

Comparative Evaluation of Methods for Isolating Small Extracellular Vesicles Derived from Pancreatic Cells

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Research

Keywords: extracellular vesicles, isolation method, ultracentrifugation, immunoaffinity capturing

Posted Date: November 19th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-110539/v1>

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Version of Record: A version of this preprint was published on February 10th, 2021. See the published version at <https://doi.org/10.1186/s13578-021-00550-3>.

Abstract

Background: Small extracellular vesicles (sEVs) are nanosized vesicles involved in cell-to-cell communication. sEVs have been widely studied for clinical applications such as early detection of diseases and as therapeutics. Various methods for sEVs isolation have been using, but different methods may result in different qualities of sEVs and impact downstream analysis and applications. Here, we compared current isolation methods and performed a comparative analysis of sEVs derived from pancreatic cancer cells.

Results: Ultracentrifugation, ultrafiltration and co-precipitation as concentration methods were firstly evaluated for yield, size, morphology and protein level of pellets. Then, isolate sEVs obtained by four different purification methods: size exclusion chromatography, density gradient ultracentrifugation, ultracentrifugation, and immunoaffinity capturing, were analysed and compared. For the concentration process, ultracentrifugation method obtained high quality and concentration pellets. For the purification process, immunoaffinity capturing method obtained the purest sEVs with less contaminants, while density gradient ultracentrifugation-based method obtained sEVs with the smallest size. Proteomic analysis revealed distinct protein contents of purified sEVs.

Conclusions: For isolating sEVs derived from pancreatic cancer cells, ultracentrifugation-based method is recommended for concentration of sEVs, density gradient ultracentrifugation-based method may be suitable for isolation of sEVs for therapeutic study, immunoaffinity capturing may be applied for studies exploring sEVs as biomarkers.

Introduction

Extracellular vesicles (EVs) are biological vesicles released by almost all types of cells. EVs have gained increasing interests over the last decade for their cell-to-cell communication properties. EVs have been emerging as attractive therapeutic tools for their content and their natural carrier role. Small EVs are able to be engineered as nano drug delivery vehicles due to their relatively small size and properties such as crossing the biological barrier, circulation stability and inherent targeting.

The methods for isolation of sEVs has been extensively studied. Conventional differential ultracentrifugation has been widely used, but may not remove all contaminants^{1,2}. To obtain sEVs with small size, 0.22 µm filtration membranes were used in some studies to remove the microvesicles^{3,4}, but the filtration membrane could not remove protein contaminants, which may mislead the study results^{5,6}. The International Society for Extracellular Vesicles (ISEV) advised that the qualities of EV from different isolation methods were different⁷. Besides, each isolation method may have disadvantages. Recently, it was reported that ultracentrifugation could not obtain EVs with high purity^{1,2}, and may have problems such as clogging and trapping of vesicles⁸. Polymer co-precipitation-based methods are simple to perform but will precipitate large vesicles and contaminant proteins in the sample^{9,10}. Size exclusion chromatography-based method and density gradient ultracentrifugation-based may be effective but

could also be time-consuming¹¹. Application of immunoaffinity capture-based method was limited by target selection¹².

It seems to be reasonable to select the isolation method according to the demand for EV qualities. Generally, EV biomarker study demanded the purest EVs for exploring the relationship between EV and diseases^{13,14}, and EV therapeutic study demanded pure and large quantities of EVs¹⁵. Pancreatic cancer-derived sEVs have shown potentials for early disease detection¹⁶ and therapeutic application¹⁷. However, there has been a lack of comparison of isolation methods of sEVs derived from pancreatic cancer cells.

This study aims to evaluate methods for isolation of pancreatic cancer-derived sEVs. A two-step isolation process was performed: concentration and purification. The concentration step aims to concentrate EVs from conditioned cell culture medium, while the purification step aims to purify crude sEVs. A details isolation process was shown in Fig. 1.

Materials And Methods

Cell culture

The Panc-1 cell line was obtained from the ScienCell (USA). Cells were maintained in DMEM (Dulbecco's modified Eagle's medium, Thermo Fisher Scientific, USA) supplemented with 10% EV-depleted fetal bovine serum (obtained via ultracentrifugation at 160,000 g for 12 h) (Thermo Fisher Scientific, USA). Cells were incubated at 37 °C with 5% CO₂.

Preparation of sEVs-containing medium

Before sEVs isolation, the supernatant from Panc-1 cells was handled as previously described¹⁸. Briefly, supernatant was centrifuged at 300 g for 10 min to remove cells, centrifuged at 2,000 g for 10 min to remove the dead cells, and then centrifuged at 10,000 g (Thermo Fisher ST 16R, USA) for 1 h to remove the cell debris and microvesicles¹⁹. The remaining supernatant was sEVs-containing medium.

Preparation of crude sEVs

The following methods were separately used for preparation of crude sEVs (Fig. 1A): (1) Ultracentrifugation (UC): 50 ml of sEVs-containing medium was centrifuged at 100,000 g (SW32Ti, Beckman Coulter XPN-100 Ultracentrifuge, USA) for 70 min at 4 °C, and the pellet was re-suspended in 200 µL of PBS¹⁹; (2) Ultrafiltration (UF): 50 ml of sEVs-containing medium was concentrated using a 100 kDa ultrafiltration centrifugal tube (Merck Millipore, USA), PBS was added to re-suspend the concentrate, resulting in a final volume of 200 µL²⁰; (3) Co-precipitation (Co-P): 50 mL of 8% PEG 6000 (Solarbio, China) solution was prepared and then mixed with 50 ml of sEVs-containing medium at 4 °C for 12 hours, and then centrifuged at 4,000 g for 60 min. The pellet was re-suspended in 200 µL of PBS. Crude sEVs were stored at -80 °C (within 2 days.) before further purification.

Purification of sEVs

Based on the results of evaluation of pellets after concentrating sEVs, ultracentrifugation was used for concentrating sEVs for further purification of sEVs. Four methods were used for purifying crude sEVs (Fig. 1B): (1) Density gradient ultracentrifugation (DGUC): 200 μ L of crude sEVs was loaded onto the top of a 12 mL discontinuous sucrose (Solarbio, China) gradient solution (15%, 20%, 25%, 30%, 40%, 60% sucrose in PBS, 2 mL for each gradient solution) and then centrifuged at 100,000 g for 16 h at 4 $^{\circ}$ C. 12 fractions (1 ml) were collected for each gradient ²¹. (2) Size exclusion chromatography (SEC): Sepharose CL-2B (Solarbio, China) was loaded into an injector (10 mL), with cotton blocked at the bottom. 200 μ L of crude sEVs was loaded onto the top of the column. Then the column was eluted by PBS, and each 1 mL of eluate was collected for 12 sequential fractions ²². (3) UC: 200 μ L of crude sEVs was washed in PBS. Then the solution was centrifuged at 10,000 g, 4 $^{\circ}$ C for 70 min. The pellet was re-suspend by 200 μ L of PBS. (4) Immunoaffinity capture (IAC): Magnetic beads (BeaverBeadsTM Protein A/G immunoprecipitation kit, Beaver, China) were washed and then activated by incubating with 100 μ L of anti-CD63 antibody (50 μ g/mL, ab134331, abcam, UK) for 15 min. After magnetic separation, the anti-CD63-conjugated beads were incubated with 200 μ L of crude sEVs at 25 $^{\circ}$ C for 1 h. The sEVs-beads complexes were separated by a magnet and eluted and re-suspended in 40 μ L of PBS. Purified sEVs were stored at -80 $^{\circ}$ C within two days before analysis.

Analysis of fraction from DGUC and SEC

Total protein level in each fraction was determined by using a BCA Protein Assay Kit (MultiSciences Biotech Co., China). The level of CD63 in each fraction was determined by using an enzyme-linked immuno- sorbent assay (ELISA) kit (CSB-E14107h, CUSABIO Biotech Co. Ltd., China). After analysis, fractions 6, 7, 8, 9, 10 from DGUC were mixed used for further analysis, while fractions 9, 10 from SEC were mixed and used for further analysis.

