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Comprehensive isolation of extracellular vesicles and nanoparticles

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There is an increasing appreciation for the heterogeneous nature of extracellular vesicles (EVs). In addition, two nonvesicular extracellular nanoparticles (NVEPs), exomeres and supermeres, have been discovered recently that are enriched in many cargo previously ascribed to EVs. The EV field has largely focused on EV isolation and characterization, while studies on NVEPs are limited. At this juncture, it is critically important to have robust and reliable methods to separate distinct populations of EVs and NVEPs to assign cargo to their correct carrier. Here, we provide a comprehensive step-by-step protocol for sequential isolation of large and small EVs, nonvesicular fractions, exomeres and supermeres from the same starting material. We describe in detail the use of differential ultracentrifugation, filtration, concentration and high-resolution density-gradient fractionation to obtain purified fractions of distinct populations of EVs and NVEPs. This protocol allows assignment and enrichment of a biomolecule of interest to its specific extracellular compartment. Compared to other isolation methods, our protocol has unique advantages, including high purity and reproducibility, with minimal expertise required. The protocol can be applied to purification of EVs and NVEPs from cell culture medium and human plasma and requires ~72 h to complete. Adoption of this protocol will help translational investigators identify potential circulating biomarkers and therapeutic targets for a host of human diseases and allow basic scientists to better understand EV and NVEP biogenesis and function. Overall, this protocol will allow those interested in isolating EVs and extracellular particles to advance scientific inquiry to answer outstanding questions in the field.

Introduction

Extracellular vesicles (EVs) and nonvesicular (NV) extracellular nanoparticles (NVEPs) play pivotal roles in both physiological and pathological conditions[1–4]. However, a major challenge in the field of EVs and NVEPs is their heterogeneity and the methods used to isolate and purify distinct populations[3,5–12]. Furthermore, the field has largely focused on studies related to EVs, while studies on extracellular amembranous NVEPs, including the recently discovered exomeres[5,10] and supermeres[13–15], are limited. However, it is becoming increasingly clear that different classes of EVs may contain specific cargo and, equally important, that NVEPs, such as exomeres and supermeres, contain many of the biomolecules, including proteins, RNA and DNA, that have previously been ascribed to exosomes[3,9,15,16]. EVs range in size from small EVs (sEVs; <200 nm), including exosomes generated from endosomal compartments, to large EVs (lEVs; >200 nm), including microvesicles and large oncosomes, which are shed from the plasma membrane[3,6,7,9,11]. NVEPs include a wide range of size of particles, including lipoproteins, exomeres and supermeres[5,15,17–19]. Fluid-phase atomic force microscopy (AFM) has revealed that supermeres have distinct morphological features in comparison to both sEVs and exomeres[15]. To understand the roles of EVs and NVEPs in basic cell biology, as well as to realize their full clinical potential, robust and reliable methods are needed to separate distinct populations of these particles. Diverse EV isolation methods have been extensively described, including differential ultracentrifugation, size-exclusion chromatography, ultrafiltration, immunocapture and microfluidics[9,20–24]. Ultracentrifugation is the gold standard for isolation of EVs and NVEPs from cells, tissues and plasma[9,10,15], and high-resolution density-gradient purification has been shown to further separate NV material from purified vesicles[9,15,18,25–28]. Centrifugation-based isolation schemes have the advantage of being relatively high yield, with ultracentrifuges being widely available to basic and clinical research laboratoriess. In the protocol herein, we provide a detailed description of how to reproducibly obtain highly purified lEVs, sEVs, exomeres and supermeres from human cell lines and human plasma[9,10,15]. In addition, we describe how to improve the

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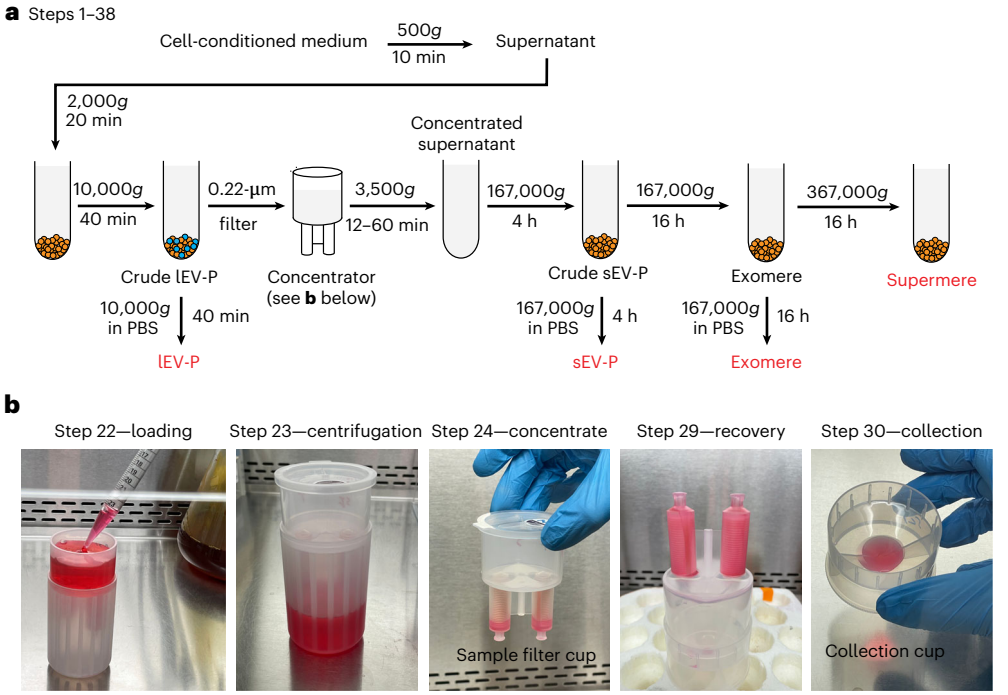

Fig. 1 | Overview of steps for isolation of EVs and NVEPs from cell-conditioned medium. a, Schematic for isolation of large EV pellets (lEV-Ps), small EV pellets (sEV-Ps), exomeres and supermeres. Serum-free conditioned medium is centrifuged (500*g* and 2,000*g*) to remove dead cells, cellular debris and apoptotic bodies. The lEV-P is obtained after centrifugation of the supernatant at 10,000*g* centrifugation for 40 min. The leftover supernatant is first concentrated and then subjected to ultracentrifugation at 167,000*g* for 4 h to obtain the sEV-P (washed one time in PBS by ultracentrifugation at 167,000*g* for 4 h). The supernatant from the previous step is centrifuged at 167,000*g* for 16 h to isolate the exomeres (washed one time in PBS by ultracentrifugation at 167,000*g* for 16 h). The supernatant from the previous step is centrifuged at 367,000*g* for 16 h to isolate supermeres. **b**, Representative photographs of the most important steps during the concentrator procedure from **a**.

purification of exomeres and supermeres from plasma by the addition of an albumin-depletion step. Methods for isolation of lipoproteins from plasma are not described here; however, methods for their isolation have been described and reviewed elsewhere[17,19,27].

Development and overview of the protocol

Major challenges in the field involve the heterogeneity of EVs and NVEPs and the various methods used to isolate and purify distinct populations[5–11]. It is increasingly clear that traditionally isolated 'exosome' or 'EV' samples contain a heterogeneous mixture of EVs and NV components[9]. Progress in the EV field has been hampered by the lack of methods to separate the various secreted vesicles from NV components. Furthermore, the focus has been on EVs and lipoproteins[6,9,17–19,26,27,29–34], while studies of the recently discovered exomeres and supermeres are very limited[5,10,15,35]. Isolation of EVs and NVEPs from plasma and other body fluids is challenging. Most EV studies have been focused on isolation of EVs from cell-conditioned media, while reports for EV and lipoprotein isolation from plasma and other body fluids are more limited, but a number of protocols using combinations of different techniques are available[18–20,26,27,30,33,34,36–39].

Our comprehensive protocol is based on methods introduced in three articles by our group[9,10,15]. These protocols entail a series of sequential centrifugation, concentration, filtration and high-resolution density-gradient centrifugation steps to sequentially isolate lEVs, sEVs, exomeres and supermeres from cell-conditioned medium (Figs. 1 and 2) and human plasma (Fig. 3). Some of the major steps and modifications are listed below:

1 Removal of cells and cell debris from cell culture medium and human plasma by a series of centrifugation steps. We also describe an albumin-depletion step (Fig. 4) that increases the purity of exomeres and supermeres from human plasma.

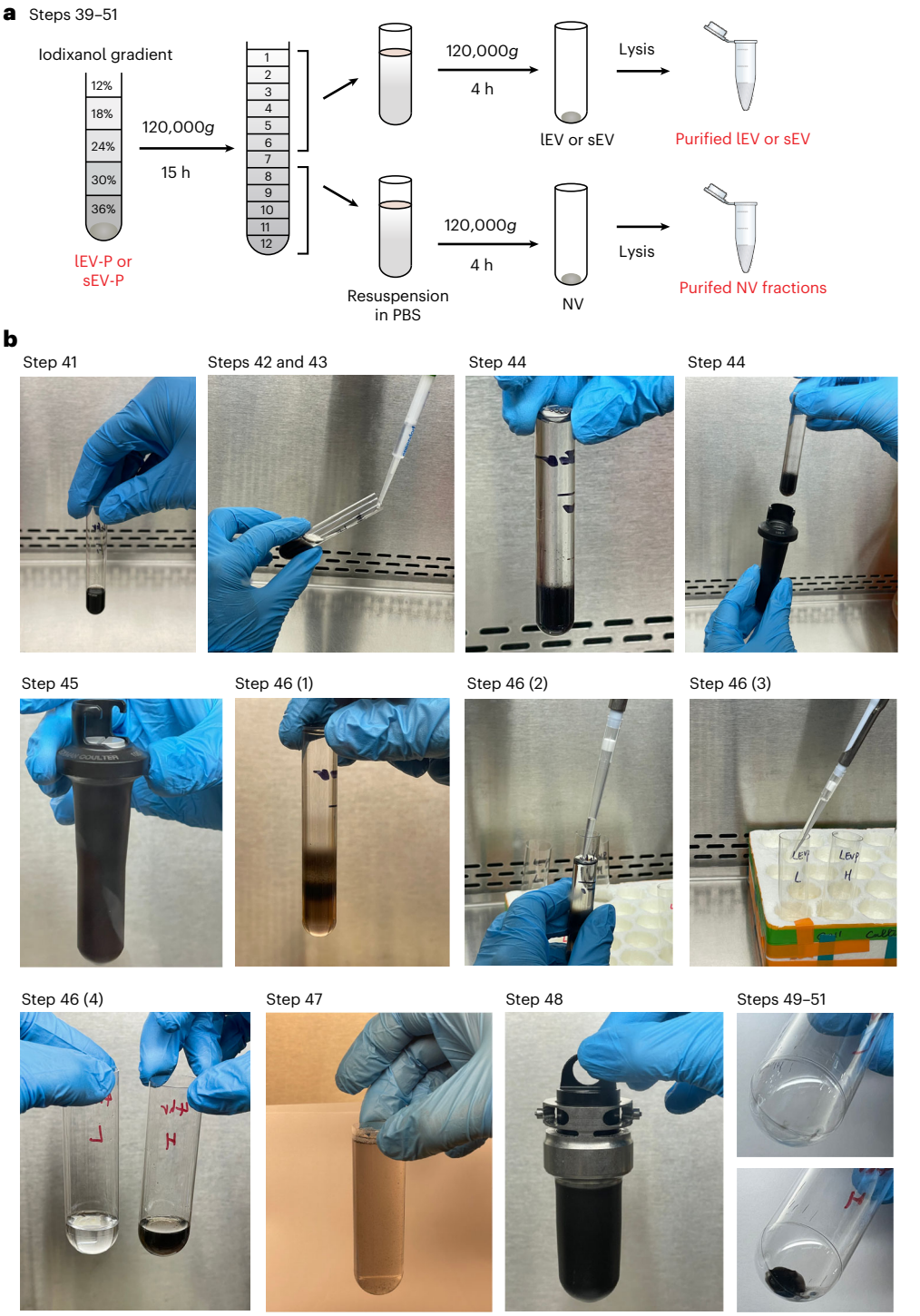

Fig. 2 | Overview of high-resolution density-gradient fractionation of EVs. a, Schematic of the generation of lEVs or sEVs and NV fractions by high-resolution iodixanol density-gradient fractionation (12–36%, wt/vol). Crude pellets of lEV-Ps or sEV-Ps were resuspended in ice-cold PBS and mixed with ice-cold iodixanol (OptiPrep)/PBS for a final 36% (wt/vol) iodixanol solution. The suspension was added to the bottom of a centrifugation tube, and solutions of descending concentrations of iodixanol (30%, 24%, 18% and 12%) in PBS were carefully layered on top, yielding the complete gradient. The bottom-loaded 12–36% (wt/vol) gradient was subjected to ultracentrifugation at 120,000*g* for 15 h. Twelve individual fractions of 1 ml were collected from the top of the gradient. The first six fractions are pooled in a tube, and the last five fractions are pooled in a second tube. The tubes are filled with PBS and mixed. After ultracentrifugation at 120,000*g* for 4 h, the two pellets represent purified EVs and NVs, respectively[9]. **b**, Representative photographs of the most important steps during the high-resolution gradient fractionation procedure from **a**. Panel **a** adapted with permission from ref. [9], Elsevier.

