particles to protein, the separation efficiency of SEC+UC is more than 10 times that of TEI and UC alone. This two-step protocol is convenient and fast, which only takes about a maximum of half a working day. Moreover, the materials and equipment required are the most commonly used, and no additional complicated equipment is required. Our results could serve as a universally applied standard method for EV-related research and clinical application.

Materials And Methods

Preparation of serum samples

C57BL/6J mice (25-30 g) were obtained from Dashuo Experimental Animal Co. Ltd (Chengdu, China). Mice were anesthetized with 250 mg/kg pentobarbital. The retro-orbital blood collection route was used and can provide moderate to large amounts of blood. The use of retro-orbital bleeding is performed by well-trained personnel. Microhematocrit tubes that hold 50-75 microliters were used to minimize the risk of injury. Only one eye was sampled at any time. Alternate between left and right eyes per session and a maximum of 3 procedures were performed per eye (up to 6 collections total). If injury and/or rupture of the eye or surrounding tissues occurs, the animal was immediately euthanized. Each 25-30 g mouse could get 250-300ul blood per time. The blood was collected in a serum collection tube (367812, BD Vacutainer). Blood samples were centrifuged at $2,500 \times g$ for 15 min at room temperature to remove cells and platelets. Then the supernatant was collected to new Eppendorf tubes and re-centrifuged at $3,000 \times g$ for 15 min. Finally, the cell-free serum was centrifuged at $10,000 \times g$ for 30 min at room temperature to remove cell debris and large vesicles. Serum samples were used immediately or frozen at -80°C .

Isolation of serum EVs using SEC

Isolation of vesicles by SEC was performed as described by Boing et al(37). Succinctly, 10 ml of Sepharose CL-4B (Sigma Aldrich, St. Louis, MO, USA) was packed in 7.0 cm long columns with a diameter of 1.5 cm (29924, Disposable Plastic Columns from Thermo Scientific), and washed with 5 column volumes of PBS (Ca^{2+} -free, Mg^{2+} -free, filtered by $0.1 \mu\text{m}$ filter membrane, Servicebio, China), 5 mM EDTA. 1 ml serum aliquot was loaded on an SEC column and 0.5 ml fractions were collected as indicated (PBS used as eluent). The washing buffer was supplemented with 1 mg/ml BSA. For maximizing yield for comparison experiments and downstream step, EV fractions about 3 ml were pooled and concentrated using MWCO 30 kDa Amicon Ultra Centrifugal Filters (Millipore, Merck, USA).

Isolation of serum EVs using UC

1 ml (for characterization experiments) or 3 ml (for SDS-PAGE and Western Blot) platelets-free serum aliquot was diluted with PBS to 3 times the original volume and ultracentrifuged for 60 min at $120,000 \times g$, 4°C to pellet the EVs (13 PA Tube 1.5x9.6cm, 332901A, HITACHI, Japan; P40ST swing rotor, himac CP 70MX, HITACHI) according to Théry et al(39, 40). The supernatant was discarded and EVs were resuspended in PBS for washing. A second UC run was performed for 60 min at $120,000 \times g$, 4°C . The EV-rich pellet was resuspended to a final volume of 100 μl with PBS and frozen at -80°C .

Isolation of serum EVs using TEI kit

The EVs isolated by Total Exosomes Isolation TEI (from serum, 4478360, Invitrogen, Thermo Fisher Scientific) was performed as the user guide provided with the product. 1 ml platelets-free serum was diluted with 1 ml PBS and then added 20% volumes of TEI reagent. Mix the serum and reagent mixture by vortexing until there is a homogeneous solution. Incubate the sample at 4°C for 30 min. After incubation, centrifuge the sample at $10,000 \times g$ for 10 min at room temperature. Discard the supernatant and use 100 μl PBS to resuspend the pellets. Keep isolated EVs at -80°C for long-term storage.