Characterization and analysis of crude sEVs and purified sEVs

Nanoparticle Tracking Analysis (NTA)

For crude sEVs, samples were diluted 100 times with PBS before NTA. For purified sEVs, samples obtained via IAC was diluted 125 times, samples obtained via SEC was diluted 2.5 times, samples obtained via UC was diluted 25 times, while samples obtained via DGUC was not diluted before NTA (NS300, Malvern, UK). The RR of particles was calculated as the following formula. The assay was repeated 3 times.

$$RR \text{ of particles} = \frac{\text{number of particles in purified sEVs}}{\text{number of particles in crude sEVs}} \quad (1)$$

Transmission Electron Microscopy

10 μ L of samples was dropped onto an ultrathin carbon film-coated 400 mesh copper grid and washed with PBS for two times. After drying excess liquid, the EVs-coated grid was stained by phosphotungstic acid (1%) and then washed with PBS for two times and then dried and imaged with a multipurpose field emission transmission electron microscope (TEM, JEM-1200EX, JEOL Ltd., Japan).

Protein level

The protein level was determined by using a BCA Protein Assay Kit. The Protein Recovery rate (RR) of the purification process was calculated as the following formula. The assay was repeated 3 times.

$$RR \text{ of protein} = \frac{\text{Total protein in purified sEVs}}{\text{Total protein in crude sEVs}} \quad (2)$$

Western blotting

5 \times SDS-PAGE Loading Buffer (New cell and Molecular Biotech Co., Ltd, China) was added into the sample. The sample was kept at 100 $^{\circ}$ C for 10 min. 6.02×10^9 EVs were loaded on each well in 12% SDS-PAGE (Lianke Bio, China). After the electrophoresis, the proteins were transferred to PVDF membrane (Millipore, USA). The membrane was blocked with 5% milk solution for 1.5 h, followed by incubation with anti-CD63 (ab134045, abcam, UK), anti-CD81 (GB111073, Servicebio, China), anti-CD9 (ab92726, abcam, UK), anti-CD47 (ab108415, abcam, UK), anti-GAPDH (AF7201, Affinity, China), and anti-ago 1(#9388, Cell Signaling, USA). The PVDF membrane was washed and then incubated with Peroxidase-conjugated Goat anti-Rabbit IgG (ZSGB-Bio, China). The membrane was incubated with ECL luminescent (New cell and Molecular Biotech Co., Ltd, China) for 3 min for detection.

Coomassie brilliant blue staining

5 \times SDS-PAGE Loading Buffer (New cell and Molecular Biotech Co., Ltd, China) was added into the sample. The sample was kept at 100 $^{\circ}$ C for 10 min. 6.02×10^9 EVs were loaded on each well in 12% SDS-PAGE (Lianke Bio, China). After the electrophoresis, the gel was incubated with 20 mL of working solution (0.0025% Coomassie brilliant blue, 45% methanol, 10% glacial acetic acid) (Solar bio, China) for 1 h, and then washed by elution solution (25% methanol, 8% glacial acetic acid) for 4 h.

ELISA

The level of CD63, CD81, TSG101, beta-actin, GAPDH, CD47 (CUSABIO Biotech Co. Ltd., China) and ago-1 (MyBiosource, Canada) in purified samples and in crude sEVs were determined by ELISA kits. The protein per EV was calculated by the following formula. The assay was repeated 3 times.

$$\text{protein per EV} = \frac{\text{total protein}}{\text{number of EVs}} \quad (3)$$

Digestion of proteins

SDT solution (4% SDS, 100 mM Tris-HCl, pH 7.6) was added into the purified sEVs. The sample was incubated under boiling water for 15 min, followed by centrifugation at 14,000 g for 15 min. The supernatant was collected as protein sample. DTT (Sigma, USA, 43819-5G) was added in the protein sample to 100 mM. The sample was incubated under boiling water for 5 min, and then cooled to room temperature. 200 μ L of UA buffer (8M Urea, 150 mM Tris-HCl, pH 8.5) was added to the sample, followed by centrifuging at 12,500 g for 15 min using a 30 kDa ultrafiltration tube and centrifuged at 12,500 g for 15 min. Then, 100 μ L of iodoacetamide (IAA) buffer (100 mM IAA in UA) was added and kept at room temperature in darkness for 30 min. The sample was centrifuged at 12,500 g for 15 min. 100 μ L of UA buffer was added to the supernatant and then centrifuged at 12,500 g for 15 min again. 100 μ L of 40 mM NH_4HCO_3 solution was added to the sample followed by centrifugation at 12,500 g for 15 min. Then, 40 μ L of Trypsin buffer was added (4 μ g Trypsin in 40 μ L of 40 mM NH_4HCO_3) to the sample and incubated at 37 °C for 16–18 h. The sample (in a filtration tube) was centrifuged at 12,500 g for 15 min, then 20 μ L of 40 mM NH_4HCO_3 solution was added and centrifuged at 12,500 for 15 min to obtain the filtrate. A C18 cartridge (WAT023590, Waters) was used to desalinate. After freeze-drying, 40 μ L of 0.1% methanol solution was added to the solid to re-solute the sample.

LC-MS/MS

Then sample was separated by Easy nLC (Thermo Fisher Scientific, USA). Solution A: 0.1% FA; Solution B: 0.1% FA, 80% ACN. Chromatographic column (Acclaim PepMap RSLC 50 μ m \times 15 cm, nano viper, P/N164943, Thermo Fisher Scientific) was balanced by 100% solution A. Velocity of flow was 300 nL/min. Gradient elution: 0–5 min, solution B 3%; 5–45 min, solution B 3%-28%; 45–55 min, solution B 28%-38%; 50–55 min, solution B 38%-100%; 55–60 min, solution B 100%.

The sample was analysed by Q Exactive (Thermo Fisher Scientific, USA). Analysis time was 1 h. The mode was positive ion mode. Range of parent ion was 350–1800 m/z. The resolution of mass spectrometry was 7000. AGC target was $3e^{6.1}$ stage Maximum IT was 50 ms. Via full scan 10 MS2 scans were acquired. MS2 Activation Type was HCD. Isolation window was 2 m/z. The resolution of 2 stage mass spectrometry was 17,500. Microscan was 1. 2 stage. Maximum IT was 45 ms. Normalized Collision Energy was 27 eV.

Data-dependent acquisition was performed. The peptides database was Uniprot_HomoSapiens_20386_20180905, downloaded in <http://www.uniprot.org>. MaxQuant 1.5.5.1 was used to qualitative analysis. Label Free Quantitation was used for quantitative analysis.

Statistical analysis

Data were presented as mean values \pm SD. One-way analysis of variance (ANOVA) and students' t test were performed at the significance level $\alpha = 0.05$.

Results

Analysis of crude sEVs

Same volume (50 ml) of the supernatant was processed to compare concentration methods. Size distribution of crude sEVs obtained via UF, UC and Co-P were presented in Fig. 2A, 2B and 2C, respectively. sEVs obtained via UC showed a smaller size distribution than other two methods. All samples showed plenty of big particles, indicating that neither single method (UC, Co-P or UF) could obtain pure sEVs. Crude sEVs obtained via UC showed significantly more total particle number and small particle (30–150 nm) number than UF and Co-P (Fig. 2D). sEVs obtained via UF showed significantly high protein levels than UC and Co-P as evaluated by BCA assay test (Fig. 2E) and coomassie brilliant blue staining (Fig. 2F), but the protein level in the control (fresh medium after UC) was also very high. TEM images for crude sEVs were presented in Fig. 2G. Big and small EVs could be observed for all crude sEVs groups. Besides, aggregation of unknown small particles (red frame) was found in crude sEVs obtained via Co-P. Based on the quality of crude sEVs, UC was used for crude sEVs collection for further purifications.

Analysis of fraction from DGUC and SEC

Total protein and CD63 levels in fractions from DGUC and SEC were shown in Fig. 3. Fractions with a relatively high level of CD63 was collected as purified sEVs^{22,23}. Hence, fractions 6, 7, 8, 9 and 10 from DGUC were mixed as the purified sample (Fig. 3A, 3B), fractions 9 and 10 from SEC were mixed as the purified sample (Fig. 3C, 3D).