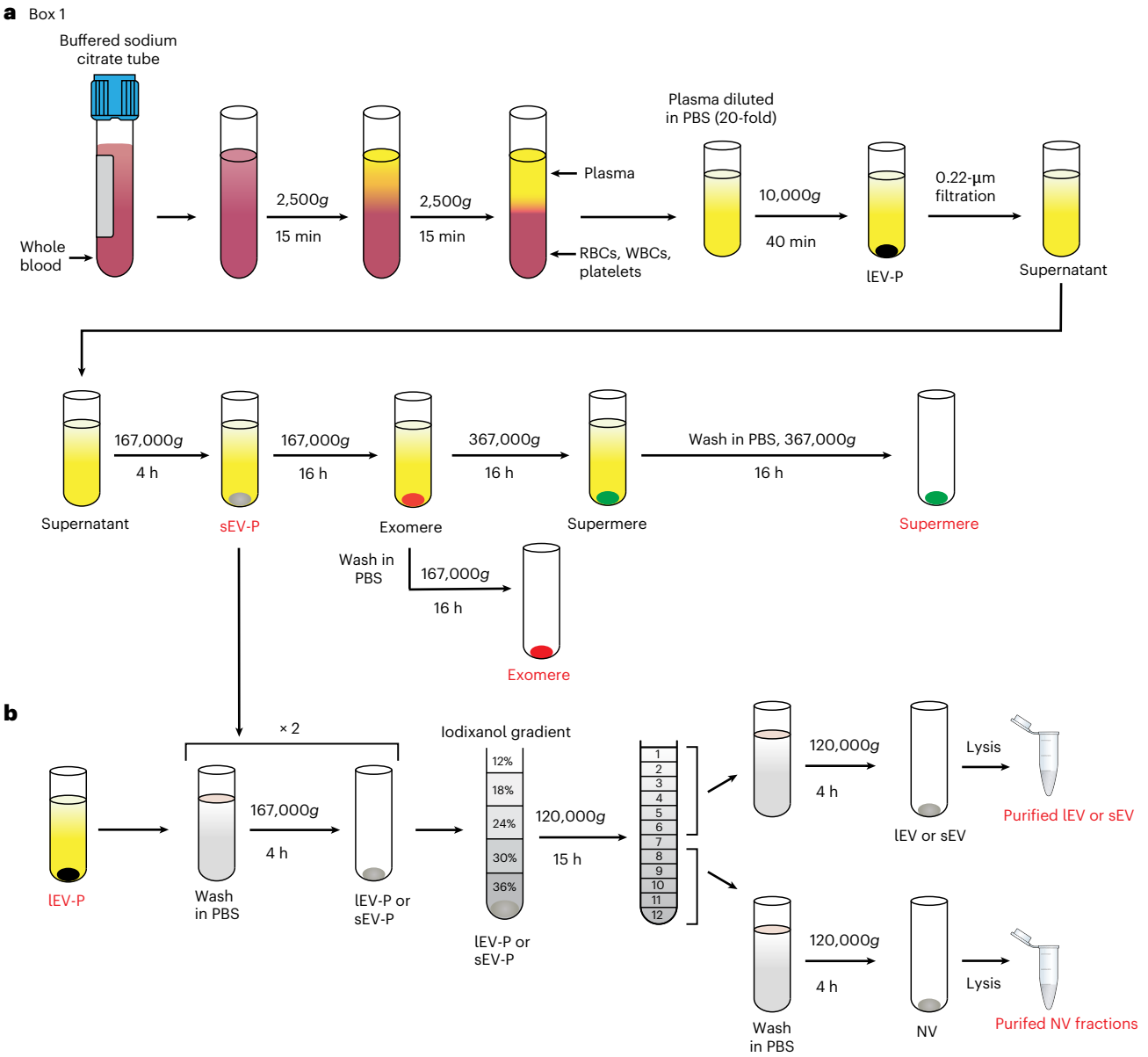

Fig. 3 | Schematic of the isolation procedure for lEVs, sEVs, exomeres and supermeres from human plasma described in Box 1. a, Schematic for isolation of different fractions from plasma. Plasma is generated by centrifugation of the blood at 2,500*g* for 15 min twice at room temperature. The resulting plasma samples are immediately diluted ~1:10–20 in ice-cold PBS-HEPES (PBS-H) and centrifuged at 10,000*g* for 40 min to pellet lEV-Ps. The supernatant is filtered through a 0.22-µm pore polyethersulfone (PES) filter, and the resulting supernatants are subjected to sequential ultracentrifugation at 167,000*g* for 4 h and 16 h and then at 367,000*g* for 16 h to isolate sEV-Ps, exomeres and supermeres, respectively. **b**, Schematic of the generation of purified plasma lEVs, sEVs and NV fractions by high-resolution iodixanol density-gradient fractionation (12–36%, wt/vol). Crude pellets of lEV-Ps or sEV-Ps are processed as described in Fig. 2. The research conducted as part of this protocol complies with all the relevant ethical regulations. The use of the human samples was approved by the Vanderbilt University Medical Center Institutional Review Board (IRB; IRB nos. 161529 and 151721). RBC, red blood cell; WBC, white blood cell. Figure adapted with permission from ref. [9], Elsevier.

2 Isolation of lEVs by a combination of ultracentrifugation and high-resolution 12–36% (wt/vol) iodixanol density-gradient fractionation (Figs. 1–3). Bottom loading of the high-resolution gradient is important to our method because it removes contaminating NV fractions from EV samples.

3 A filtration step to ensure that any remaining lEVs are removed so that only sEVs, exomeres and supermeres remain for subsequent steps.

4 A dual-purpose concentration step. A 100,000-molecular-weight cutoff concentrator is used to concentrate the sample from a large to a small volume and to facilitate the removal of free proteins. This step is omitted from most published EV protocols but is crucial to maximize the yield of

exomere and supermere fractions[15] and to remove free proteins that would otherwise contaminate exomere and supermere samples.

5 Isolation of sEV samples by a combination of high-speed ultracentrifugation and high-resolution 12–36% (wt/vol) iodixanol density-gradient fractionation[9,15] (Figs.1–3). The bottom-loaded high-resolution gradient has been demonstrated to remove contaminating NV fractions from sEV samples, including contaminating vault structures and nucleosomes[9]. Bottom loading is necessary because the centrifugation time (15 h) and speed (120,000*g*) is insufficient for NV components to reach their buoyant densities if samples are top loaded.

6 Isolation of exomeres by 167,000*g* ultracentrifugation[10,28]. This simple step is an alternative to asymmetric flow field-flow fractionation (AF4)[5,35], which is more costly and results in a lower yield (Figs. 1 and 3).

7 Isolation of supermeres by 367,000*g* high-speed ultracentrifugation[15]. This method of purification was used in our recent article[15] that described the discovery of supermeres and is the only published method for their isolation (Figs. 1 and 3).

8 Albumin-depletion using a commercially available kit. When isolating sEVs, exomeres and supermeres from plasma, the biggest challenge for downstream analysis is albumin contamination. The albumin-depletion step greatly improves the purity of exomere and supermere samples, thereby aiding downstream analysis and characterization.

The protocol we previously described[15] allows reasonable quantities of supermeres to be isolated from cell-conditioned medium and plasma. We detail the necessary steps, including crucial filtration and concentration steps, to maximize yield (Figs. 1 and 3 and Steps 1–38).

A further development of the EV purification schema is the use of a high-resolution density gradient[9,15] to increase the resolving power of separating both lEVs and sEVs from NV contaminants such as nucleosomes, vault structures and nanoparticles (Fig. 2 and Steps 39–51). For purification of EVs from human plasma, the gradient allows separation of EVs and HDL particles[9]. We provide a detailed description of the steps in our 12–36% (wt/vol) iodixanol gradient protocol, with the rationale for both the concentrations that we use and for bottom loading of the sample in contrast to the widely practiced top-loading method.

New to our protocol is the addition of an albumin-depletion process for purification of sEVs, exomeres and supermeres from plasma that contains large amounts of albumin (Fig. 4 and Box 1). The removal of contaminating albumin greatly improves downstream analysis, such as immunoblotting, thereby allowing detection of low-abundance target proteins as potential biomarkers and/or therapeutic targets.

Applications of the method

It is of utmost importance for the future therapeutic potential and design of treatment interventions to correctly identify the compartment and mechanisms by which specific DNA, RNA and proteins are secreted in human disease[9,15,40]. A strength of this protocol is that it allows investigators to use the same starting material to identify the compartment(s) in which specific secreted cargo resides. The methods can be applied to isolate lEVs, sEVs, exomeres and supermeres from conditioned medium of cells in culture and from plasma.

We recently developed a high-speed ultracentrifugation procedure to isolate and characterize a novel small extracellular NVEP that we have termed 'supermere' (supernatant of exomeres)[15]. Supermeres display a markedly greater uptake in various organs in vivo compared with sEVs and exomeres. Supermeres are highly enriched in cargo involved in multiple disease states, including cancer, Alzheimer's disease and cardiovascular disease. Cancer-derived supermeres play important biological functions, including transfer of cetuximab resistance[15]. Thus, supermeres are a distinct functional new NVEP replete with potential circulating biomarkers and therapeutic targets for a host of human diseases.

In our recent publication on exomeres[10,28], we described a high-yield ultracentrifugation-based method for exomere isolation and demonstrated that exomeres contain and transfer functional cargo. These findings should accelerate advances in determining the composition and biological functions of exomeres along with their potential use in biomarker discovery and therapeutic applications. In our third key supporting research manuscript[9], we used differential ultracentrifugation together with a unique high-resolution density-gradient fractionation to precisely characterize the RNA, DNA and protein constituents of sEVs and NV material. We showed that extracellular RNA, RNA-binding proteins and other cellular proteins are differentially expressed in sEVs and NV compartments. This study provides a framework for a clearer understanding of EV heterogeneity.

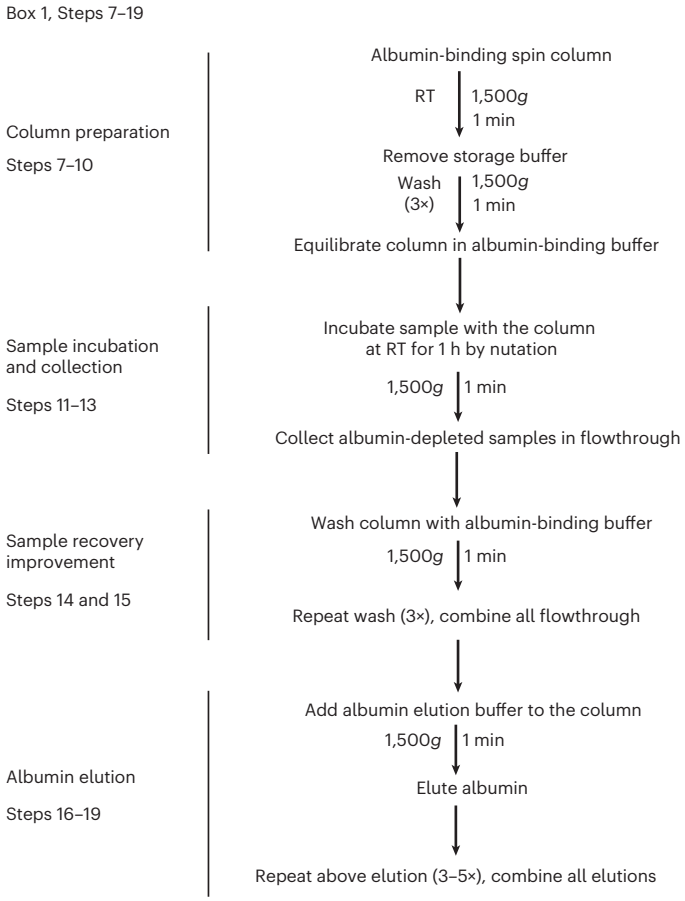

Fig. 4 | Flowchart of albumin depletion steps from human plasma-derived sEV-Ps, exomeres and supermeres described in Box 1. Albumin spin columns are equilibrated in albumin-binding buffer by centrifugation at 1,500*g* for 1 min at room temperature (RT). The samples are then applied to the albumin-binding columns and incubated for 1 h at RT with rotation. The columns are centrifuged, and the albumin-depleted samples are collected in the flowthrough. Albumin is eluted by centrifugation by using albumin-elution buffer. This protocol was modified from the albumin depletion kit procedure (Abcam). The research conducted as part of this protocol complies with all the relevant ethical regulations. The use of the human samples was approved by the Vanderbilt University Medical Center IRB (IRB nos. 161529 and 151721).

Isolation of crude EV pellets (EV-Ps), exomeres and supermeres has been performed in multiple human cancer cell lines and normal human kidney epithelial cells[15]. The EV and NVEP isolation protocol has also been performed on plasma from both healthy individuals and patients with colorectal cancer. The final yield, purity and characteristics of these EVs and NVEPs have been examined in diverse sets of cell lines and patient conditions. Optimization is necessary on the basis of cell type and growth conditions, patient conditions and plasma volumes.

These refined isolation methods for parsing of lEVs, sEVs (a subset of which are exosomes), exomeres and supermeres are vital to the advancement of basic science investigation, as well as clinical translation, for many disorders such as cancer, Alzheimer's disease, cardiovascular disease and infectious diseases like COVID-19. Overall, these techniques will improve biomarker discovery and identification of therapeutic targets. Protocols for isolation of exomeres and supermeres from other biofluids, including urine and milk, have not yet been developed but will likely be detailed in the near future because of the importance of these nanoparticles in health and disease.

We cannot exclude that other subsets of EVs and NVEPs will be identified as methods are refined and new technologies are developed. However, we have provided a comprehensive and detailed protocol for isolation of lEVs, sEVs, exomeres and supermeres from the same starting material. The protocols for isolation of the different extracellular compartments require the use of high-speed ultracentrifuges and rotors capable of reaching the required high centrifugal forces. This complete protocol requires ~3 d to complete.

Box 1 | Isolation of lEV-Ps, sEV-Ps, exomeres and supermeres from human plasma and albumin depletion ● Timing 3 d, 4.5 h

EVs, exomeres and supermeres can also be purified from human plasma by using the procedure described in this protocol. Rather than collect cell-conditioned medium, blood is drawn, plasma is prepared and sample is diluted as outlined in the procedure below. After isolation of EVs, exomeres and supermeres, albumin depletion is performed to increase the purity of the samples.