SDS-PAGE, Western blotting and protein concentration measurement

Protein concentrations were determined by BCA protein assay (KeyGen BioTECH, KGP902, China), according to the manufacturer's instructions. For total protein analysis, 15 μ l of each fraction was mixed with 5 μ l 4-fold concentrated reducing sample buffer, boiled for 5 minutes, and loaded on a 10% gradient gel for SDS-PAGE. Proteins were stained with Bio-Safe Coomassie G-250 Stain and detected using a Bio-Rad ChemiDoc imager. Pre-stained protein marker (Bio-Rad) was used as a molecular weight standard.

For Western blotting, proteins were separated either by 6% or 10% SDS-PAGE and transferred by electrophoresis to PVDF membranes (Millipore, Billerica, MA). The blots were blocked for 1 h in PBS containing 0.1% Tween-20, and 5% non-fat milk powder, and incubated overnight with antigen-specific antibodies in the same buffer. CD9 was detected using rabbit anti-mouse CD9 (220642, Zen Bio, 1:1,000); CD63 with rabbit anti-mouse CD63 (510953, Zen Bio, 1:2,000); CD81 with rabbit anti-mouse CD81 (381296, Zen Bio, 1:1,000); apoA1 with rabbit anti-mouse apoA1 (381145, Zen Bio, 1:1,000). Primary antibodies were labeled for 1 h with HRP-conjugated goat anti-rabbit Ig (ab205718, Abcam, 1:10,000). HRP was detected with High-sig ECL Western Blotting Substrate (Tanon,180-501) using a Bio-Rad Chemidoc imager. Relative signal intensities were determined using Bio-Rad image lab 5.1 software. All antibodies used revealed specific protein signals as determined by appropriate molecular weights and lack of signal with negative control primary antibodies.

Nanoparticle tracking analysis (NTA)

The particle concentration and size distribution of extracellular vesicles were determined by NTA using Particle Metrix Zeta View® Nanoparticle Tracking Analysis (Particle Metrix, Germany). The experiment operation is as described in the manufacturer's manual. The samples are diluted 1:100-1:10000 in ultrapure water. All samples were measured in duplicate and using the same instrument settings. The samples were measured for size and concentration in scatter mode (488 nm laser) and the resulting videos were analyzed with the Zeta View® software 8.05.11 (Particle Metrix, Germany).

Transmission electron microscopy (TEM)

For TEM, ~2 μ l of each sample droplet was placed and adsorbed on Formvar-carbon electron microscopy grids, incubated for 10 min at room temperature to allow non-specific particle binding. Subsequently, the grids were washed with MilliQ water. After washing, the grids were transferred to a drop of uranyl acetate solution, pH 7 for ~3 min. Excess fluid was removed using filter paper and the grids were dried on air. Samples were imaged using a Jeol 1010 electron microscope (JEM-1400PLUS, Jeol Ltd, Peabody, USA).

Statistical analysis

Experimental results were statistically analyzed using unpaired t-test. All the statistical analysis was performed using the software package GraphPad Prism 8.4.0 and values of $\alpha=0.05$ were used for hypothesis testing as statistically significant levels. The data in the graphs are presented as mean \pm SD. * $p\leq 0.05$; ** $p\leq 0.01$; *** $p\leq 0.001$.

Abbreviations

EVs: extracellular vesicles; SEC: Size Exclusion Chromatography; UC: ultracentrifugation; TEI: Total Exosomes Isolation; NTA: Nanoparticle Tracking Analysis; TEM: Transmission Electron Microscopy; PBS: Phosphate Buffered Saline.

Declarations

Ethics approval and consent to participate

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Sichuan University. This study was reviewed and approved by the Committee of Ethics of West China Hospital of Stomatology, Sichuan University (NO. WCHSIRB-D-2018-100).

Consent for publication

The manuscript does not contain individual person's data. The consent for publication does not apply.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LL and WDT conceived, designed and supervised the study. JY designed and performed the experiments. JY, XG, HSH and XTX processed and analyzed the data. JY, XSM, XX, CL, MJL and QT collected and prepared the serum samples. LL and JY wrote the manuscript. All authors reviewed the manuscript. All authors read and approved the final manuscript.