Analysis of purified sEVs

The size distribution of purified samples via UC, SEC, IAC and DGUC were shown in Fig. 4A, 4B, 4C and 4D, respectively. The big particles in sEVs for all samples were removed substantially. The size of the sample via DGUC showed the smallest size, and the sample via IAC showed a relatively small size with less microvesicles. The sample via UC showed more particle and sEVs numbers than other methods (Fig. 4E). The sample via SEC showed the least particles and sEVs. Besides, samples via DGUC or IAC showed a relatively high proportion of sEVs in all particles (Fig. 4E). The sample via UC showed the highest RR (Fig. 4F). TEM images of purified sEVs were shown in Fig. 4G. sEVs could be observed for all samples, while samples obtained via UC show relatively more particles. Samples obtained via IAC and DGUC showed clearer background under TEM. Contaminants were observed in samples obtained via SEC.

Protein evaluation

Purified sEVs showed lower total protein levels for all methods than crude sEVs (Fig. 5). The protein level of the sample via UC was higher than other methods as evaluated by BCA assay (Fig. 5C) and coomassie brilliant blue staining (Fig. 5A). The sample purified by IAC showed the least total protein level and recover rate (Fig. 5C, 5D).

WB results were shown in Fig. 5B. Three EVs marker proteins (CD81, CD63, CD9) and CD47 were detected in the purified samples. Compared to the crude sEVs, ago-1 as contaminant protein was significantly

decreased in purified samples, which was consistent with the WB results (Fig. 5E). Results of ELISA were summarized in Fig. 5F. Samples purified by UC showed less CD47, CD81, GAPDH and β -actin. The sample via IAC showed the least TSG101. The sample via IAC had a higher CD63 level as the sample was isolated by anti-CD63-conjugated beads. RR of CD63 was almost 100% for IAC method, indicating that almost all particles expressing CD63 were extracted. For the samples purified by SEC and DGUC, levels of most proteins tested were similar.

Proteomics

A total of 817 proteins were detected in proteomic study. There were 631 proteins in the crude sEVs sample via UC, 383 proteins in the purified sEVs sample via UC, 78 proteins in the sample via SEC, 154 proteins in the sample via DGUC and 76 proteins in the sample via IAC, 25 proteins were identified for all five groups (Fig. 6A). Heat map analysis of all proteins was summarized in Fig. 6B. Purified sEVs obtained via UC showed significantly more protein content, which was consistent with our results of protein evaluation (Fig. 5). Reported potential protein biomarkers for cancer development²⁴, metastasis²⁵ and drug resistance²⁶ were also analysed in our proteomic study. Further analysis revealed distinct protein contents in samples (Fig. 6). High contents of both overexpressed and downexpressed proteins associated with metastasis and drug-resistance were detected in crude sEVs (Fig. 6C to 6H). Purified sEVs via IAC retained most pancreatic cancer-overexpressed (Fig. 6C), but not downexpressed (Fig. 6D), proteins (compared to normal adjacent pancreatic tissue). sEVs obtained via SEC showed the least contents of proteins associated with metastasis (Fig. 6E) and drug resistance (Fig. 6G).

Discussion

Despite progress in technique, isolation of sEVs has been challenging. It would be wise to choose a strategy and develop isolation protocols depending on experiment purpose. Three concentration methods and four purification methods were evaluated in our study. A summarization of advantages, disadvantages and possible suggestions for isolation methods used in this study was shown in Table 1. For the concentration of sEVs, UC-based method showed high yield and purity of sEVs and was used to concentrate the sample before purification. For the purification of sEVs, IAC was effective and time-saving and yield purest sEVs among all methods evaluated, thus was recommended to be suitable for biomarker study^{13,14}. DGUC method effectively produced the smallest and also purified sEVs with relatively high yield, thus may be suitable for EV therapeutic study as pure and large number of sEVs are required for therapeutic application^{15,27-29}. More importantly, sEVs obtained via DGUC method could avoid aggregation and precipitation³⁰ and were superior for cellular uptake³¹.

In the presented study, the size, yield, morphology and protein were assessed for quality of sEVs. The size and yield of sEVs were crucial for therapeutic use, especially for drug delivery. It has been reported that smaller EVs could be uptaken by cells more efficiently³¹. The morphology showing the presence of EV was observed, sEVs obtained in this study was saucer-shaped under TEM images as reported³². CD63 was a marker for EVs and was used in IAC for purification^{9,33,34}. Besides, in our fraction analysis, CD63

level was used to reflect EV-containing fractions, this method was also reported in previous studies^{21,23,35}. However, for SEC, CD63 may not fully represent the sEVs fractions as expected. Possibly, CD63 was not exclusive to the sEVs. Recent studies reported that microvesicles also expressed CD63^{36,37}. A combined strategy of several sEVs marker proteins such as CD63, TSG101 and CD9 may reflect the sEVs-containing fractions more accurately.

Our study included the majority of current sEVs isolation methods, except commercial kits and microfluidic-based methods. Because of unstable quality of the extracted EVs, high price and unknown solutions³⁸, commercial kits were not included in our study. The microfluidic technology was popular but not included in our study as the technique was mostly used for methodological studies, such as sEVs detection, instead of therapeutic application study, even if it was potentially available for isolation^{39,40}.

In the presented study, an equal number of EVs particles was evaluated for each method in case of contaminant protein influencing the results. The level of target protein could be weak as detected if equal protein, with a high amount of contaminant protein, was loaded onto each well in the gel. This may explain why the level of EV marker protein was weak in study⁴¹.

The UC-based technique remains the most common method for sEVs isolation⁴²⁻⁴⁴. However, our study results demonstrated that DGUC and IAC methods produced sEVs with better purity than UC. Besides, the interpretation of results should be cautious when commercial sEVs isolation kits based on the Co-P method was used^{38,45}. The combination of several isolation methods may produce purer sEVs. Jeppesen et al. used ultracentrifugation-based technique, density gradient ultracentrifugation-based technique and immunoaffinity capture-based technique to purify sEVs⁴⁶. But more isolation steps may produce fewer sEVs, and combined isolation protocols were often hard to follow as the strategy could be complicated and fussy. The technique for evaluation of sEVs has been advancing. Tian et al. splendidly used nanoflow cytometry to evaluate the quality of sEVs³⁸. But nanoflow cytometry-based analysis of sEVs needs further refinement of methods. For better comparisons between groups and future replication, we applied basic characterization of sEVs isolated by different methods^{23,35,47}.

sEVs are a heterogenous group of vesicles⁴⁸. Single EV may carry distinct proteins, isolation method based on EV marker may lose EV subtypes of potential interest. For example, anti-CD63 conjugated-beads could be used to obtain CD63-enriched EVs, but effects of other vesicles may be neglected during subsequent experiments. This may explain why several metastasis and drug resistance-associated proteins were not highly expressed in purified sEVs obtained via IAC. However, future basic and clinical studies are likely to provide valuable information regarding their heterogeneity and advance our understanding of biological functions, thus reveal and harness their potentials for disease detection and therapy.

Conclusions

Current methods are useful for isolating sEVs. Recommendations for choosing sEVs isolating method depend on the aim of study. For isolation of Panc-1 cell-derived sEVs, UC was advised to concentrate the medium. IAC method is effective for isolating sEVs with high purity, thus was suitable for biomarker study. Meanwhile, DGUC was recommended for EV therapeutic study due to high yield and small size distribution.

Declarations

Authors' contributions

Conceptualization, JM Wang, YJ Li and DX Xiang; Methodology, JM Wang, YJ Li, JY Wu; Investigation, JM Wang, JX Cai, J Wen, XB Hu; Formal analysis, JM Wang, YJ Li and WQ Li; Writing—original draft, JM Wang; Writing—review and editing, YJ Li; Supervision, DX Xiang; Funding Acquisition, DX Xiang. All authors read and approved the final manuscript.

Funding

This work was supported by the Hunan Provincial Technology Plan (2016TP2002)

Availability of data and materials

Not applicable

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table

Table 1. Summary of concentration and purification methods for isolating sEVs from pancreatic cancer cells

Process	Method	Advantages	Disadvantages	Possible Suggestions	Recommendation
Concentration	UC	✓High yield	*High equipment cost	NA	Suitable for concentrating medium
	Co-P	✓Convenient	*Non-exosomal contaminants	NA	NA
	UF	✓Convenient	*Non-exosomal contaminants	NA	NA
Purification	UC	✓High yield ✓Feasibility	*High equipment cost	♦Fully re-suspend the crude sEVs	NA
	SEC	✓High purity ✓Up-scale isolation	Require methodological validation	♦Lengthen the column and increase the sepharose ♦Slow down the flow rate Lower the temperature	NA
	DGUC	✓Small EVs ✓High purity	*Require methodological study *Time-consuming centrifugation *High equipment cost	♦Pilot test for exploring suitable gradient solutions	Suitable for therapeutic study
	IAC	✓Small EVs ✓Time-saving ✓High purity	*Low yield *Subtype of sEVs	♦Personalized customization depending on aim of study	Suitable for biomarker study

Abbreviations: Co-P: Co-precipitation; DGUC: Density gradient ultracentrifugation; EV: Extracellular vesicles; IAC: Immunoaffinity capture; NA: Not available; SEC: Size exclusion chromatography; UC: Ultracentrifugation; UF: Ultrafiltration.