For this study, blood was collected from individuals at Vanderbilt University Medical Center. A group of 13 patients with colorectal cancer ranged in age from 30 to 68 years, with a mean age of 52.2 years. Three healthy individuals ranged in age from 44 to 71 years, with a mean age of 56 years. All procedures on human peripheral blood specimens were approved and performed in accordance with the Vanderbilt University Medical Center IRB (IRB nos. 161529 and 151721). All human participants provided informed consent (clinical trial registration number: NCT03263429). Participants did not receive compensation. Consent to publish this information was provided.

Procedure

Isolation of lEV-Ps, sEV-Ps, exomeres and supermeres from human plasma

1 Draw the blood into BD Vacutainer (9NC) blood-collection tubes (BD Bioscience) containing buffered sodium citrate as an anticoagulant. The first tube collected should be discarded.
▲ **CRITICAL** One tube of 4.5 ml of blood normally yields ~2.2 ml of plasma, from which we can isolate enough EVs and NVEPs for downstream analysis including immunoblotting and flow cytometry, especially for exomeres and supermeres.

2 Transfer the blood to a new 15-ml centrifuge tube (CellStar) with a 5-ml pipette and centrifuge the blood at 2,500*g* for 15 min at RT by using a swinging-bucket centrifuge (Thermo Fisher Scientific, Sorvall LYNX 4000) to pellet red blood cells, white blood cells and platelets.
▲ **CRITICAL STEP** Tubes are kept at RT and should be processed within 2 h after collection. If a blood sample shows any significant hemolysis after the initial pellet, it will be difficult to remove contaminating hemoglobin.

3 Transfer the plasma from Step 2 to a new 15-ml centrifuge tube (CellStar) with a 5- or 10-ml pipette and centrifuge the plasma at 2,500*g* for 15 min at RT by using a swinging-bucket centrifuge (Thermo Fisher Scientific, Sorvall LYNX 4000) to isolate platelet-poor plasma.
! CAUTION Be careful when pipetting off the plasma to avoid the red and white blood cell pellet. Leave a small amount of supernatant on top of the pellet to avoid contamination. The type of pipette used depends on the initial blood volume collected.
■ **PAUSE POINT** The platelet-poor plasma can be frozen at −80 °C (long-term storage).

4 If isolation of lEV-Ps is not required, pass the plasma collected in step 3 above through a 0.22-μm syringe filter (Millipore) connected to a syringe (BD) to remove large vesicles, cell debris and other contaminants.

5 Dilute the resulting plasma samples immediately ≥1:10 in ice cold PBS-H. Mix well by pipetting up and down.
■ **PAUSE POINT** The diluted plasma can either be kept at 4 °C (short-term storage for 1–2 weeks) or used immediately.

6 Isolate lEV-Ps, sEV-Ps, exomeres and supermeres from plasma as described in Steps 11–18 and 31–38 for EV and NVEP isolation from cell-conditioned medium.
▲ **CRITICAL STEP** Optionally, further isolate plasma lEVs and sEVs on high-resolution density gradients as described in Steps 39–51 if highly purified EVs are required.
? TROUBLESHOOTING

Albumin depletion from sEV-Ps, exomeres and supermeres

▲ **CRITICAL** Samples of sEV-Ps, exomeres and supermeres isolated from human plasma have high levels of albumin that can interfere with downstream analyses such as immunoblotting. Therefore, depletion of albumin and other highly abundant proteins from plasma-derived exomeres and supermeres will improve downstream analysis. We performed albumin depletion from isolated exomeres and supermeres by using an albumin depletion kit (Abcam) according to the manufacturer's instructions with some modifications as follows.

7 Warm up the binding buffer, elution buffer and the column to RT before use.

8 Conduct a 1:5 dilution of plasma-derived exomeres and supermeres isolated from step 6 above by mixing 40 μl of sample with 160 μl of albumin-binding buffer.
▲ **CRITICAL STEP** Although the kit recommends diluting unprocessed plasma samples 10-fold with the maximum sample loading volume being 100 μl, we have found that dilution of the isolated sample fivefold with a maximum sample loading volume of 200 μl can efficiently remove albumin contamination. We found that this kit also works for unprocessed plasma samples by diluting the sample 10-fold. The decision to use unprocessed plasma or the isolated extracellular samples as the starting material for albumin depletion will depend on the downstream sample analysis.
! CAUTION The maximum sample protein concentration is 5–7 mg/ml; thus, the sample concentration needs to be adjusted to within the loading limit.

9 Remove the bottom closure of the column by hand and slightly loosen the column cap. Put the column in a 1.5-ml sterile microcentrifuge tube (Fisher Scientific) and centrifuge at 1,500*g* for 1 min at RT on a benchtop microcentrifuge (Thermo Scientific, Sorvall Legend Micro 17R) to remove the storage buffer. Discard the storage buffer.
! CAUTION Be careful to not let the column touch the liquid on the side of the tube.

10 Remove the column carefully and place it in a new 1.5-ml sterile microcentrifuge tube, add 200 μl of albumin-binding buffer and centrifuge as above to wash the column. Discard the flowthrough and repeat the wash step for a total of three times.

11 Add 200 μl of diluted sample from step 8 above onto the spin column. Close the cap tightly to avoid losing sample from the bottom of the column. Invert the column two or three times.

12 Incubate the column for 1 h on a sample mixer at RT (Invitrogen) by 360° end-over-end rotation with vortexing at 10 rpm periodically.
! CAUTION At each step, make sure that there is no residual solution at the bottom of the column before placing the column in a new microcentrifuge tube.
▲ **CRITICAL STEP** We have observed that a 200-μl volume of sample can cover the column better than 100 μl, allowing more albumin to bind the column. If the volume is limited, then the sample will not contact the column well. In addition, longer incubation time (1 h) helps albumin bind better with the column than the 30 min recommended by the manufacturer.

13 Transfer the column to a new microcentrifuge tube, loosen the cap and centrifuge at 1500*g* for 1 min at RT. Keep the flowthrough (this is the albumin-depleted sample).

14 Optionally, to recover more albumin-depleted sample, add 200 μl of albumin-binding buffer to the column that was transferred to a new microcentrifuge tube to wash the column; then, repeat step 13 above. Repeat this wash several times based on the protein concentrations in the flowthrough.

15 Combine the initial flowthrough with the flowthrough from the washes and concentrate by using Amicon Ultra-15 50-kDa (Millipore) or 30-kDa (Millipore) centrifugal filters or other available concentration methods.

16 Add 200 μl of albumin elution buffer to the column and spin down at 1,500*g* for 1 min at RT to elute the bound albumin from the column.

Box 1 | Continued

17 (Optional) To elute more bound albumin from the column, repeat the elution step three or four times by transferring the column to a new microcentrifuge tube each time. Combine the initial elution with the elution from the other repeats and concentrate by using an Amicon Ultra-15 centrifugal filter or other available concentration methods.
▲ **CRITICAL STEP** The number of washes (step 14) or elutions (step 17) need to be optimized on the basis of the downstream analyses.
18 Measure the OD_{280} of the albumin-depleted exomere and supermere samples by using Nano Drop One (Thermo Scientific). Also quantify the proteins in the samples by using a Direct Detect spectrometer or the BCA or Bradford methods.
19 Run 30 µl of pre-albumin depletion exomere and supermere samples together with post-albumin depletion exomere and supermere samples on a NuPAGE 4–12% (wt/vol) Bis-Tris gel (Invitrogen). Stain with SimplyBlue (Invitrogen) to confirm the albumin-depletion efficiency or perform immunoblotting to confirm the enrichment of specific cargo in exomere and supermere samples after albumin depletion.
? TROUBLESHOOTING

Comparison with other methods

Our high-resolution iodixanol density-gradient fractionation of lEV and sEV pellets (lEV-Ps and sEV-Ps) reliably separates EVs from contaminating NV fractions[9]. A key feature of this method is bottom loading of a 12–36% (wt/vol) gradient, which allows EVs to float upward while higher-density components remain at the bottom portion of the gradient. Previous studies have used different percentages of iodixanol density gradients (for example, 5–40% wt/vol) and top-loaded iodixanol density gradients to isolate EVs from NV fractions. However, these methods cannot be assumed to produce the same results. Our method of fractionation reliably separates lEV or sEVs from NV fractions to ensure that cargo can be assigned to their correct compartments[9,15].

Our ultracentrifugation method for isolation of exomeres has several advantages over the alternative AF4 protocol that has been used by other groups[5,35]. Besides the centrifugation method to isolate exomeres[10,28] detailed in this protocol, AF4 is the only other published method for isolation of exomeres[5,35]. AF4 represents a step forward by fractionating exomeres from exosome subpopulations on the basis of their size and hydrodynamic properties[5,35]; however, the technique relies on a specialized instrument[8], and the input and yield are limited. The loading capacity of AF4 is limited and typically involves ultracentrifugation to concentrate EVs and NVEPS before AF4 isolation, making comprehensive characterization and functional analysis challenging[41]. The exomere yield using ultracentrifugation can be comparatively high (Table 1). AF4 also requires costly instrumentation and considerable technical expertise that may limit its widespread adoption[8].

Although differential ultracentrifugation has been widely used for isolation of EVs based on size and density, it has some disadvantages. For example, the EVs and NVEPs isolated by using ultracentrifugation may be heterogeneous, including subpopulations of different sizes. In addition, some soluble proteins and nucleic acids may be co-isolated with each fraction. Other disadvantages include the cost of high-speed ultracentrifuges, along with the time and labor involved in processing the samples. However, to date, high-speed ultracentrifugation is the only method described to isolate biologically functional supermeres[15].

Expertise needed to implement the protocol

Previous experience with cell culture and high-speed ultracentrifugation using different rotors is needed to perform the basic isolation protocol. First-hand isolation of EVs, exomeres and supermeres from plasma requires experience in handling and collecting human blood samples.

Limitations

In the isolation of exomeres and supermeres from human plasma, the albumin-depletion step may not remove all albumin. In addition, during the depletion process, some albumin-binding proteins may be lost and thus will not be detected during downstream analysis. Therefore, the compatibility of the albumin-depletion step with a specific target protein in mind should be empirically validated to ensure that the target is not unintentionally depleted along with the albumin.

This protocol allows separation of plasma EVs from HDL particles[9]; however, other lipoprotein particles, such as LDL, VLDL, intermediate-density lipoprotein and chylomicrons present in human plasma, are not removed. Therefore, if minimizing the presence of these other lipoprotein particles is a priority, other published protocols may be more suitable[18,19,26,27,33]. In addition, we cannot exclude the possibility that other types of NVEPs, including lipoproteins, may be co-isolated with exomeres and supermeres from human plasma. Other biofluids, including urine, milk and stool, may present unique

Table 1 | Protein concentrations and ratios of the sEV-P, exomeres and supermeres produced from cell lines in equal volumes

Cell line	Cancer	Concentration (μg/μl)			Ratio		
		sEV-P	Exomere	Supermere	sEV-P	Exomere	Supermere
DiFi	Colorectal	8.7	4.5	13.8	1	0.52	1.59
LIM1215	Colorectal	14.1	8.9	36.4	1	0.63	2.58
LS174T	Colorectal	10.1	7.5	20.8	1	0.74	2.06
CC	Colorectal	4.5	2.2	15.4	1	0.49	3.42
CC-CR	Colorectal	7.2	3.1	21.5	1	0.43	2.99
SC	Colorectal	7.3	3.7	21.1	1	0.51	2.89
MDA-MB-231	Breast	16.0	9.6	36.0	1	0.60	2.25
PANC-1	Pancreas	17.7	12.8	33.8	1	0.72	1.91
Calu-3	Lung	1.3	1.2	2.1	1	0.92	1.62
HREC	Normal kidney	3.2	1.8	6.7	1	0.56	2.09

Note that the size of the sample preparations (number of cell culture plates) was not equal between different cell lines. Adapted from ref. [15].

challenges (such as the highly abundant Tamm-Horsfall protein in urine[30]) for isolation of EVs and NVEPs, and, in those instances, other published protocols may represent better alternatives[18,30,31,34].

Experimental design

Cell line choice

Our laboratory has used a human colorectal cancer cell line, DiFi, as a standard for isolation and characterization of EVs and NVEPs[10,15]. The Extracellular RNA Communication Consortium Phase 2 is using secreted fractions from this line for its benchmarking studies[42]. A typical preparation from DiFi cells consists of 80 culture dishes (15 cm) with ~1.34×10^8 cells per dish at the time of harvest. The typical protein yield is ~4 mg for sEV-P, 2.5 mg for exomeres and 7 mg for supermeres. Depending on the downstream application, our methods can be applied to any cell type; we have applied this method to human cancer cell lines (colorectal, breast, lung, pancreas and glioblastoma) and human primary renal proximal tubule epithelial cells (Table 1). The protocol is suitable for cell culture medium from both conventional 2D cultures and bioreactor (3D) cultures[32,43,44]. However, the yield and purity of different extracellular fractions may be influenced by cell type, cell growth conditions, culturing methods, serum-free or exosome-depleted serum-containing medium, cell density, cell stress and drug exposures. Additional factors, including the number of culture dishes and the serum-starvation times, will also influence yield.

Isolation of lEV-Ps, sEV-Ps, exomeres and supermeres from cell-conditioned medium (Steps 1–38) or plasma (Box 1)

From the same starting material, we present an optimized protocol that sequentially isolates crude lEV-Ps, crude sEV-Ps, exomeres and supermeres, which are released from cells, by using sequential centrifugation, concentration and filtration (Fig. 1a and Steps 1–38).