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Table

Table 1 Summarizes and evaluates the results of all the above methods

Method	Protein conc. (mg / ml)	Particle conc. (particles / ml)	Particle-to-Protein ratio (10^8 particles / ug)	Median of size (nm)	EV markers	Lipoprotein marker	Evaluation of quality and purity
SEC	1.2 ± 0.01	1.3×10^{12}	11.18	85	✓	Not detected	Median
UC	0.05 ± 0.02	4.4×10^{10}	0.74	112	✓	Not detected	Low
TEI	2.6 ± 0.4	2.4×10^{12}	0.93	117	✓	✓	Low
SEC+UC	0.1 ± 0.07	6.1×10^8	12.31	132	✓	Not detected	High
SEC+TEI	0.19 ± 0.06	9.2×10^9	42.53	85	✓	✓	Low

Figures

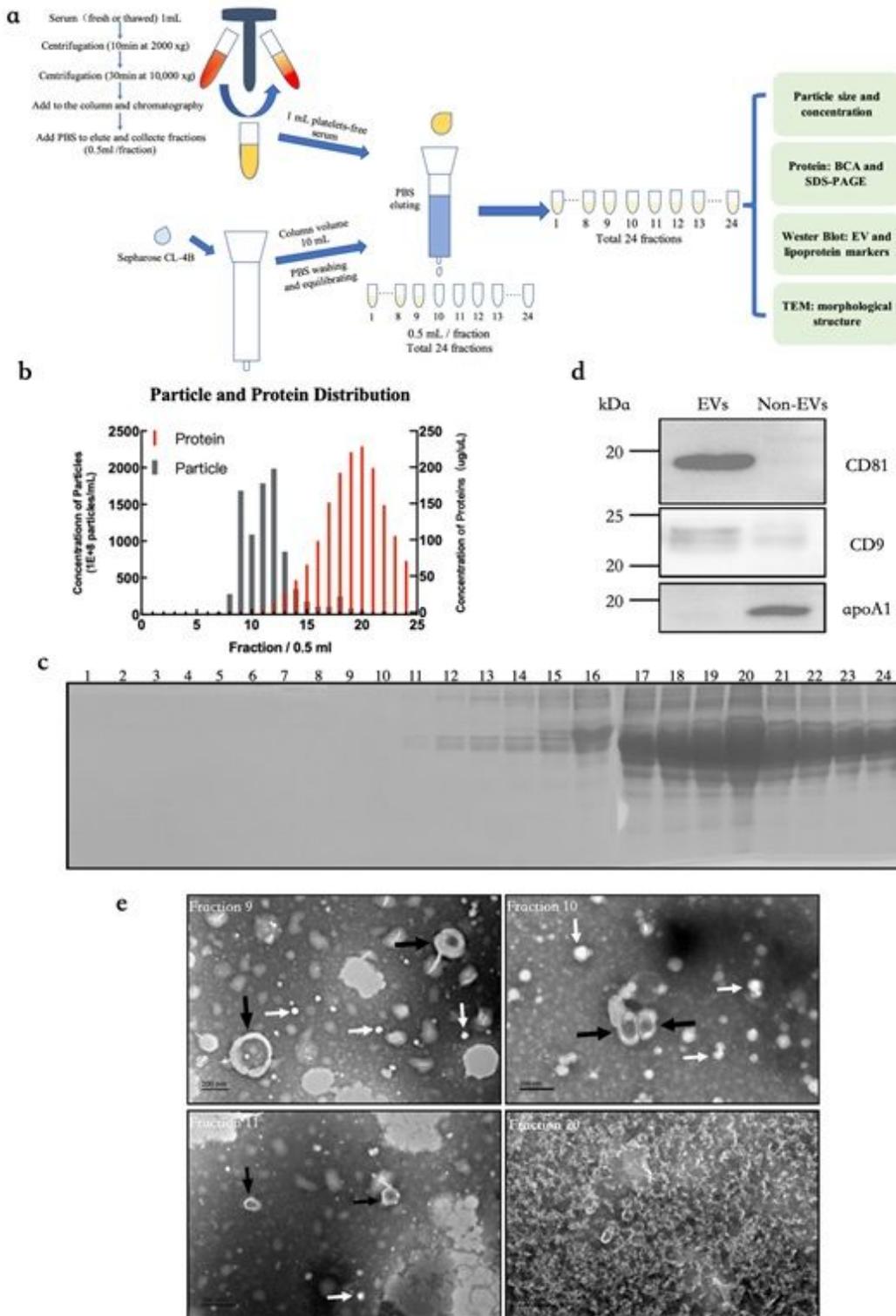


Figure 1

Procedures and characteristics of SEC for separating EVs from serum. 1 ml of prepared platelet-free serum was loaded onto 10 ml Sepharose CL-4B columns, and 24 fractions of 500 µl were collected from each column. (a) Schematic overview of the experimental workflow. (b) Concentrations of particles and

proteins in the SEC fractions were determined with nanoparticle tracking analysis (gray) and BCA (red), respectively. Data shows all 24 fractions. (c) SDS-PAGE was used to determine to directly visualize the relative presence of proteins in the all collected fractions, 15 μ l of each fraction was mixed with 5 μ l 4-fold concentrated reducing sample buffer, boiled for 5 minutes, and loaded on a 10% gradient gel. (d) The presence of the vesicle marker CD9, CD81, and the HDL marker apoA1 was determined in pooled EVs concentrates of fractions 8-13 and pooled Non-EVs concentrates fractions 14–24 with Western blot. (e) Droplets of fractions 9-11 and 20 were loaded onto grids, negative stained, and evaluated with transmission (electron microscopy (TEM). Examples of EV-like structures (cup-shaped) are indicated by black arrows, and examples of lipoprotein particle-like structures (white solid spherical structures) are indicated by white arrows. Scale bars are 200 nm.