Figures

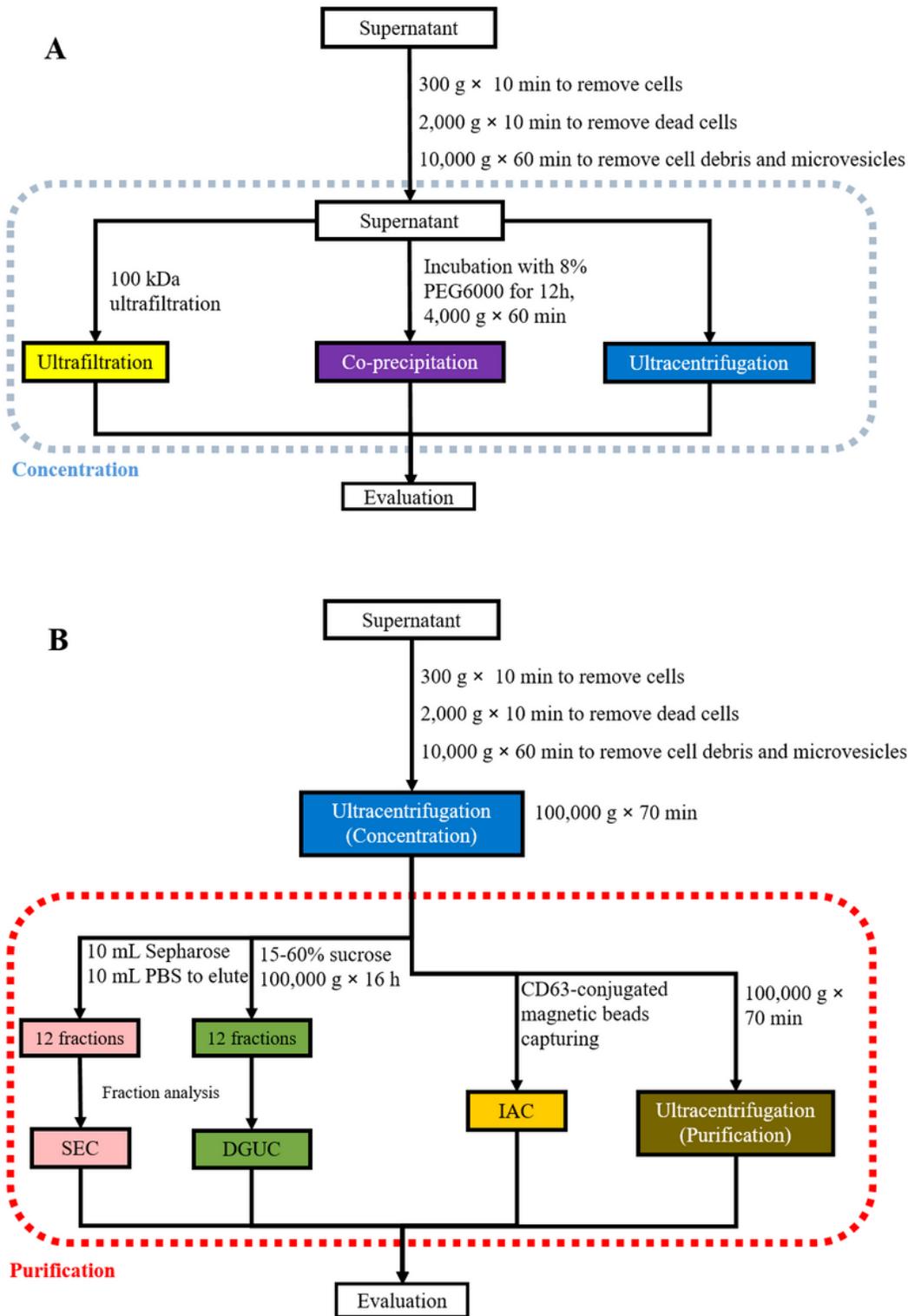


Figure 1

The workflow of the study. (A) Three different methods for concentrating sEVs from cell culture medium. (B) Four different methods for purification of sEVs after standard differential ultracentrifugation.