In our published protocol, serum-free conditioned medium was centrifuged for 10 min at 500*g*, followed by 2,000*g* for 20 min at 4 °C to remove cellular debris and apoptotic bodies. The resulting supernatant was then subjected to 10,000*g* for 40 min to isolate lEV-Ps. Then, the supernatant was filtered through a 0.22-μm polyethersulfone filter (Millipore) to reduce potential lEV contamination of the later sEV samples (Steps 1–18).

The supernatant filtrate was concentrated by using a centrifugal concentrator with a 100,000-molecular-weight cutoff (Millipore) (Fig. 1b and Steps 19–30). A 100,000-molecular-weight cutoff concentrator can concentrate the sample from a large (several hundreds of milliliters to several liters depending on the number of cultured dishes) to a smaller volume (<37 ml depending on the preparation size). The concentration procedure serves two purposes: to facilitate the next centrifugation steps by reducing the sample volume and to reduce the amount of potentially contaminating free soluble proteins by allowing them to pass through the concentrator cores. The extent to which the conditioned medium is concentrated (fold-concentration) will depend both on the volume of the medium collected and on the producer cells. Some cell types will produce more EVs and NVEPs; thus,

Box 2 | Optional protocol modification to further homogenize sEV-Ps, exomeres and supermeres by sequential syringing

For many types of downstream analyses, the samples in PBS-H can be resuspended and adequately dispersed by pipetting up and down by using a P1000 manual pipette. However, for some downstream analyses, such as flow cytometry or functional assays, having the samples fully monodispersed may be required or deemed beneficial.

Procedure

To fully homogenize the sample solution containing sEV-Ps, exomeres or supermeres, pass the pelleted material in PBS-H through sequential needles of decreasing pore size: 22G × 1 1/2 (0.7 mm × 40 mm), 27G × 1 1/2 (0.4 mm × 30 mm) and 30G × 1 1/2 (0.3 mm × 13 mm) attached to a 3-ml syringe (BD Plastipak). The syringing is performed at least seven times per needle to obtain a more homogeneous, monodispersed particle preparation.

the medium will need to be concentrated less because the starting concentration is higher. Conversely, cells that release lesser amounts of EVs and NVEPs will require a greater degree of concentration because the starting conditioned medium is more dilute. The procedure is flexible in terms of the fold-concentration and can be determined by the investigator to suit their individual needs and concerns.

The concentrate, containing sEV-Ps, exomeres and supermeres, is then subjected to high-speed ultracentrifugation at 167,000*g* for 4 h at 4 °C in an SW32 Ti swinging-bucket rotor (*k* factor of 133; Beckman Coulter). The resulting pellet is resuspended in PBS-HEPES (25 mM) (PBS-H) and washed by centrifuging again at 167,000*g* for 4 h. The washed pellet contains sEVs and NV components and is designated as 'sEV-P'. In many studies, sEV-Ps are isolated by ultracentrifugation for shorter times, including 1 h, 70 min or 2 h, although some studies have also used extended overnight centrifugation. Short centrifugation times may not completely pellet all the sEVs[32]. Longer centrifugation times will allow more complete pelleting of sEVs but at the cost of co-sedimenting increasing amounts of NV material, including exomeres. Likewise, the choice of centrifugation *g* force (and the *k* factor of the chosen rotor type) will influence the efficiency of sEV sedimentation, with higher *g* forces leading to more effective pelleting of sEVs but at the potential cost of increased levels of co-sedimented NV material[29,32]. We have previously used centrifugation *g* forces ranging from 120,000 to 167,000*g* for 4–6 h with good yields of intact sEVs[9,10,15,45]. Yield and purity of sEV-Ps are two important factors to consider during this step of isolation, but the protocol is flexible enough to allow *g* forces from 100,000 to 167,000*g* and spin times from 70 min to 16 h (overnight), depending on the needs of the investigator.

We have developed and optimized a simple but high-yield method of separating exomeres from sEV-Ps. To isolate exomeres, the supernatant collected from the 4-h ultracentrifugation, which is normally discarded, is ultracentrifuged at 167,000*g* for 16 h at 4 °C in an SW32 Ti swinging-bucket rotor (*k* factor of 133; Beckman Coulter). The resulting pellet is resuspended in PBS-H and washed by centrifuging again at 167,000*g* for 16 h. The washed pellet represents biologically active exomeres[10].

To isolate supermeres, the supernatant from the pelleting of exomeres is subjected to ultracentrifugation at 367,000*g* by using a Beckman Coulter SW55 Ti rotor (*k* factor of 48) for 16 h at 4 °C. The resulting pellet is resuspended in PBS-H and is designated as 'supermeres'.

An optional step in the protocol (Box 2) is to pass pelleted samples sequentially through increasingly narrow-pore-size syringes when monodispersion of the sample is required (Steps 35, 37 and 38).

High-resolution density gradient fractionation of lEVs and sEVs from NV fractions (Steps 39–51)

In our protocol, we demonstrate separation of lEVs and sEVs from NV components by a combination of high-speed ultracentrifugation and bottom-loaded high-resolution iodixanol density-gradient fractionation[9] (Fig. 2 and Steps 39–51). The bottom-loaded high-resolution gradient (12–36%, wt/vol) allows EVs to float upward in the gradient and reach their buoyant densities in the top portion of the gradient while the NV components remain in the bottom portion of the gradient because of their higher density. This approach has been shown to remove contaminating NV fractions, including vault structures, nucleosomes and non-EV RNA-binding proteins[9]. Bottom loading of iodixanol gradients has also been shown to be preferable for isolation of EVs from urine[30,31]. The bottom-loaded high-resolution (12–36%, wt/vol) gradient can be used to separate HDL from EVs because of the relatively higher density of HDL particles[9]. However, because of their relatively lighter density, other lipoprotein particles, such as LDL, VLDL, intermediate-density lipoprotein and chylomicrons, will have overlapping buoyant densities with EVs. For plasma samples in which these lipoproteins are abundant, a top-loading gradient approach may be preferable if minimizing the presence of these lower-density lipoprotein particles is a priority[18,26,27]. Most circulating Argonaute 2

(AGO2)-miRNA complexes are NV and can be separated from EVs in plasma by bottom-loading the samples for the high-resolution gradient[9,46].

Isolation of sEV-Ps, exomeres and supermeres from human plasma and a step to deplete albumin (Box 1)

In addition to EVs, exomeres and supermeres, plasma contains other types of particles (including lipoproteins) and proteins (including albumin, globulins and clotting factors such as fibrinogen). Other published protocols have implemented various combinations of centrifugation, size-exclusion chromatography and density-gradient fractionation to improve isolation of EVs from plasma[19,26,27,33]. However, in this protocol, we focus on isolation of EVs and the newly identified exomeres and supermeres from plasma by using ultracentrifugation and density-gradient methods[9,10,15]. Unlike cell-conditioned medium, plasma samples are not concentrated, but rather diluted ≥10-fold before isolation of exomeres and supermeres because of the inherently high viscosity of plasma. Albumin is the most abundant protein in plasma, accounting for roughly 60% of the total protein. We describe a method to isolate lEV-Ps, sEV-Ps, exomeres and supermeres from plasma by sequential ultracentrifugation[15] (Fig. 3 and Box 1). However, because of the abundance of albumin in exomeres and supermeres, it is difficult to perform downstream analysis of these NVEPs by using proteomics or immunoblotting. We describe a method to deplete albumin from isolated exomeres and supermeres by using an albumin-depletion kit (Abcam) (Fig. 4 and Box 1). We can also deplete albumin from plasma before EV and NVEP isolation, but plasma should be diluted ≥10-fold before performing the albumin-depletion step; however, because of the low plasma input volume on the column (each column can hold 100 µl) and the high volume of diluted plasma, it may be too costly to routinely dilute the plasma before isolation of EVs and NVEPs. The sEV-P isolated from plasma can be further fractioned into sEV and NV fractions[9] by using high-resolution 12–36% (wt/vol) iodixanol gradient fractionation (Steps 39–51) to further increase the purity of sEVs for downstream analysis. Our comprehensive isolation of EVs, exomeres and supermeres from plasma, including albumin depletion, can enhance biomarker discovery.

Characterization of isolated EVs and NVEPs

After isolation, we recommend characterization of EVs and NVEPs in compliance with guidelines outlined in MISEV2018[47] before further analyses to validate the quality of isolated fractions. Protocols are available to assess the size, morphology and ultrastructure by AFM[48] and electron microscopy[49], and standard protocols for immunoblotting or ELISA can be used to assess the presence of expected marker proteins for lEVs, sEVS, exomeres and supermeres[6,9,10,15,47]. Nanoparticle tracking analysis (NTA) can be used to count total particle numbers and estimate size distribution on the basis of the scattering of light[50], with the addition of detergents improving the discrimination between EVs and NVEP[9]. However, it should be noted that both exomeres and supermeres are likely to be below the size that can reliably be detected with NTA, and caution should therefore be taken when normalizing by particle number for downstream analysis or functional assays. Normalization by protein quantification is the preferred option at this time.

Controls

For depletion of albumin, the plasma before albumin depletion is used as a control to compare with the plasma obtained after albumin depletion to determine if the depletion has been successful.

Materials

Biological materials

- *Human plasma samples from healthy individuals and patients with colorectal cancer*. We have isolated EV and NVEPs from human plasma from both healthy individuals and patients with colorectal cancer at Vanderbilt University Medical Center. **! CAUTION** The research conducted as part of this protocol complies with all the relevant ethical regulations. The use of the human samples was approved by the Vanderbilt University Medical Center Institutional Review Board (IRB; IRB nos. 161529 and 151721).
- The DiFi (RRID: CVCL_6895) colorectal cancer cell line, developed and characterized by the Robert Coffey laboratory
- The DKO-1 (RRID: CVCL_9798) colorectal cancer cell line, obtained from T. Sasazuki at Kyushu University

- The Gli36 (RRID: CVCL_RL88) human glioblastoma cell line, obtained from Xandra Breakefield at Harvard Medical School
- The B16-F1 (RRID: CVCL_0158) mouse melanoma cell line, obtained from the American Type Culture Collection

General laboratory reagents

- PBS without calcium and magnesium, pH 7.4 (Gibco, cat. no. 14190-144)
- HEPES (Sigma, cat. no. H0887)
- DMEM (Corning, cat. no.10-017-CV)
- Non-essential amino acid solution (100×, Cytiva HyClone; Fisher Scientific, cat. no. SH3023801)
- Penicillin-streptomycin (100×, Cytiva HyClone; Fisher Scientific, cat. no. SV30010)
- L-Glutamine (Cytiva SH3003401)
- Bovine calf serum (BCS) (Cytiva HyClone; Fisher Scientific, cat. no. SH3054103)
- Distilled H_2O (Corning, cat. no. 46-300-cl)
- Ethyl alcohol, 200 proof (Pharmco, cat. no. 111000200) **! CAUTION** Ethanol is a highly flammable liquid and vapor. It causes serious eye irritation. Keep the container tightly closed and store it in a well-ventilated place.
- Iodixanol (OptiPrep) (Sigma-Aldrich, cat. no. D1556)
- Albumin-depletion kit (Abcam, cat. no. ab241023)
- SimplyBlue SafeStain (Invitrogen, cat. no. LC6060)
- Trypan blue solution, 0.4% (wt/vol) (Cellgro, cat. no. 25-900-cl)
- TGFBI (RRID: AB_2202311) antibody (Proteintech, cat. no.10188-1-AP)

Equipment

General equipment

- Microcentrifuge (Sorvall Legend Micro 17R; Thermo Scientific, cat. no. 75-002-543)
- Centrifuge (Sorvall LYNX 4000, superspeed, swing bucket; Thermo Fisher Scientific, cat. no. 75006580)
- Benchtop swinging-bucket centrifuge (Sorvall Legend XTR; Thermo Fisher Scientific, cat. no.75-217-420)
- Optima XPN-100 ultracentrifuge (Beckman Coulter, cat. no. A94469)
- Optima XE-100 ultracentrifuge (Beckman Coulter, cat. no. A94516)
- SW 41 Ti swinging-bucket rotor, titanium (Beckman Coulter, cat. no. 331362); the *k* factor of this rotor is 124, and the maximum speed is 41,000 rpm
- SW 32 Ti swinging-bucket rotor, titanium (Beckman Coulter, cat. no. 369694); the *k* factor of this rotor is 133, and the maximum speed is 32,000 rpm
- SW 55 Ti swinging-bucket rotor, titanium (Beckman Coulter, cat. no. 342194); the *k* factor of this rotor is 48, and the maximum speed is 55,000 rpm
- Direct Detect spectrometer (Millipore, cat. no. DDHW00010-WW)
- Direct Detect cards (Millipore, cat. no. DDAC00010-GR)
- Balance (Explorer Pro Precision; Ohaus, cat. no. 80108933)
- Cabinet (Class II, Type A2 biological safety cabinet, Nuaire, cat. no. NU-425-400)
- Nutating mixer (Barnstead International, cat. no. M26125)
- Electronic pipettor (Repeater E3x - electronic multi-dispenser pipette; Eppendorf, cat. no. 4987000410)
- Sample mixer (HulaMixer; Invitrogen, cat. no. 15920D)
- NanoDrop One (Thermo Scientific, cat. no. 13-400-518)
- T-175 cell culture flask (175 cm^2, CELLSTAR; Greiner, cat. no. 660-175)
- T-75 cell culture flask (Falcon 75 cm^2 rectangular straight neck cell culture flask with vented cap; Corning, cat. no. 353-110)
- 145/20-mm cell culture dish (CELLSTAR; Greiner, cat. no. 639160)
- 60/15-mm cell culture dish (Falcon; Corning, cat. no. 353002)
- 50-ml conical centrifuge tube (Corning, cat. no. 430829)
- 15-ml conical centrifuge tube (CellStar, cat. no. 188261)
- 1.5-ml centrifuge tube (Fisher Scientific, cat. no. 05-408-129)
- 10-ml pipette (CELLSTAR serological pipette; Greiner, cat. no. GN607180)
- 5-ml pipette (CELLSTAR serological pipette; Greiner, cat. no. GN606180)
- 25-ml pipette (CELLSTAR serological pipette; Greiner, cat. no. GN760180)
- 22G × 1 1/2 needles (0.7 mm × 40 mm, precision glide needle; BD, cat. no. 305156)
- 27G × 1 1/2 needles (0.4 mm × 30 mm, precision glide needle; BD, cat. no. 305136)