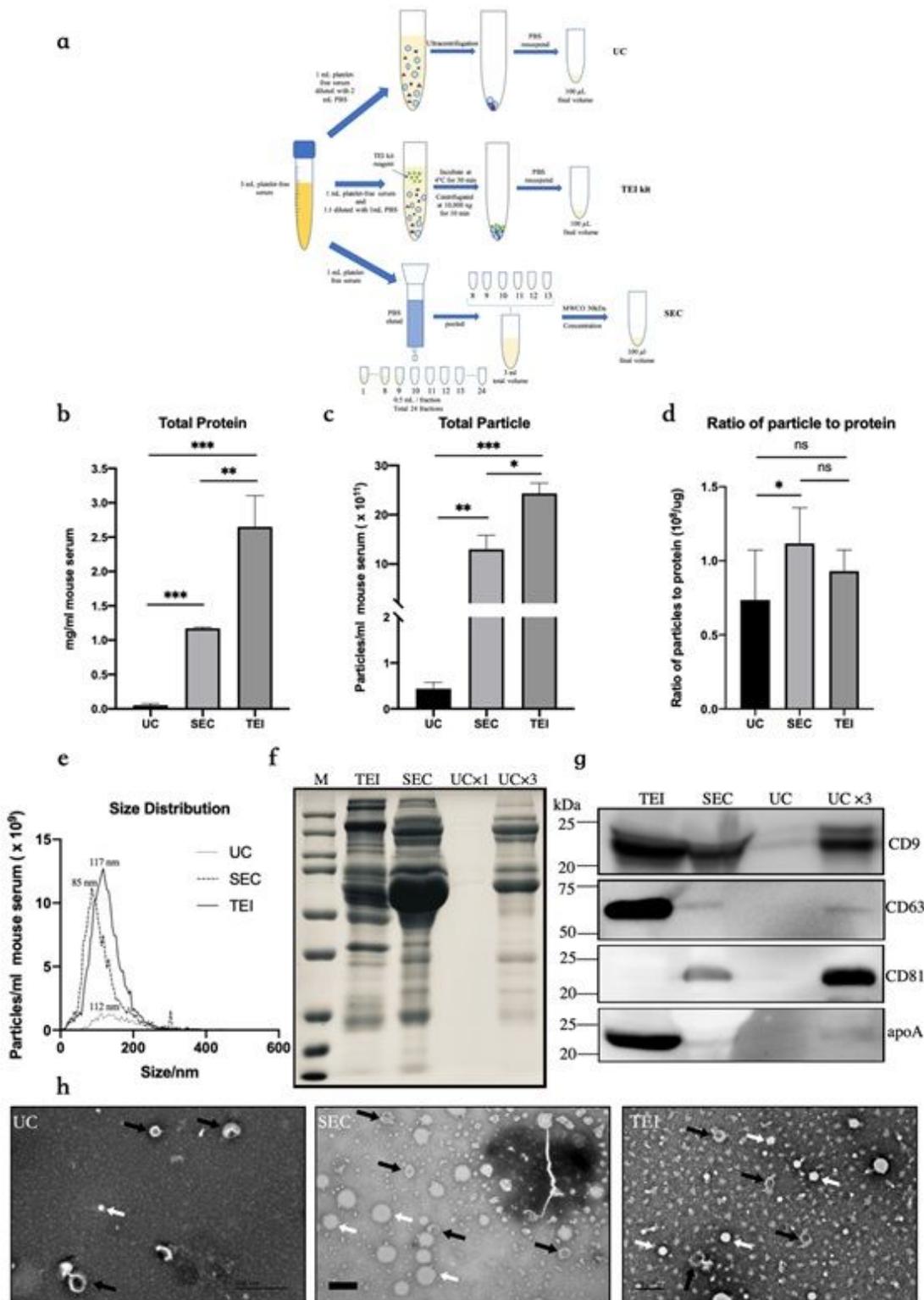


Figure 2

Systematically compare the separation efficiency of SEC, UC, and TEI. (a) Schematic overview of the experimental workflow. (b) total protein was determined by BCA (expressed as mg/ml originating serum; mean \pm SD, n = 3). (c) the concentration of particles as detected by NTA (particles/ml originating serum; mean \pm SD, n = 3). (d) the ratio of particle to protein for SEC, UC, and TEI (e). size distribution of particles detected in B (representative for n = 3). The particle size at peak optimum is indicated. (f) samples were

analyzed by SDS-PAGE followed by Bio-Safe Coomassie G-250. (g) the same samples were analyzed by Western blotting for the presence of the EV markers CD9, CD81, and CD63 or the HDL marker apoA1. The experiment shown is representative of 3 independent experiments. (f and g) SEC, UC, and TEI samples were normalized to equivalent volumes of originating plasma, or, where indicated, 3 × concentrated. (h) The whole amount of TEM images of particles collected by SEC, UC, or TEI. Examples of EV-like structures (cup-shaped) are indicated by black arrows, and examples of lipoprotein particle-like structures (white solid spherical structures) are indicated by white arrows. Scale bars are 500 nm.