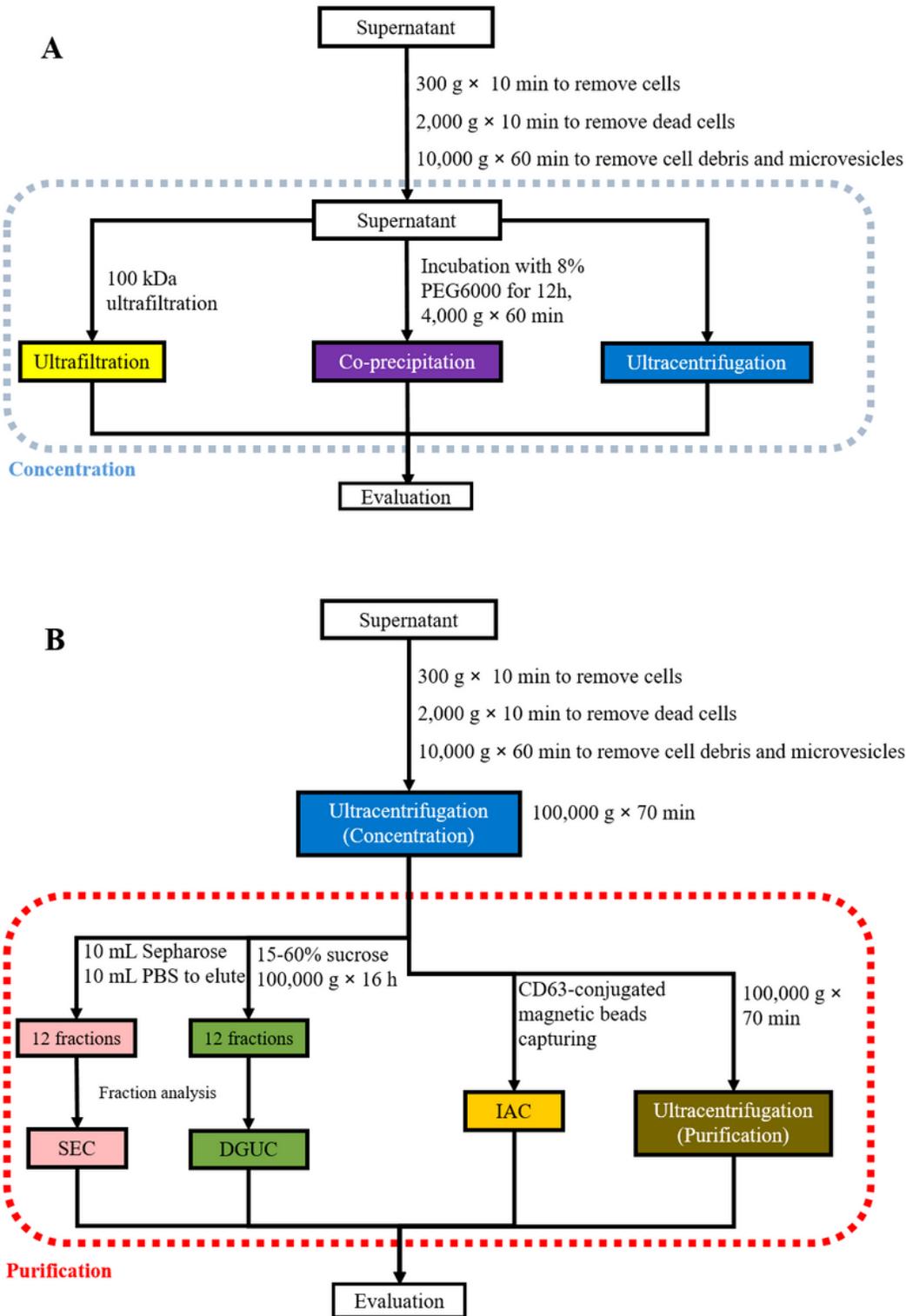


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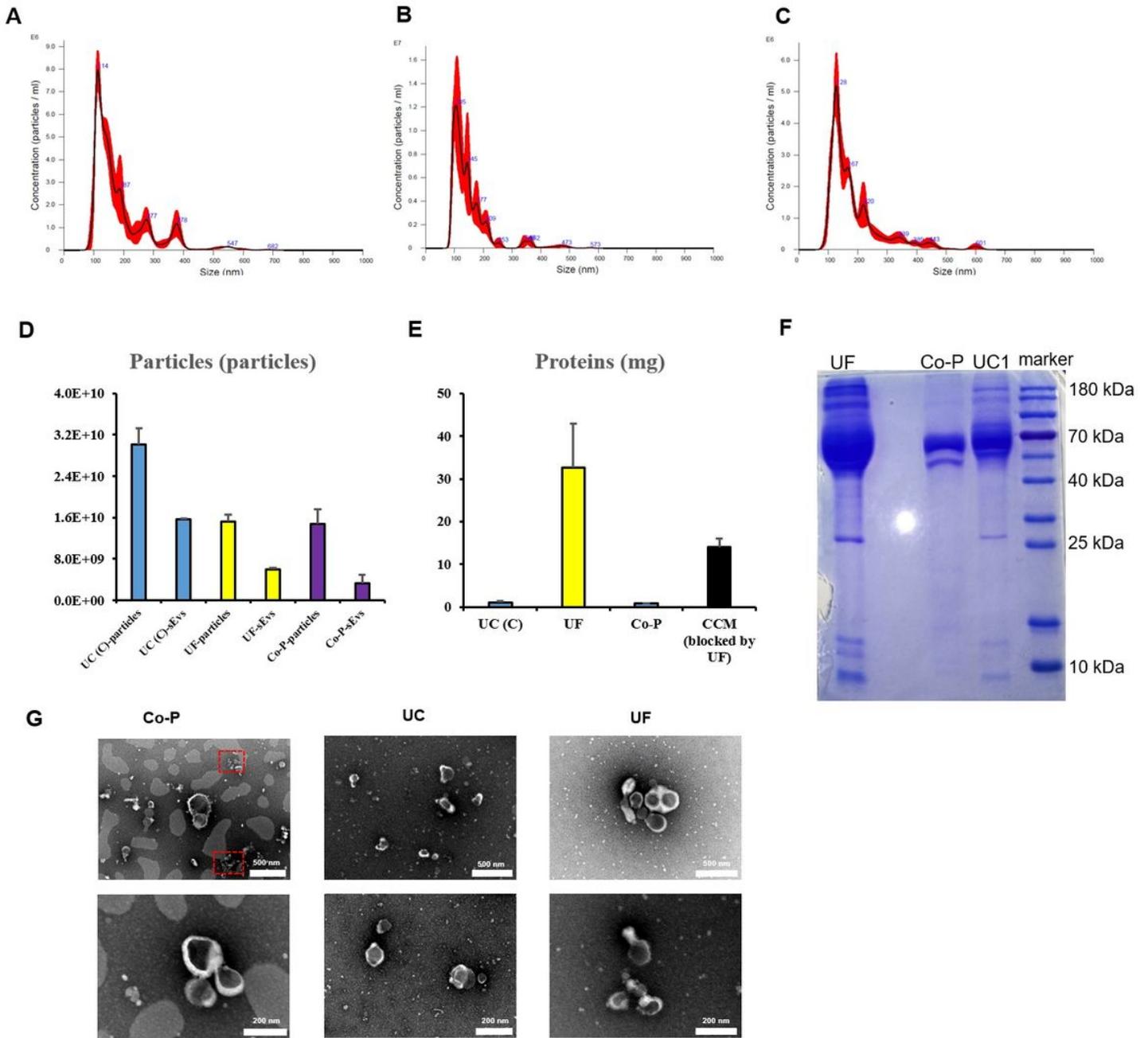


Figure 2

Evaluation of crude sEVs obtained via three different concentration methods. (A) Size distribution of pellets via ultrafiltration (n=3). (B) Size distribution of pellets via ultracentrifugation (n=3). (C) Size distribution of pellets via co-precipitation (n=3). (D) The numbers of total particles and sEVs (30-150nm) in the pellets (n=3). (E) The yield of the protein in pellets after concentration process (n=3). (F) Coomassie brilliant blue staining of pellet. (G) TEM images of pellets. Contaminants of aggregated small particles (red frame) could be observed in the pellet obtained via co-precipitation method.