- 30G × 1 1/2 needles (0.3 mm × 13 mm; precision glide needle; BD, cat. no. 305106)
- 3-ml syringe (Plastipak; BD, cat. no. 309657)
- 5-ml syringe (Plastipak; BD, cat. no. 309646)
- Centricon Plus-70 (100,000-MV cutoff; Millipore, cat. no. UFC710008)
- SETON open-top polyclear centrifuge tubes, 1 × 3 1/2 inch (25 × 89 mm) (SETON, cat. no. 7052)
- Ultra-clear centrifuge tubes (13 × 51 mm) (for supermere isolation) (Beckman Coulter, cat. no. C14295)
- Ultra-clear centrifuge tubes (25 × 89 mm) (for sEV-P and exomere isolation) (38.5 ml, sterile, open-top thin wall; Beckman Coulter, cat. no. C14292)
- Ultra-clear centrifuge tubes (14 × 89 mm) (for gradient purification of EVs) (Beckman Coulter, cat. no. C14293)
- Stericup quick release (express plus 0.22-uM polyethersulfone (PES); Millipore, cat. no. S2GPU11RE)
- Syringe filter (Millex-GP syringe filter unit, 0.22 µm, PES, 33 mm, gamma sterilized; Millipore, cat. no. SLGP033RS)
- Blood-collection tubes (BD Bioscience, cat. no. 369714)
- Amicon Ultra-15 centrifugal filters (, 50-kDa molecular-weight cutoff; Millipore, cat. no. UFC905024)
- Amicon Ultra-15 centrifugal filters (30-kDa molecular-weight cutoff; Millipore, UFC903024)
- NuPAGE Bis-Tris gel (4–12%; Invitrogen, cat no. NP0322BOX)

Reagent setup

70% (vol/vol) sterile ethanol

To prepare 1 liter of 70% (vol/vol) ethanol, mix 700 ml of 200 proof ethyl alcohol with 300 ml of Milli-Q H_2O, pass through a 0.22-µm filter and store at room temperature (RT; 22 °C) for ≤6 months.

Complete DMEM medium (10% (vol/vol) BCS)

In a tissue culture hood, to a bottle of 450 ml of DMEM medium, add 50 ml of bovine growth serum, 5 ml of non-essential amino acid solution (100×), 5 ml of L-glutamine (100×, 200 mM) and 5 ml of penicillin-streptomycin (100×, 10,000 U/ml), mix well and keep at 4 °C for ≤1 week.

Serum-free DMEM medium

In a tissue culture hood, to a bottle of 500 ml of DMEM medium, add 5 ml of non-essential amino acid solution (100×), 5 ml of L-glutamine (100×) and 5 ml of penicillin-streptomycin (100×), mix well and keep at 4 °C for ≤1 week.

PBS-H (25 mM) (vol/vol)

Add 12.8 ml of 1 M sterile HEPES (pH 7.0–7.6) to a bottle of 500 ml of sterile PBS, mix well and keep at 4 °C for ≤1 month.

Iodixanol (OptiPrep) 12–36% (wt/vol)

To make the density solutions, mix 60% (wt/vol) iodixanol with sterile PBS on the basis of the volumes listed in Table 2. Mix well. Solutions should be made fresh before the experiment and kept at 4 °C for up to a few days.

Procedure

Isolation of lEV-Ps, sEV-Ps, exomeres and supermeres from conditioned medium of cell culture ● Timing variable, depending on the growth rate of the cells and the size of the preparation

▲ CRITICAL The DiFi colorectal cancer cell line has been used as a standard in this protocol. This protocol is applicable to various cell lines from different organs and origins (see Experimental design for further discussion).

▲ CRITICAL The procedure below is for cell-conditioned medium, but the isolation of lEV-Ps, sEV-Ps, exomeres and supermeres can also be performed for human plasma (Box 1). For plasma samples, skip

Table 2 | Iodixanol (OptiPrep) solutions

Final iodixanol solution concentration (%, wt/vol)	Volume of 60% iodixanol stock (ml)	PBS volume (ml)
50	20	4
36	14.4	9.6
30	12	12
24	9.6	14.4
18	7.2	16.8
12	4.8	19.2

the medium collection (Steps 1–10) and medium concentration (Steps 19–30) procedures below and instead perform the steps outlined in Box 1.

Medium collection

▲ **CRITICAL** Perform Steps 1–6 in a Class II, Type A2 biological safety cabinet (Nuaire).

1 Seed cells in 175T flasks in 35 ml of warm (37 °C) DMEM complete medium (listed in the reagent setup) in the tissue culture hood. The number of flasks of cells needed depends on the size of the preparation needed for downstream applications.

! CAUTION Cells should be routinely tested for mycoplasma contamination. We routinely assess cell viability by using trypan blue exclusion (Cellgro). Only medium collected from cells with >95% viability is used for isolation of EVs and NVEPs.

▲ **CRITICAL** The number of cells per flask depends on the specific cell line because they vary in size. A visual estimation that the cells cover ~70–80% of the flask is sufficiently precise.

2 Grow cells in the tissue culture incubator with 5% CO_2 at 37 °C.

3 Seed cells into 15-cm dishes in 18 ml of DMEM complete medium (or T175 culture flask in 25–30 ml of medium) when the cells reach ~70–80% confluency and grow them in the tissue culture incubator with 5% CO_2 at 37 °C until they reach 70–80% confluency. Normally, it takes ~48 h to reach 70–80% confluency, depending on the growth rate and the seeding density of the cells.

▲ **CRITICAL STEP** The number of dishes (or T175 flasks) needed depends on the size of the preparation and the downstream application. A standard preparation for comprehensive profiling and functional in vivo assays normally uses 80–120 dishes, but smaller setups can be used for biochemical and molecular characterization.

4 Wash the cells twice with ≥12 ml of pure DMEM medium without any supplements.

▲ **CRITICAL STEP** Tilt the plate for 1 min after aspirating the medium to be sure that all the medium is completely removed.

5 Starve the cells in 18 ml of serum-free DMEM medium (without BCS but include all the other supplements listed in the reagent setup) for 48 h in the tissue culture incubator with 5% CO_2 at 37 °C.

▲ **CRITICAL STEP** The EVs and NVEPs are isolated from serum-free medium to avoid any bovine serum–derived EV contamination. If bovine growth serum is used, EV-depleted FBS should be prepared (Box 3).

6 Collect the serum-free conditioned medium into 50-ml conical tubes.

! CAUTION Tilt the plate for 1 min after aspirating the medium to be sure that the medium is completely removed.

lEV-P isolation ● Timing 2 h

7 Centrifuge the conditioned medium at 500*g* for 10 min at 4 °C to pellet cells and cell debris.

8 Transfer the supernatant to a new 50-ml conical tube by decanting or pipetting the supernatant in the tissue culture hood. Discard the pellet.

▲ **CRITICAL STEP** Perform this step as soon as the centrifugation is completed, to avoid dispersion of the pellet. If transferring by pipetting, leave 0.5 ml of the supernatant above the pellet to avoid possible contamination.

9 Centrifuge the supernatant at 2,000*g* for 20 min at 4 °C to remove cell debris and apoptotic bodies.

10 Transfer the supernatant to a new 50-ml conical tube by decanting or pipetting the supernatant in the tissue culture hood. Discard the pellet.

Box 3 | Optional protocol modification for using medium containing EV-depleted bovine serum

As an alternative to using serum-free medium for harvesting EV- and NVEP-containing cell-conditioned medium, medium containing EV-depleted serum can be used. It is important to note that while bovine EVs can be mostly eliminated, some contaminating EVs may remain[52], and bovine NVEPs and bovine RNA-protein complexes[53] will also still be present. In addition, growth factors present in the serum that support cellular viability may be removed by the procedure to eliminate bovine EVs[53]. Of course, the use of serum-free medium will also influence cellular growth and stress, with possible consequences for subsequent isolation and content of EV and NVEPs. Thus, no perfect solution is currently available, and investigators will have to choose serum-free or EV-depleted serum on the basis of the most important downstream analyses that they wish to perform.

Procedure

1 Dilute BCS or FCS 1:5-fold in DMEM.
2 Centrifuge the diluted serum-DMEM at 100,000*g* for 16–18 h at 4 °C in an SW 32 Ti swinging-bucket rotor (Beckman Coulter).
3 After centrifugation, the EV-depleted medium is contained in the supernatant, whereas the bovine serum EVs will form the pellets at the bottom of the tubes. Remove the EV-depleted medium by decanting or pipetting. Discard the pellets.
4 Pass the EV-depleted medium through a 0.22-μm PES filter (Millipore). Add the filtered EV-depleted medium (20% serum) to DMEM to obtain the desired final concentration of serum in the medium.

▲ **CRITICAL STEP** Perform this step as soon as the centrifugation is completed, to avoid dispersion of the pellet. If transferring by pipetting, leave 0.5 ml of the supernatant above the pellet to avoid possible contamination.

11 Centrifuge the supernatant at 10,000*g* for 40 min at 4 °C to collect the lEV-P.
▲ **CRITICAL STEP** Depending on the experimental design, if only the sEV-Ps, exomeres and supermeres are to be isolated but not lEV-Ps, then skip Steps 11–17, continuing at Step 18 to filter the medium supernatant collected in Step 10 through a 0.22-μm pore PES filter.

12 Transfer the supernatant to a T175 flask by pouring off carefully or pipetting the supernatant. Tilt the tube and carefully transfer the rest of the supernatant out to avoid disturbing the pellet. This pellet is the lEV-P.
▲ **CRITICAL STEP** Perform this step as soon as the centrifugation is completed, to avoid dispersion of the pellet. If transferring by pipetting, leave 0.5 ml of the supernatant above the pellet to avoid possible contamination.

13 Repeat Steps 11 and 12 until there is no more medium left.
▲ **CRITICAL STEP** The number of times this needs to be repeated will depend on the total volume of conditioned medium collected. A higher volume of medium will take a longer time to process.

14 Keep all the supernatant in the T175 flasks at 4 °C for downstream isolation of sEVs, exomeres and supermeres, starting from Step 19.

15 Wash the lEV-P pellet with PBS-H. Add 1 ml of PBS-H to each pellet in the 50-ml tubes and resuspend the pellet thoroughly by pipetting to wash away free proteins and other contaminants trapped in the lEV-P. Combine the resuspended pellets into a new 50-ml centrifuge tube by pipetting. Rinse each tube by adding 1 ml more of PBS-H and transfer the liquid to the 50-ml tube containing the resuspended lEV-P. Fill this tube with PBS-H until it reaches 50 ml.

16 Centrifuge the resuspended lEV-P at 10,000*g* for 40 min at 4 °C to collect the washed lEV-P. Discard the supernatant by decanting or pipetting. Tilt the tube and carefully transfer the rest of the supernatant out by using a 1-ml pipette to avoid disturbing the lEV pellet.

17 Resuspend the pellet in PBS-H and transfer the lEV solution to a 1.5-ml microcentrifuge tube by using a P1000 manual pipette. Take out 10 μl of the lEV-P material and quantify the protein concentration by using a Direct Detect spectrometer as described by the manufacturer's protocol (Millipore) or by using alternative assays for protein quantification such as bicinchoninic acid (BCA) or Bradford[51].
▲ **CRITICAL STEP** The amount of buffer added depends on the size of the preparation, the host cell line and the downstream applications. For example, if the preparation size is eighty 15-cm plates of DiFi cells, we resuspend the pellet in 500 μl of PBS-H or lysis buffer.
■ **PAUSE POINT** Depending on the downstream application purpose, the lEV-Ps can be kept at either 4 °C (short-term storage) for characterization including immunoblotting, electron microscopy and NTA, as well as for functional studies, or −80 °C (long-term storage) or used immediately.
? TROUBLESHOOTING

18 Pass the medium supernatant from Step 14 through a 0.22-μm pore PES filter (Stericup 1,000 ml, Millipore express plus 0.22-μm PES) to remove any remaining lEVs. Keep the supernatant in the filter bottle at 4 °C for up to a few days or continue immediately to the next step for sEV-P isolation.

■ **PAUSE POINT** The filtered medium supernatant can be kept at 4 °C for a short time (1–2 d) before sEV-P isolation.

Cell-conditioned medium supernatant concentration by using a concentrator

● **Timing variable, depends on the total volume of medium and the host cells**

19 Rinse the centrifugal filter device by adding 15 ml of 70% (vol/vol) ethanol to the sample filter cup and shake the sample filter cup several times upside down to sanitize the device (Centricon Plus-70, Millipore, 100,000 NMWL (nominal molecular weight limit)).
▲ **CRITICAL STEP** 70% (vol/vol) ethanol needs to be filtered through a 0.22-μm pore PES filter (Stericup 1,000 ml, Millipore express plus 0.22-μm PES).

20 Centrifuge the filter device at 3,500*g* for 5 min at 4 °C by using a benchtop swinging-bucket centrifuge (Thermo Fisher Scientific, Sorvall Legend XTR). Shake the bottom filtrate collection cup with 70% (vol/vol) ethanol to sanitize the device and pour off the ethanol.
! **CAUTION** The maximum centrifugal force is 3,500*g* for a concentration spin using the filter device. Do not use a fixed-angle rotor.

21 Rinse the filter device by adding 15 ml of sterile PBS-H into the top sample filter cup. Shake it well. Spin the filter device at 3,500*g* for 5 min at 4 °C. Shake the bottom filtrate collection cup several times and pour off the PBS-H. The device is ready for sample concentration.
▲ **CRITICAL STEP** Keep the membrane wet once it is rinsed with 70% (vol/vol) ethanol and PBS-H. Leave the fluid on the membrane until it is used.