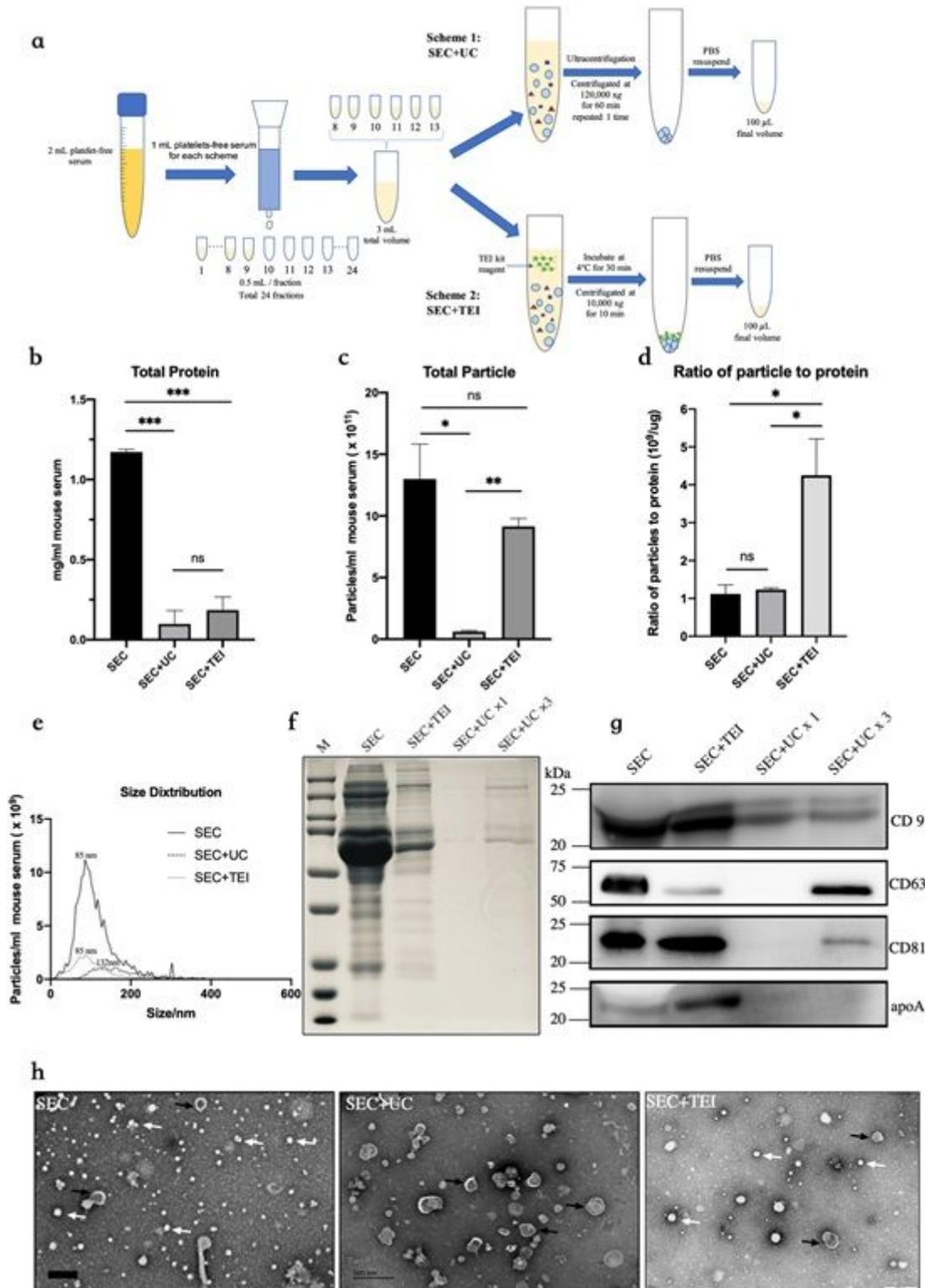


Figure 3

Comparison of two different combination methods and SEC. (a) Schematic overview of the experimental workflow. (b) total protein was determined by BCA (expressed as mg/ml originating plasma; mean \pm SD, n = 3). (c) the concentration of particles as detected by NTA (particles/ml originating plasma; mean \pm SD, n = 3). (d) the ratio of particle to protein for SEC, SEC+UC, and SEC+TEI. (e) size distribution of particles detected in B (representative for n = 3). The particle size at peak optimum is indicated. (f) samples were analyzed by SDS-PAGE followed by Bio-Safe Coomassie G-250. (g) the same samples were analyzed by Western blotting for the presence of the EV markers CD9, CD81, and CD63 or the HDL marker apoA1. The experiment shown is representative of 3 independent experiments. (f and g) SEC, SEC+UC, and SEC+TEI samples were normalized to equivalent volumes of originating serum, or, where indicated, 3 \times concentrated. (h) The whole amount of TEM images of particles collected by SEC, SEC+UC, or SEC+TEI. Examples of EV-like structures (cup-shaped) are indicated by black arrows, and examples of lipoprotein particle-like structures (white solid spherical structures) are indicated by white arrows. Scale bars are 500 nm.