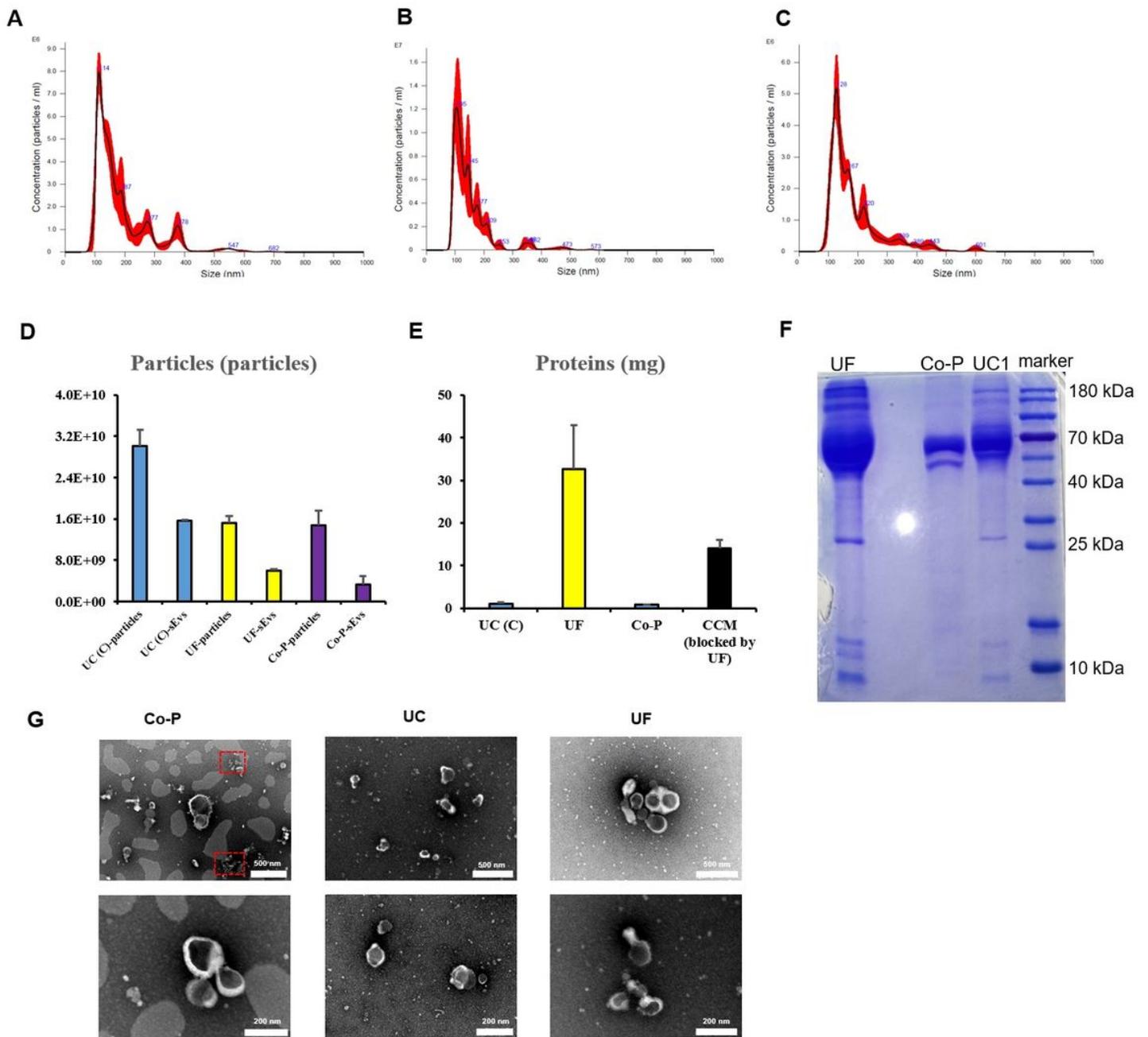


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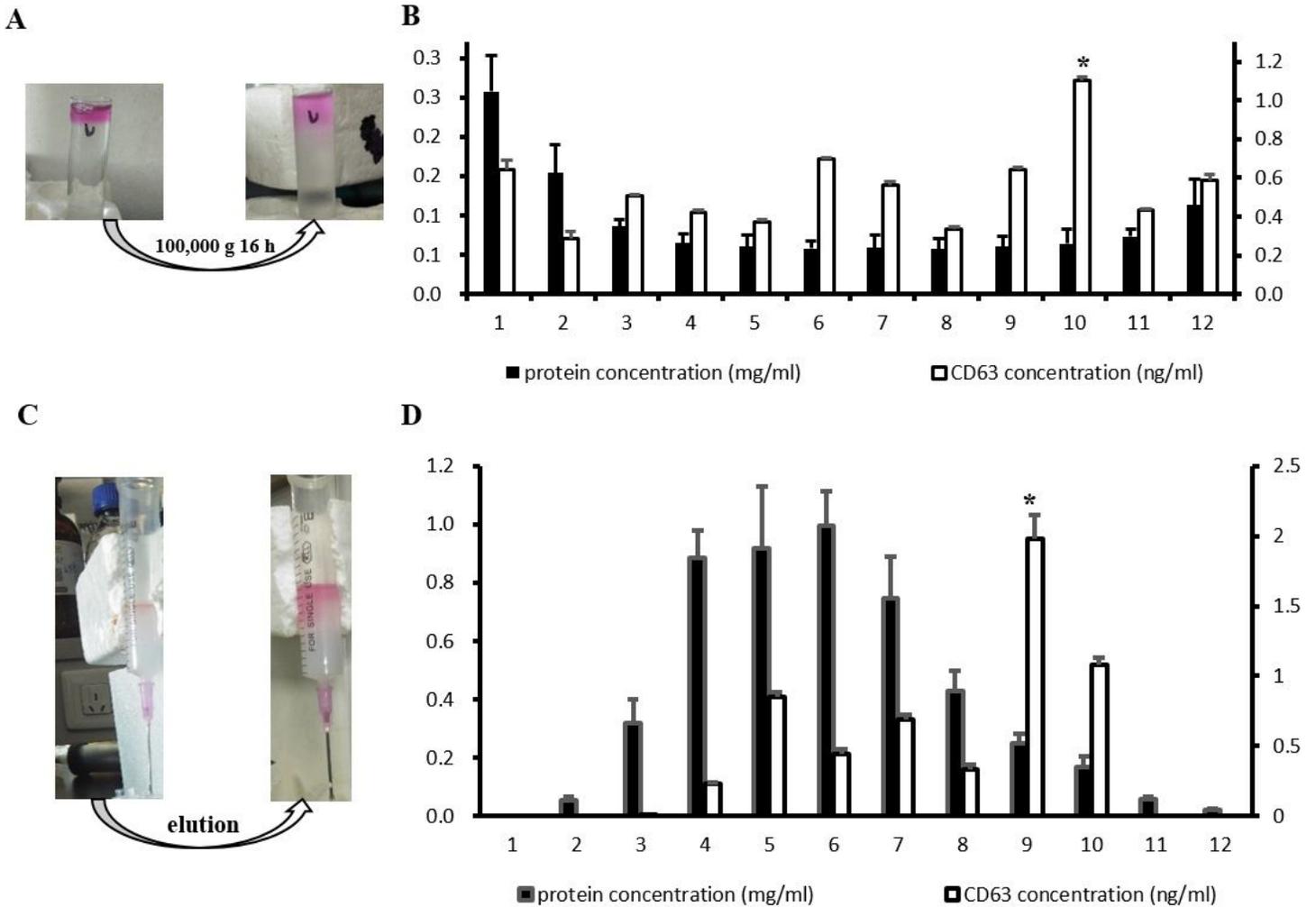


Figure 3

SEC purification process of the pellet obtained via ultracentrifugation (A) The picture of the ultracentrifuge tube before and after ultracentrifugation. The border of the concentrate and ultracentrifugation rose solution became vague after ultracentrifugation. B. Fraction analysis of DG ultracentrifugation (n=3). Because of high CD63 concentration, the fraction 6, 7, 8, 9 and 10 were merged as the purified sample. C. The picture of SEC, the EVs concentrate was eluted. D. Fraction analysis of SEC (n=3). Because of high CD63 concentration, the fraction 9 and 10 were merged as the SEC purified sample. * P < 0.05.