22 Shake the bottle with medium from Step 18 and load 60 ml to the sample filter cup. Carefully put on the cap.
! **CAUTION** The maximum volume capacity of the collection cup is 70 ml. Add no more than 70 ml of medium to the collection cup, each time.

23 Centrifuge the filter device at 3,500*g* at 4 °C until no medium is above the top of the filter cores. Proteins with a molecular weight smaller than 100 kDa will go through the filter.
! **CAUTION** Balance the centrifuge with a second filter device and an equal volume of PBS or medium.
▲ **CRITICAL STEP** The centrifugation time varies from 12 to 60 min depending on the host cell line and medium protein concentration.

24 Remove the filter device and separate the sample filter cup from the filtrate-collection cup. Carefully pour the medium out of the filtrate-collection cup and discard it.

25 Repeat Steps 22–24 until no medium is remaining in the top of the sample filter cup.
! **CAUTION** If the medium flow rate slows to <1 ml/min, then a new filter device should be used. Typically, a filter device can filter 1 liter of medium, but this depends on the number of EVs and NVEPs released into the condition medium. The final volume of the concentrate depends on the fold concentration desired.

26 Add 60 ml of PBS-H to the sample filter cup and centrifuge at 3,500*g* for 10 min at 4 °C to wash the concentrate. Pour off the flowthrough in the filtrate-collection cup and discard it.

Concentrate recovery ● Timing 5–10 min

27 Rinse the concentrate-collection cups (Fig. 1) with 70% (vol/vol) ethanol, dry in the tissue culture hood with air for ≥30 min and then expose the cups under the UV light in the tissue culture hood for ≥30 min to sterilize.

28 Turn the concentrate-collection cup upside down and place it on top of the sample filter cup.

29 Invert the cups carefully and place them in a benchtop centrifuge; then, centrifuge at 1,000*g* for 2 min at 4 °C.
! **CAUTION** The maximum centrifugal force for the recovery spin is 1,000*g*. Sample may be lost if higher than a 1,000*g* spin force is used.
▲ **CRITICAL STEP** Wipe off any excess fluid on the side of the filter cup.

30 Remove the concentrate-collection cup containing the concentrated sample from the sample filter cup. Transfer the sample with a 1-ml pipette to a 50-ml conical tube. Rinse the collection cup with PBS-H several times and then combine into the 50-ml tube.
■ **PAUSE POINT** The concentrated samples can be kept at 4 °C for ≤16 h or can be immediately subjected to downstream sEV-P isolation.

Sequential high-speed ultracentrifugation to isolate sEV-Ps, exomeres and supermeres

● Timing 3 d

31 Load the concentrate from Step 30 to ultra-clear centrifuge tubes (25 × 89 mm, 38.5 ml, sterile; Beckman Coulter). Add PBS-H to reach a total volume of ~37–37.5 ml. Mix by pipetting up and down.

! CAUTION Each tube can hold a maximum of 38.5 ml of solution but is normally filled to 37–37.5 ml. If the solution volume is less than that, the tubes may be crushed because of the high-speed ultracentrifugation power. The tubes should be filled to the recommended volume with PBS-H.

! CAUTION Incorrect use of an ultracentrifuge may result in equipment failure and damage and cause loss of the sample. Always ensure that tubes are filled nearly to the top, that rotor buckets are correctly closed and attached to the rotor and that opposing buckets with filled tubes have the same weight to ensure balance.

▲ CRITICAL STEP If the centrifuge tubes are not sterile (e.g., SETON open-top Polyclear centrifuge tubes 1 × 3 1/2 inches (25 × 89 mm)), they need to be sanitized. Rinse the tubes with 70% (vol/vol) 0.22-μm-filtered ethanol first, pour off the ethanol, air-dry the tubes in the tissue culture hood and then expose the tubes to UV light to sanitize. These centrifuge tubes are used for sEV-P and exomere isolation.

▲ CRITICAL STEP The centrifuge tubes must be carefully balanced by weight. Tubes numbered 1 and 4, 2 and 5 and 3 and 6 should be balanced in pairs.

32 Carefully load the tubes on the rotor and centrifuge the concentrate at 167,000*g* for 4 h at 4 °C in an SW 32 Ti swinging-bucket rotor (Beckman Coulter).

! CAUTION The tube number must match the number on the rotor. For example, put tube 1 onto the rotor that is labeled '1'. Be careful to be sure that the tubes are secured on the rotor.

▲ CRITICAL STEP The rotor and holders should be kept at 4 °C when not in use.

33 Transfer the supernatant to a new sterile ultracentrifuge tube by decanting carefully or pipetting off the supernatant and keep at 4 °C for exomere and supermere isolation. If pouring off, tilt the tube and remove the extra liquid above the pellet by pipetting with a 1-ml manual pipette to avoid disturbing the pellet and then discard the supernatant. If transferring the supernatant by pipetting, use a 10- or 25-ml sterile disposable pipette and leave ~1 ml of the supernatant over the pellet. Transfer the residual volume by using a 1-ml pipette.

▲ CRITICAL STEP Perform this step as soon as the centrifugation is completed, to avoid dispersion of the pellet.

34 To wash the pellet, add 1 ml of PBS-H and pipette up and down several times by using a 1-ml pipette. Transfer the resuspension to a new sterile ultracentrifuge tube and add 36.5 ml of PBS-H to the residual pellet to transfer the rest of the pellet to the tube. Then, repeat Steps 31 and 32.

35 Discard the supernatant (PBS-H) by decanting or pipetting as soon as the centrifugation is completed. Tilt the tube, carefully transfer the rest of the supernatant off by using a 1-ml pipette to avoid dispersing the pellet. The resulting washed crude pellet is designated the 'sEV-P'. Resuspend the sEV-P in PBS-H or lysis buffer depending on downstream analysis and transfer the solution to a 1.5-ml microcentrifuge tube by using a 1-ml pipette. Optionally, the crude sEV-P can be further homogenized by sequential passage through syringes (Box 2). Pipette out a small amount (2 μl per measurement) of the sEV-P material and quantify the protein concentration by Direct Detect spectrometer as described by the manufacturer's protocol (Millipore) or by using alternative assays for protein quantification such as BCA or Bradford.

! CAUTION Perform this step as soon as the centrifugation is completed, to avoid dispersion of the pellet.

▲ CRITICAL STEP The amount of buffer added depends on the size of the preparation/pellet and the donor cell line. For example, if the preparation size is eighty 15-cm dishes of DiFi cells, resuspend the pellet in 1 ml of PBS-H or lysis buffer.

■ PAUSE POINT Depending on the downstream applications, the sEV-P sample can be kept at either 4 °C (short-term storage) or −80 °C (long-term storage) or used immediately.

? TROUBLESHOOTING

36 To isolate exomeres, ultracentrifuge the supernatant collected from the 4-h ultracentrifugation in Step 33 at 167,000*g* for 16 h at 4 °C in an SW 32 Ti swinging-bucket rotor (Beckman Coulter). After the first centrifugation is completed, keep the supernatant at 4 °C for later supermere isolation.

37 Fully resuspend and wash the pellet in 37.5 ml of PBS-H and centrifuge at 167,000*g* for 16 h at 4 °C in an SW 32 Ti swinging-bucket rotor. The resulting washed pellet is designated as 'exomeres'. Resuspend the exomere pellet in 0.5–1.0 ml of PBS-H or lysis buffer depending on downstream analysis and transfer the solution to a 1.5-ml microcentrifuge tube by using a 1-ml pipette. Optionally, the exomeres can be further homogenized by sequential passage through syringes (Box 2). Take out a small amount of the exomere material (2 µl per measurement) and quantify the protein concentration by Direct Detect spectrometer (Millipore) or alternative assays for protein quantification such as BCA or Bradford.

PAUSE POINT Depending on the downstream applications, the exomere solutions can be kept at either 4 °C (short-term storage) or −80 °C (long-term storage) for future use or used immediately.

? TROUBLESHOOTING

38 To isolate supermeres, load the supernatant collected from the pelleting of exomeres (Step 36) to ultra-clear centrifuge tubes (13 × 51 mm; Beckman Coulter). Subject the supernatant to ultracentrifugation at 367,000*g* by using a Beckman Coulter SW 55 Ti rotor for 16 h at 4 °C. Each ultracentrifuge tube (13 × 51 mm; Beckman Coulter) can hold 5 ml of supernatant, and the rotor can hold six tubes, resulting in a total of 30 ml of the supernatant that can be used for isolation of the supermeres. The tubes need to be balanced and loaded on the rotor as stated in Step 31. Discard the supernatant. The resulting pellet is designated as 'supermeres'. Optionally, wash the supermere in PBS-H by an additional round of 367,000*g* centrifugation for 16 h at 4 °C. Resuspend the supermere pellet in 0.5–1.0 ml of PBS-H or lysis buffer depending on downstream analysis and transfer the supermere solution to a 1.5-ml microcentrifuge tube by using a 1-ml pipette. Optionally, the supermeres can be further homogenized by sequential passage through syringes (Box 2). Take out a small amount of the supermere material (2 µl per measurement) and quantify the protein concentration by Direct Detect spectrometer (Millipore) or alternative assays for protein quantification such as BCA or Bradford.

PAUSE POINT Depending on the downstream applications, the supermere sample can be kept at either 4 °C (short-term storage) or −80 °C (long-term storage) for future use or used immediately.

? TROUBLESHOOTING

High-resolution (12–36%, wt/vol) iodixanol density-gradient fractionation of crude EV samples ● Timing 1 d

▲ CRITICAL The crude lEV-Ps and sEV-Ps samples isolated in Steps 18 and 35 above are heterogeneous, containing both EVs and NV material in separate fractions. To obtain highly purified EVs that have been separated from contaminating NV fractions, a bottom-loaded high-resolution (12–36%, wt/vol) iodixanol density-gradient fractionation procedure is performed. Note that the individual fractions can be processed either individually for downstream analyses or pooled (highlighted as optional in the steps below) to represent purified EVs and NV fractions, respectively (Fig. 2).

39 Prepare the iodixanol (OptiPrep) (Sigma-Aldrich) density solutions as outlined in Table 2 in ice-cold PBS immediately or shortly before use. The amounts listed are enough for four to five gradients.

! CAUTION The iodixanol density solutions should be made fresh each time before use or shortly before use and kept at 4 °C.

40 Carefully mix 700 µl of a PBS suspension containing crude lEV-Ps and/or sEV-Ps, isolated in Steps 18 and 35, with 1,700 µl of ice-cold 50% (wt/vol) iodixanol in a 15-ml tube to obtain a final volume of 2.4 ml of 36% (wt/vol) iodixanol EV-P solution.

▲ CRITICAL STEP To ensure complete mixing of the 50% (wt/vol) iodixanol solution with the crude EV suspension in PBS to generate the 36% (wt/vol) iodixanol EV-P solution, slowly pipette (using a P1000 manual pipette) up and down in the 15-ml tube until the mixture looks completely homogeneous.

41 Use a P1000 manual pipette to carefully transfer the 2.4 ml of the 36% (wt/vol) iodixanol EV-P suspension from the 15-ml tube to the bottom of a 14 × 89-mm ultra-clear centrifuge tube (Beckman Coulter), taking care not to deposit material on the sides of the tube.

42 Tilt the centrifugation tube sideways to an angle of 30–45° and carefully dispense 2.4 ml of the 30% (wt/vol) iodixanol solution on top of the previous layer by using an automatic pipettor (Eppendorf Repeater E3x - electronic multi-dispenser pipette) set to the lowest dispensing speed with a Combitips advanced 2.5-ml pippete tip. Alternatively, manually dispense the solution by using a P1000 manual pipette, taking care not to perturb the previous layer.

43 Repeat Step 42 three more times in sequence by dispensing 2.4 ml of 24% (wt/vol) iodixanol solution, then 2.4 ml of 18% (wt/vol) iodixanol solution and then 2.4 ml of 12% (wt/vol) iodixanol solution each on top of the previous layer to complete the gradient.

44 Weigh the loaded centrifugation tube containing 12 ml of 12–36% (wt/vol) iodixanol gradient on a balance (Ohaus, Explorer Pro Precision) to make sure that it matches exactly the second tube that will run opposite in the ultracentrifugation rotor. The second tube can be another gradient containing an EV sample, or it can be an empty 12–36% (wt/vol) gradient that can be used for later determination of fraction densities by refractometry. If an empty (no EV-P sample) gradient is used, instead of loading the 2.4 ml of 36% (wt/vol) iodixanol EV-P sample at the bottom of the centrifugation tube, dispense 2.4 ml of 36% (wt/vol) iodixanol solution to the bottom of the tube (Table 2) and proceed to add the lower-density steps as detailed. For the tube weights to match exactly, carefully add a few drops of 12% (wt/vol) iodixanol solution on top of either tube to make sure that the weights of both tubes match exactly.

45 Place the two tubes opposite each other in cooled (4 °C) buckets of an SW 41 TI swinging-bucket rotor (*k* factor of 124; Beckman Coulter) and centrifuge at 120,000*g* for 15 h at 4 °C.

! CAUTION Incorrect use of an ultracentrifuge may result in equipment failure and damage and cause loss of the sample. Always make sure that tubes are filled nearly to the top, that rotor buckets are correctly closed and attached to the rotor and that opposing buckets with filled tubes have the same weight to ensure balance.

46 Remove the tubes from the bucket and slowly and carefully collect 12 individual fractions of 1 ml each from the top of the gradient by using a P1000 pipette, taking care not to disturb the underlying fractions. Dispense each individual 1-ml fraction in a labeled 14 × 89-mm ultra-clear centrifuge tube. In total, there are 12 fractions.

▲ CRITICAL STEP Optionally, instead of isolating each of the 12 individual fractions, the first 6 fractions (fractions 1–6 from the top) representing EVs can be pooled in an ultra-clear centrifuge tube (13 × 51 mm; Beckman Coulter). The 7th fraction is discarded, and the last 5 fractions (fractions 8–12 from the top) representing NV material can then be pooled in a second tube (Fig. 2).

47 Add 11 ml of PBS to each of the 12 tubes containing a 1-ml fraction and mix (by pipetting or tube inversion) the total volume of 12 ml until the solution appears homogeneous. Adjust with PBS to make sure that the total volume in each tube is equal and to make sure that the weights of the tubes match exactly.

▲ CRITICAL STEP Optionally, if fractions have been pooled in two tubes, add 30 ml of PBS to each of the two tubes and mix. Adjust with PBS to make sure that the total volume in each tube is equal and that the weights of both tubes match exactly.

48 Wash the first six fractions (fractions 1–6, taken from the top of the gradient) in a cooled (4 °C) SW 41 TI swinging-bucket rotor (*k* factor of 124; Beckman Coulter) by centrifugation at 120,000*g* for ≥4 h at 4 °C.

▲ CRITICAL STEP Optionally, if fractions have been pooled in two tubes, wash the two pools in a cooled (4 °C) SW 32 TI swinging-bucket rotor by centrifugation at 120,000*g* for ≥4 h at 4 °C.

49 Discard the supernatant by decanting or pipetting, resuspend the individual pellets of highly purified lEVs or sEVs that are at the bottom of each of the first six centrifugation tubes in a small volume (30–100 µl) of PBS and transfer to a microcentrifuge tube.

▲ CRITICAL STEP Optionally, if fractions have been pooled in two tubes, discard the supernatant and resuspend the two pellets representing the final purified EVs and NV fraction, respectively, in a small volume (30–100 µl) of PBS. Transfer the samples to microcentrifuge tubes. For pooled samples, this concludes the procedure, and Steps 50 and 51 are not applicable.

? TROUBLESHOOTING

50 Wash the second six fractions (fractions 7–12, taken from the top of the gradient) in a cooled (4 °C) SW 41 TI swinging-bucket rotor (*k* factor of 124; Beckman Coulter) by centrifugation at 120,000*g* for ≥4 h at 4 °C.

51 Discard the supernatant by decanting or pipetting and resuspend the individual pellets of NV material that are at the bottom of each of the second six centrifugation tubes in a small volume (30–100 µl) of PBS. Transfer the samples to microcentrifuge tubes.

■ PAUSE POINT The gradient-purified lEV (fractions 1–6) or sEV (fractions 1–6) and NV (fractions 7–12) samples can be used immediately or stored at 4 °C for a short duration. If the samples are extracted in lysis buffer, they can be stored long term at −80 °C.

Troubleshooting

Troubleshooting advice can be found in Table 3.

Table 3 | Troubleshooting table

Step	Problem	Possible reason	Solution
17	Low lEV-P yield	Low cell density when the conditioned medium was collected	Starve cells when 70–80% confluent
		The lEV-P was lost after 10,000*g* centrifugation	Carefully collect and transfer the supernatant once the spin is complete
			The pellet may be firmly attached to the tube; thus, it may be necessary to dislodge and solubilize it by using the pipette tip. Simply rinsing the tube may not be sufficient
35, 37 and 38	Low yields of sEV-P, exomeres and supermeres	Low cell density when the conditioned medium was collected	Starve cells when 70–80% confluent
		The sEV-P and NVEPs were lost during the concentration step because of filter membrane leakage	Save the flowthrough and keep it at 4 °C and re-concentrate it with a new filter device
		The sEV-P and NVEPs were lost during the concentrate-recovery step due to high-speed centrifugation	Spin the concentrate in the collection cup at 1,000*g*
		The sEV-P, exomeres and supermeres were lost after ultracentrifugation at 167,000*g* for 4 h and 16 h for sEV-Ps and exomeres, respectively, and 367,000*g* for 16 h for supermeres	Carefully collect and transfer the supernatant once the spin is complete
			The pellet may be firmly attached to the tube; thus, it may be necessary to dislodge and solubilize it by using the pipette tip. Simply rinsing the tube may not be sufficient
37 and 38	Very high yield of exomeres and supermeres from cell culture	The cell-conditioned medium harvested from cells contains bovine serum	Shift to serum-free medium 48 h before harvest of cell-conditioned medium and wash supermeres in PBS by 367,000*g* centrifugation
49	Low lEV or sEV yield	The initial input of lEV-Ps or sEV-Ps is too low because the sample will be dispersed across several fractions	Increase the amount of input lEV-P or sEV-P material
		Insufficient dilution of the harvested iodixanol-containing fractions in PBS may cause a decrease in the recovered material after the wash step	Increase the volume of PBS used to dilute the fractions for the wash step
Box 1, step 6	Low yield of EV and NVEP derived from human plasma	Blood was kept for too long at room temperature or 4 °C	Human blood needs to be processed at room temperature within 2 h after collection
		The plasma was not diluted enough before EV and NVEP isolation, making the plasma sample too viscous and dense, thus preventing efficient pelleting	Dilute the plasma ≥10-fold before isolation of EVs and NVEPs
		The sEV-P, exomeres and supermeres were lost after ultracentrifugation at 167,000*g* for 4 h and 16 h for sEV-Ps and exomeres, respectively, and 367,000*g* for 16 h for supermeres	Carefully collect and transfer the supernatant once the spin is complete
			The pellet may be firmly attached to the tube; thus, it may be necessary to dislodge and solubilize it by using the pipette tip. Simply rinsing the tube may not be sufficient
Box 1, step 19	Albumin was not sufficiently depleted	Too much sample was placed on the column. The column was saturated	Reduce the input load; each column can bind only ~2–3 mg of albumin
	Low yield of albumin-depleted samples	Residual albumin bound to the column after elution	Wash the column several more times with albumin-binding buffer to elute residual albumin
		The column was not washed enough with albumin-binding buffer; some of the albumin-depleted samples still bind the column	Wash the column several times with albumin-binding buffer to release more samples

Timing

Steps 1–6, cell growth and medium collection: variable (depending on the number of dishes with cells that will be used for medium collection)
Steps 7–17, lEV-P isolation: 2 h (depending on the size of the preparation)
Step 18, medium filtration: 5–10 min
Steps 19–26, medium concentration using a concentrator: variable (depending on the volume of the medium collected and the host cells)
Steps 27–30, medium concentrate recovery: 5–10 min

Steps 31–35, isolation of sEV-Ps from cell-conditioned medium: 8–9 h
Steps 36 and 37, isolation of exomeres from cell-conditioned medium: 33–34 h
Step 38, isolation of supermeres from cell-conditioned medium: 16–17 h
Steps 39–51, high-resolution iodixanol gradient fractionation of crude EV samples: 1 d
Box 1, isolation of sEV-Ps, exomeres and supermeres from human plasma, and albumin depletion: 3 d, 4.5 h

Anticipated results

We have provided a comprehensive protocol for the sequential isolation of lEVs, sEVs, NV fractions, exomeres and supermeres from cells and human plasma. We describe in step-by-step detail the use of differential ultracentrifugation, filtration, concentration and high-resolution density-gradient fractionation to obtain pure EVs, exomeres and supermeres. Representative AFM images of these distinct fractions derived from DiFi cells are shown in Fig. 5a. Similar samples isolated from other cell lines and plasma are expected to have broadly similar morphology as those from DiFi cells. Proteomic analyses have revealed that lEVs, sEVs, NV fractions, exomeres and supermeres have distinct proteome profiles[9,10,15]. Representative immunoblots of select proteins in the distinct fractions are shown in Fig. 5b. Characteristically, the EV protein flotillin-1 is restricted to gradient-purified sEVs, and the vault protein MVP is restricted to NV fractions. The expression of the metabolic enzyme LDHA is

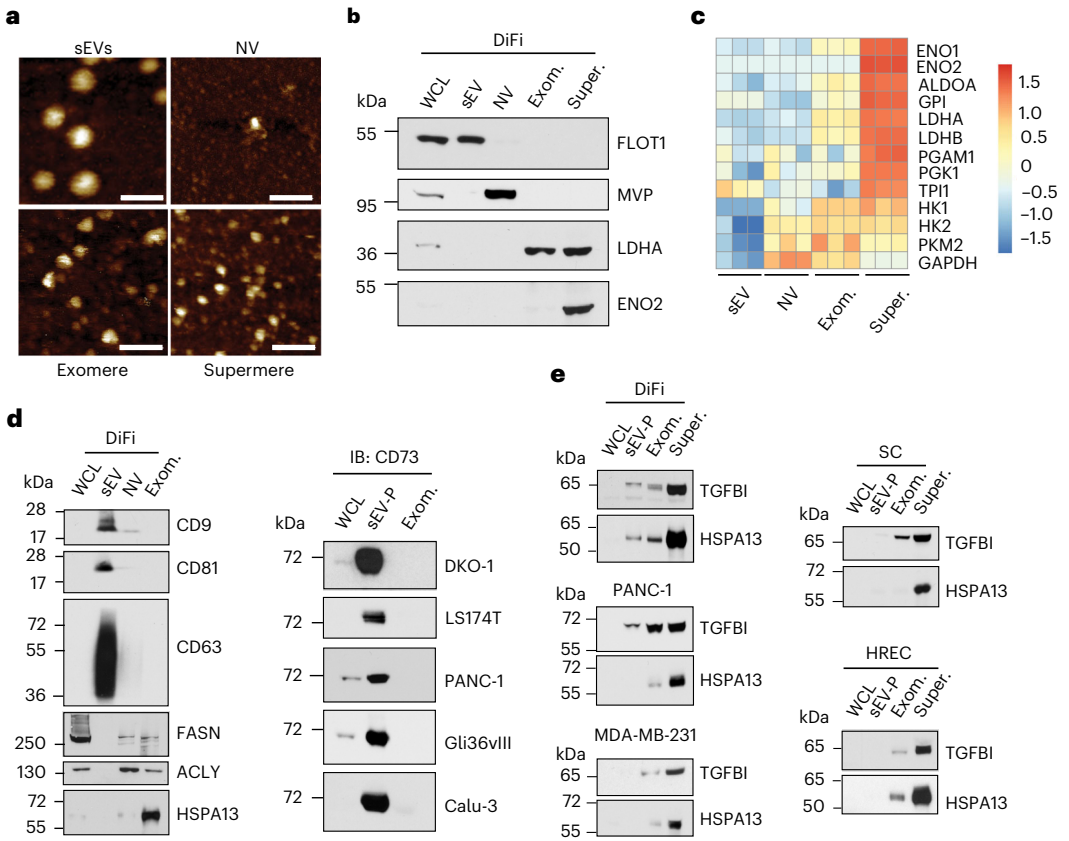

Fig. 5 | Anticipated results. a, Representative fluid-phase AFM topographic images of all the fractions derived from DiFi cells. sEVs (top left), NV fractions (top right), exomeres (bottom left) and supermeres (bottom right). Twenty microliters of isolated sEVs, NV fractions, exomeres and supermeres were diluted 1:1 with PBS and then incubated over (3-aminopropyl) triethoxysilane–modified mica substrates (Ted Pella) for 3 min. The substrates were washed twice with 50 µl of PBS to remove unbound particles and imaged in PBS at RT. Scale bars, 100 nm. **b**, Representative immunoblots of the proteins FLOT1, MVP, LDHA and ENO2 in distinct fractions obtained from DiFi cells. Equal amounts of proteins from each fraction were used. **c**, Heatmap of normalized spectral counts for select proteins and enzymes involved in glycolysis in sEVs, exomeres and supermeres from DiFi cells. sEVs, NV fractions, exomeres and supermeres were isolated and fractionated as described in the protocol (Steps 1–51). Equal amounts of proteins from each fraction were used for proteomic analysis, and selected metabolic proteins and enzymes were further analyzed. **d**, Representative immunoblots of CD9, CD81, CD63, FASN, ACLY and HSPA13 in distinct fractions obtained from DiFi cells (left) and immunoblots for CD73 in fractions obtained from indicated cell lines (right). Equal amounts of proteins from each fraction were used. **e**, Representative immunoblots of TGFBI and HSPA13 in distinct fractions obtained from indicated cell lines. Equal amounts of proteins from each fraction were used. Exom., exomere; IB, immunoblot; Super., supermere; WCL, whole-cell lysate. Figure adapted with permission from ref. [15], Springer Nature Ltd.