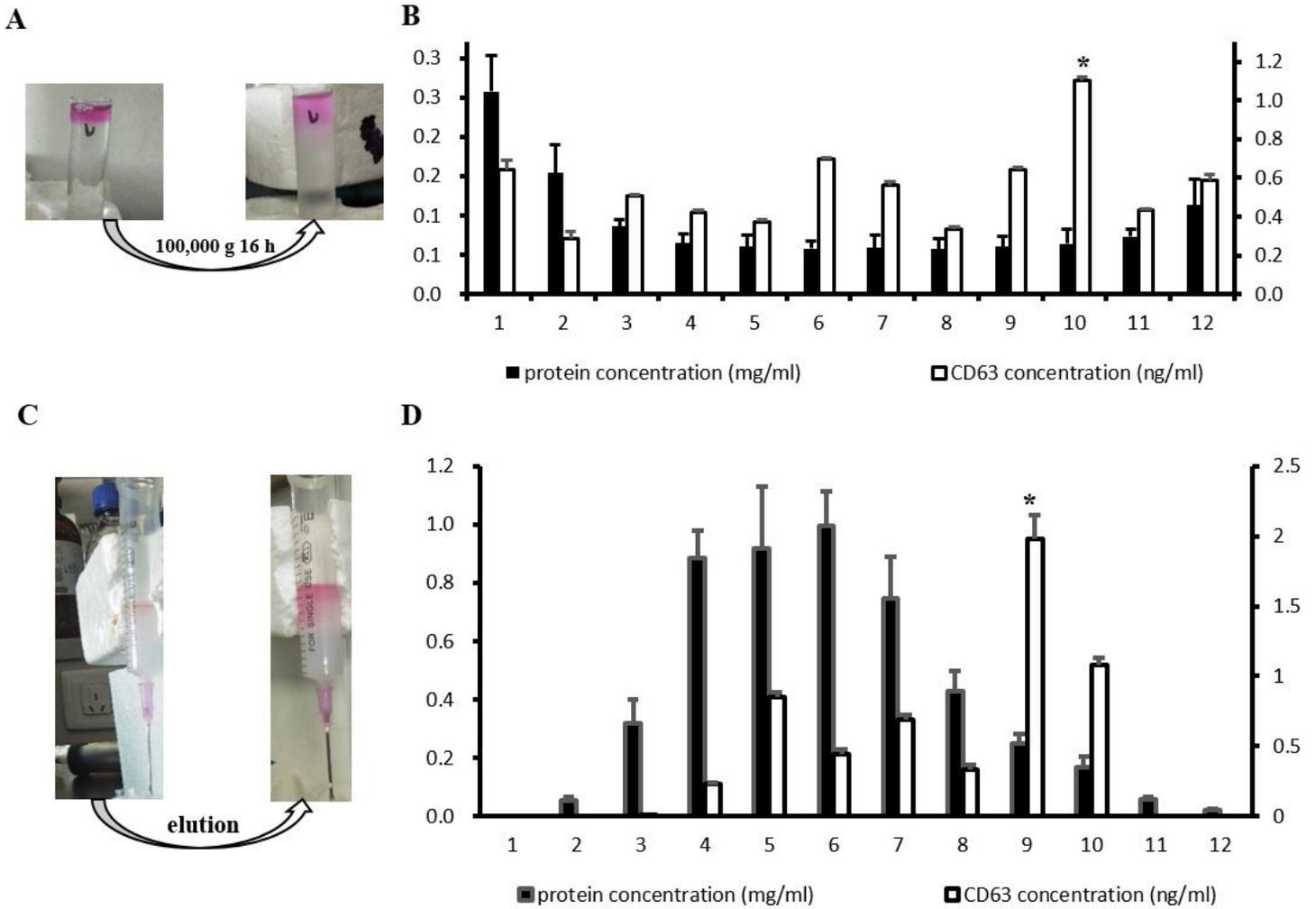


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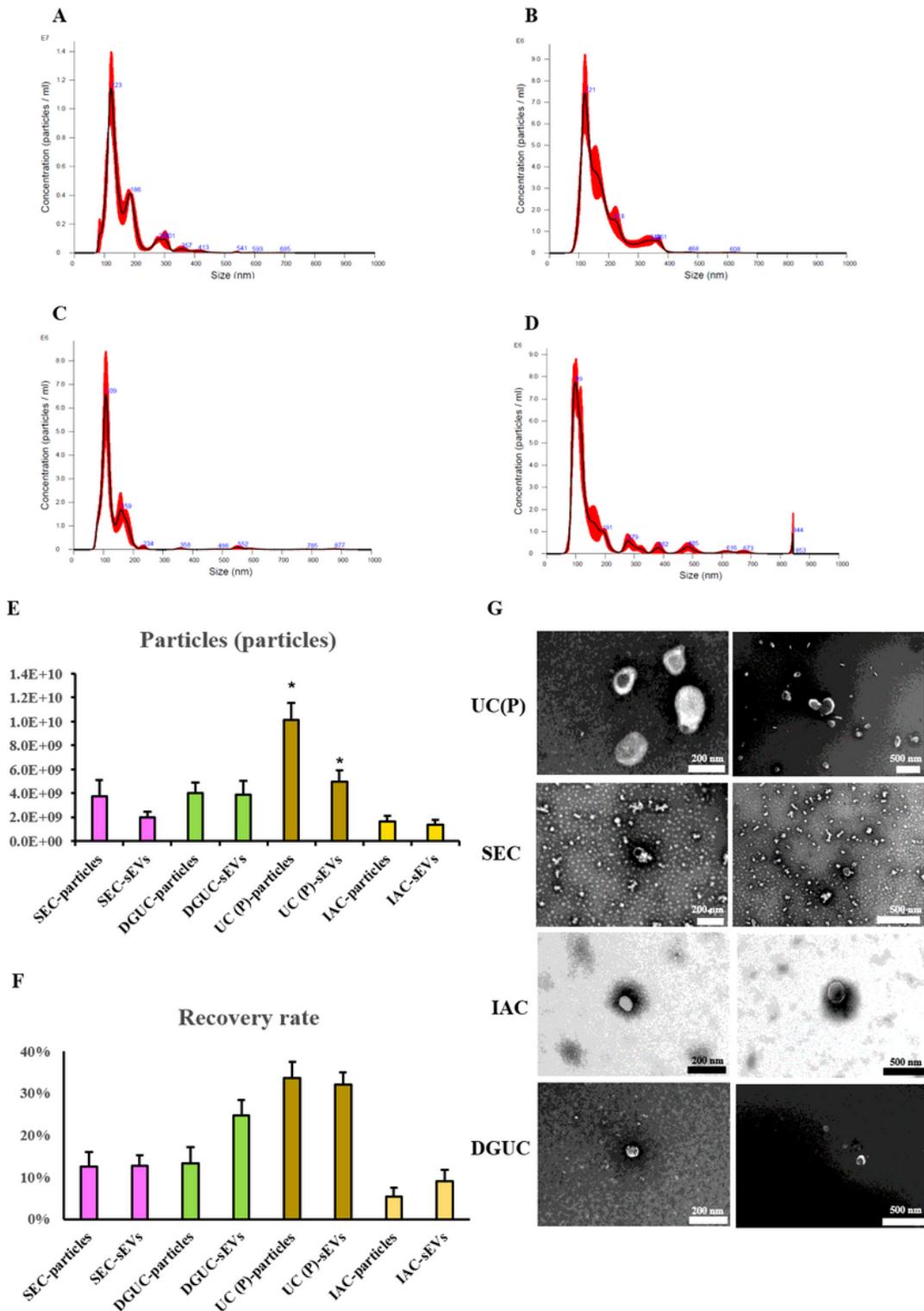


Figure 4

Evaluation of particles of the purified samples. (A) Size distribution of pellets via ultracentrifugation (n=3). (B) Size distribution of pellets via SEC (n=3). (C) Size distribution of pellets via IAC (n=3). (D) Size distribution of pellets via DGUC (n=3). (E) The numbers of total particles and sEVs (30-150 nm) in the pellets (n=3). (F) RR of the particle and sEVs. (G) TEM images of the purified samples. E. Dyed-gel. Compared of the concentrates, the purified samples showed lower protein content. * P < 0.05.