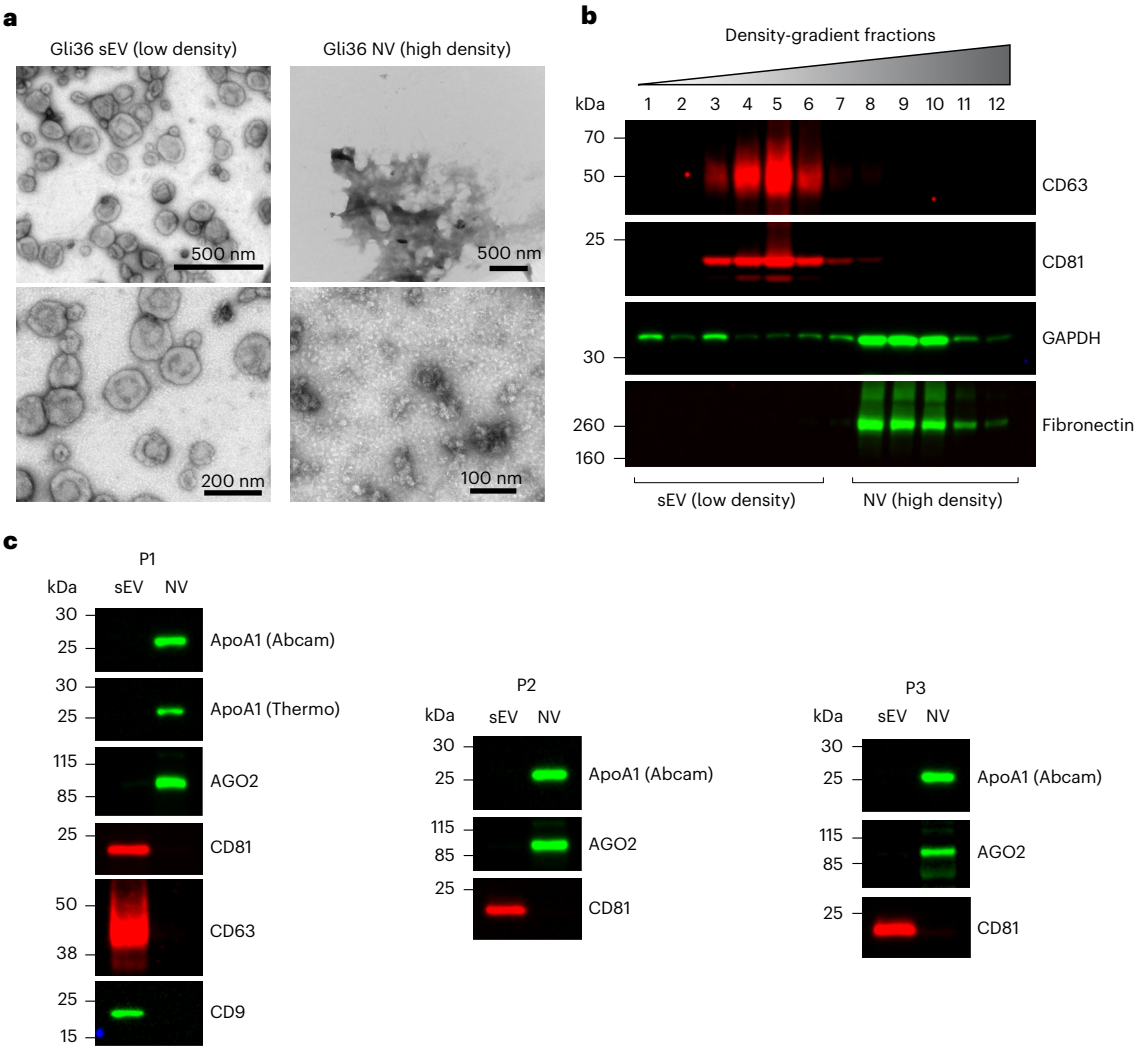

Fig. 6 | High-resolution density-gradient fractionation separates sEVs from NV components. a, Negative stain transmission electron microscopy of pooled sEV (low-density) and NV (high-density) fractions derived from Gli36 cells obtained from high-resolution density gradients. The purified sEVs display the expected cup-shaped morphology for EVs, whereas the NV fractions display few distinct structures. **b**, Density-gradient fractionation of crude sEV-Ps derived from DKO-1 cells. After flotation of the sample in high-resolution iodixanol gradients (12–36%, wt/vol), equal volumes of each fraction were loaded on SDS-PAGE gels, and membranes were immunoblotted with indicated antibodies. **c**, High-resolution density-gradient fractionation of crude human plasma sEV-Ps to obtain purified sEV and NV fractions. Immunoblots of plasma samples from three healthy human individuals (P1–P3) after fractionation. ApoA1 (marker for HDL particles) and AGO2 are enriched in the NV fraction, whereas CD81, CD63 and CD9 (markers for sEVs) are enriched in the sEV fraction. Figure adapted with permission from ref. [9], Elsevier.

high in both exomeres and supermeres, whereas the metabolic enzyme ENO2 is highly enriched in supermeres (Fig. 5b). Metabolic enzymes are a category of proteins enriched in exomeres and supermeres (Fig. 5c). Characteristically, the specific EV marker proteins including CD9, CD81, CD63 and CD73 will also be enriched in sEVs (Fig. 5d), and TGFBI and HSPA13 are highly expressed in exomeres and supermeres (Fig. 5e).

In our modified protocol, we first isolate the crude lEV-P, sEV-P, exomeres and supermeres sequentially. However, crude lEV-Ps and sEV-Ps are heterogeneous and include both EVs and contaminating NV material[9]. Performing additional high-resolution 12–36% (wt/vol) iodixanol density-gradient fractionation of crude EV-P samples with bottom loading of samples successfully separates the EVs from NV components as assessed by electron microscopy and immunoblotting (Figs. 5 and 6). Fractions from the gradient can be either pooled to represent purified EVs and NV fractions, respectively (Fig. 6a,c), or each fraction can be analyzed individually (Fig. 6b). For crude plasma sEV-P samples, performing the high-resolution density-gradient fractionation partitions HDL particles and AGO2 complexes in the NV fractions away from the purified sEVs (Fig. 6c). By using

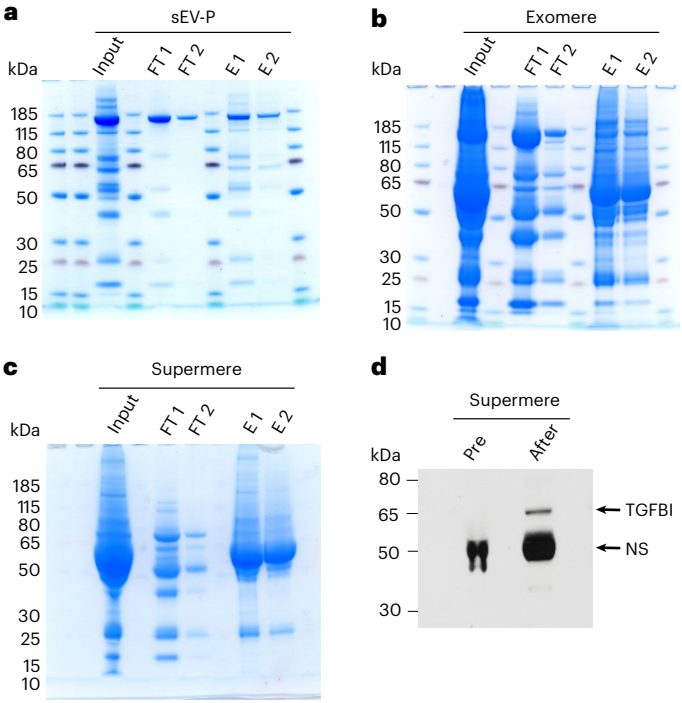

Fig. 7 | Depletion of albumin from sEV-Ps, exomeres and supermeres isolated from plasma from patients with colorectal cancer. The extracellular samples were isolated from plasma from patients with colorectal cancer as described in Box 1, with concentrations of 4.8, 78.3 and 83.3 μg of proteins/μl for sEV-Ps, exomeres and supermeres, respectively. Equal volumes (40 μl) of each fraction were diluted 5-fold in albumin-binding buffer and applied to the column for albumin depletion by using the albumin depletion kit (Abcam) as described in Box 1. Equal volumes (12 μl) of input (pre-albumin depletion), albumin-depleted samples (two flowthroughs, FT1 and FT2) and eluted-albumin samples (two elutions, E1 and E2, mainly containing albumin) were analyzed by 4–12% (wt/vol) SDS-PAGE and stained by Coomassie blue. **a**, Albumin depletion from sEV-Ps. **b**, Albumin depletion from exomeres. **c**, Albumin depletion from supermeres. **d**, Immunoblot analysis of TGFBI levels in albumin-depleted supermeres derived from plasma from patients with colorectal cancer. Albumin was depleted in supermeres as described above and analyzed by immunoblotting for TGFBI expression. Equal amounts (30 μg) of pre- and post-albumin-depleted supermeres were used for immunoblot analysis with the antibody indicated. Research conducted as part of this protocol complied with all the relevant ethical regulations. The use of the human samples was approved by the Vanderbilt University Medical Center IRB (IRB nos. 161529 and 151721). NS, nonspecific background.

this optimized protocol, cargo that has previously been claimed to be in exosomes or EVs can be assigned to their correct extracellular compartment[15].

DiFi cells have been used as a standard for our isolation method. A standard production lot of DiFi consists of 80 culture dishes (15 cm) with ~1.34×10^8 cells per dish at time of harvest. The typical protein yield is ~4 mg for sEV-P, 2.5 mg for exomeres and 7 mg for supermeres. Yields and ratios of different fractions derived from other types of cells are listed (Table 1)[15]. The yields for sEV-Ps, exomeres and supermeres can vary on the basis of host cells, growth conditions, number of culture dishes, etc. The yields presented in Table 1 are from cells that are switched to, and incubated in, serum-free medium for the purpose of harvesting cell-conditioned medium for isolation of EVs and NVEPs. If serum-containing medium is used, even if this medium has been depleted for bovine EVs, the presence of bovine serum proteins is a likely contaminant in exomere and supermere samples. Washing the supermeres in PBS by including an extra 367,000*g* step may improve the result, but the measured protein yield will probably be an overestimation of supermere content because of the presence of bovine serum proteins.

EV and NVEP isolation from human plasma is complicated because of the inherent complexity of blood. The yield of sEV-P samples from plasma is lower relative to exomeres and supermeres when compared to the yield from cells in culture. One of the reasons for this is the high abundance of albumin and other soluble blood proteins present in plasma samples that will contaminate EV and NVEP samples. With the albumin-depletion step, the protein yield of exomeres dropped to about one-third, while the yield of supermeres dropped to about one-quarter (Fig. 7a–c). Albumin depletion dramatically enriched low-abundance proteins in exomeres and supermeres, which would otherwise

be masked in plasma. The albumin-depletion step may aid in biomarker discovery for plasma-derived nanoparticles such as exomeres and supermeres. As an example, TGFBI is the most abundant protein in supermeres derived from DiFi cells, and it can also be detected in plasma-derived NVEPs by ELISA[15]. However, it was not possible to detect TGFBI by immunoblotting in supermeres derived from plasma from patients with colorectal cancer without albumin depletion, probably because of high levels of albumin contamination in the supermere samples. After albumin depletion, TGFBI was clearly detectable in supermeres by immunoblotting (Fig. 7d), confirming that removal of albumin can help reveal proteins that may serve as potential biomarkers for various diseases.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data used to generate protein expression heatmaps are provided in the supporting primary research article by Zhang et al.[15].

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Author contributions
Q.Z. and D.K.J. conceived the study; designed the experimental methodology; performed the experiments; analyzed, interpreted and visualized the data; and wrote the manuscript. J.N.H. conceived the study and developed, designed and performed the experiments. J.L.F. analyzed data. R.J.C. supervised the research and edited the manuscript.

Competing interests
The authors declare no competing interests.

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Zhang, Q. et al. *Cell Rep.* **27**, 940–954.e6 (2019): https://doi.org/10.1016/j.celrep.2019.01.009
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Sampling strategy	
Data collection	
Timing	
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Methods

n/a	Involved in the study
☐	☐ ChIP-seq
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Antibodies

Antibodies used: The primary antibody used for immunoblot: Anti-TGFBI (10188-1-AP) was from Proteintech. Anti-AGO2 (clone EPR10411, ab186733),

Validation: All the antibodies are commercially available and has been validated by the manufacturer.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s): DiFi and SC cells were maintained in the Coffey lab, DKO-1 cells were obtained from Dr. T. Sasazuki at Kyushu University, Gli36

Authentication: Cell lines were authenticated using short tandem repeat (STR) analysis.

Mycoplasma contamination: All cell lines were tested negative for mycoplasma contamination.

Commonly misidentified lines (See ICLAC register): No commonly misidentified cell lines were used.

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Ethics oversight: The animal experiments described in this study were carried out with the approval of Vanderbilt University Medical Center Institutional

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Population characteristics: The human participants were listed in the original supporting research papers, and age and sex matched.

Recruitment: See details in the original supporting research papers

Ethics oversight: The study protocol was approved by the Vanderbilt University Medical Center Institutional Review Board (IRB#161529 and

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Policy information about clinical studies

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Clinical trial registration: registration number: NCT03263429

Study protocol	clinicaltrail.gov
Data collection	Blood samples were collected at Vanderbilt University Medical Center
Outcomes	

Dual use research of concern

Policy information about dual use research of concern

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Could the accidental, deliberate or reckless misuse of agents or technologies generated in the work, or the application of information presented in the manuscript, pose a threat to:

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ChIP-seq

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Methodology

Replicates	
Sequencing depth	
Antibodies	
Peak calling parameters	
Data quality	
Software	

Flow Cytometry

Plots

Confirm that:

☑ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☑ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☐ All plots are contour plots with outliers or pseudocolor plots.

☑ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Small EV pellet (sEV-P) derived from DiFi cells were stained and sorted as described in the method. For FAVS staining and
Instrument	All FAVS analysis and sorting were performed on a BD FACS ARIA IIIu instrument with FSC-PMT.
Software	All samples were acquired with BD FACSDiva 8.1.3. software.
Cell population abundance	Cells were not used for flow cytometry in this study. Extracellular vesicles and nanoparticles were used.
Gating strategy	See methods section for specific details.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Magnetic resonance imaging

Experimental design

Design type	
Design specifications	
Behavioral performance measures	

Acquisition

Imaging type(s)	
Field strength	
Sequence & imaging parameters	
Area of acquisition	

Diffusion MRI ○ Used ○ Not used

Preprocessing

Preprocessing software	
Normalization	
Normalization template	
Noise and artifact removal	
Volume censoring	

Statistical modeling & inference

Model type and settings	
Effect(s) tested	

Specify type of analysis: ○ Whole brain ○ ROI-based ○ Both

Statistic type for inference (See Eklund et al. 2016)	
Correction	

Models & analysis

n/a | Involved in the study

- ○ Functional and/or effective connectivity
- ○ Graph analysis
- ○ Multivariate modeling or predictive analysis

Functional and/or effective connectivity	
Graph analysis	
Multivariate modeling and predictive analysis	