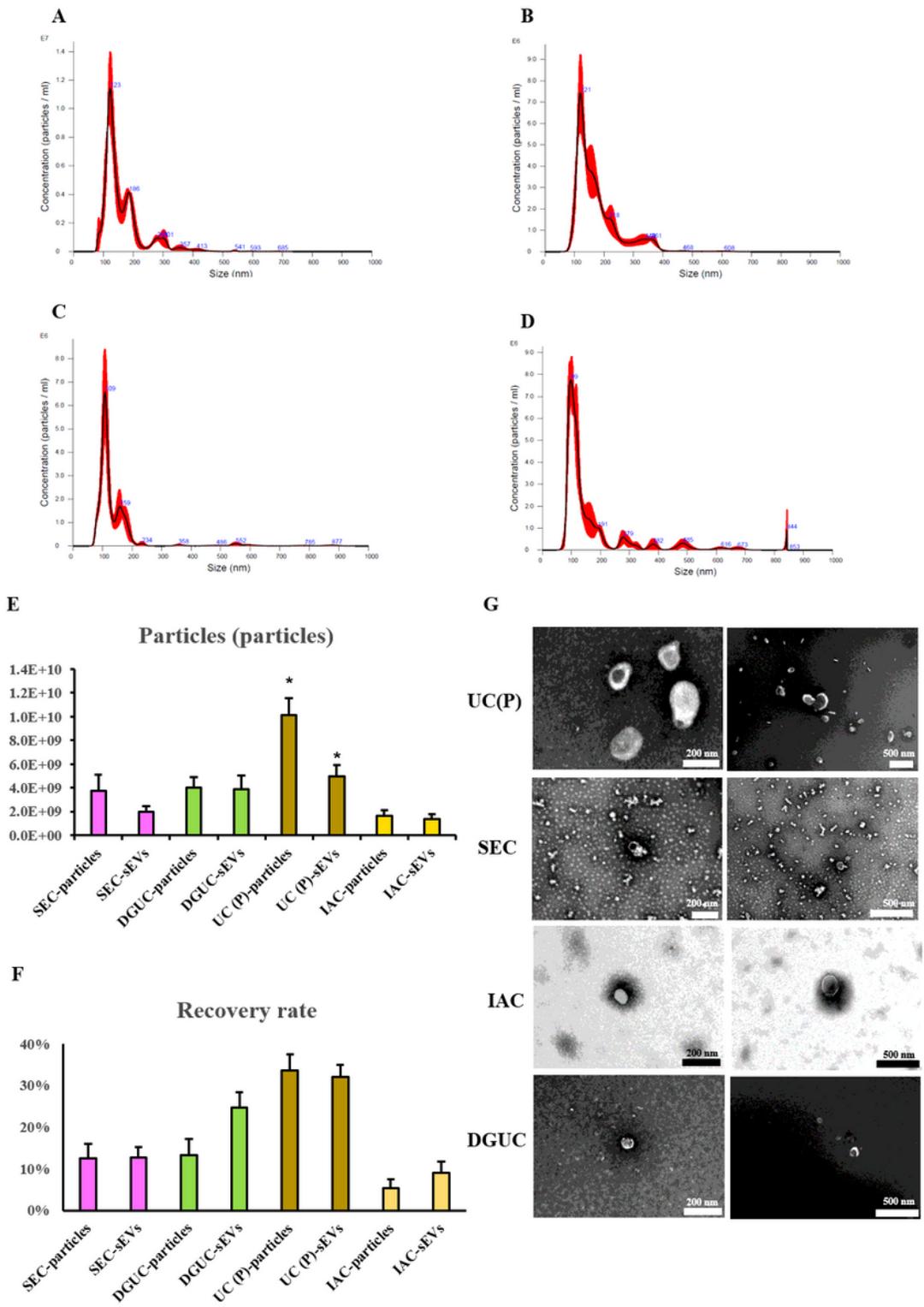


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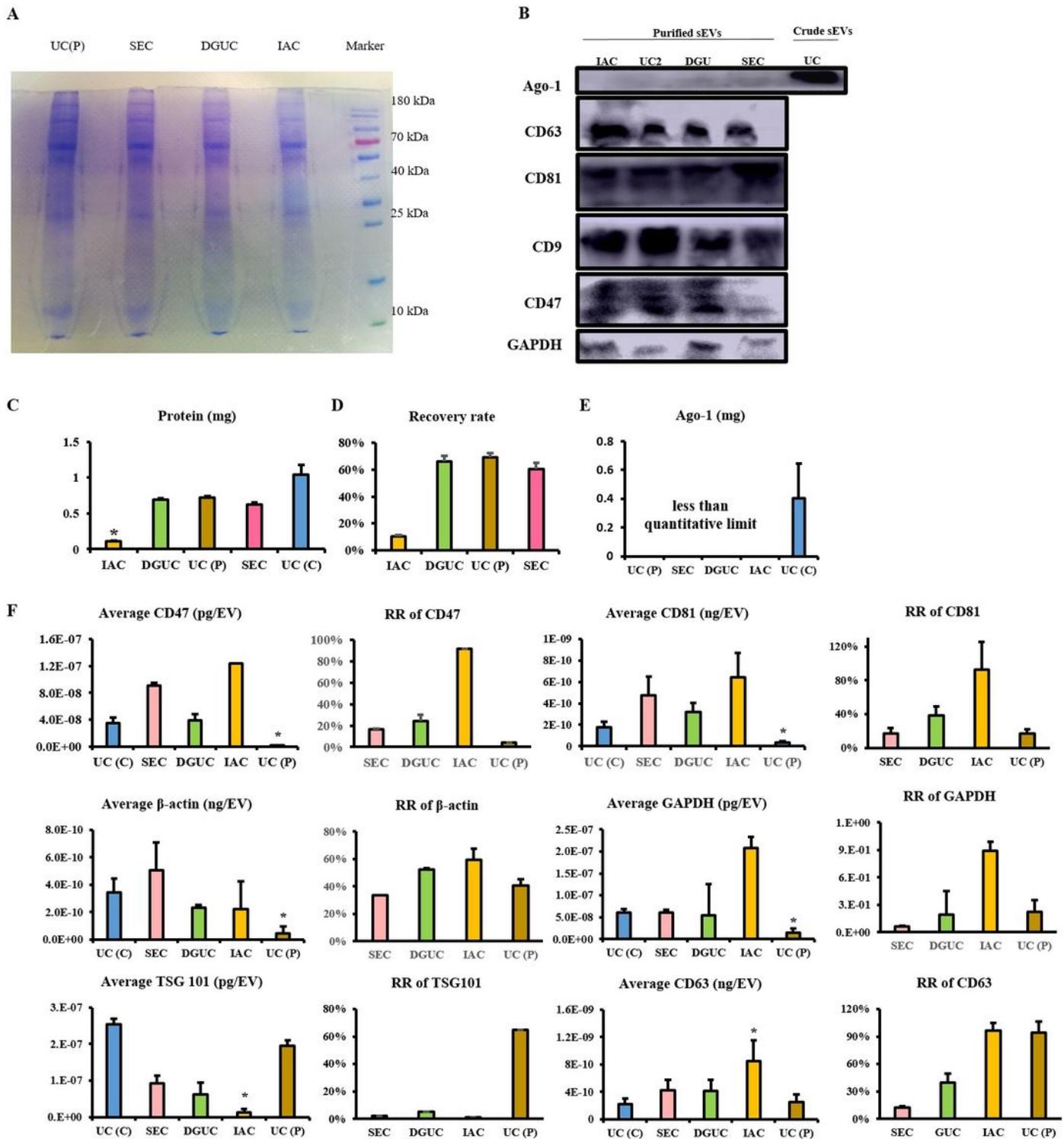


Figure 5

Evaluation of proteins of the purified samples. (A) Coomassie brilliant blue staining of pellet. (B) Western blot analysis of purified sEVs sample. (C) Levels of protein in purified sample (n=3). (D) Recover rate of protein via different purification method. (E) Ago-1 level in purified samples. (F) Level of various proteins and recover rate in purified sample. * P < 0.05.

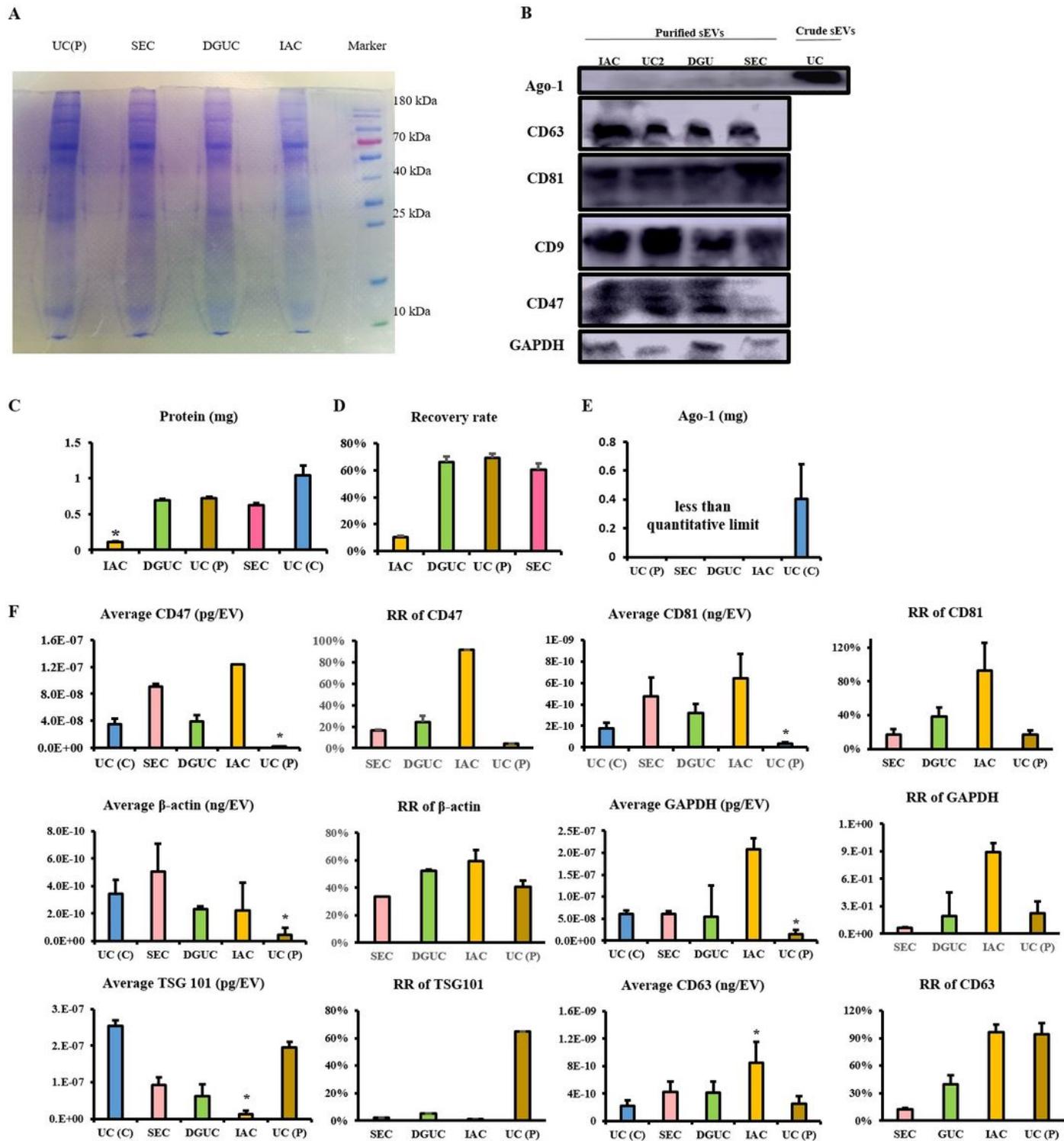


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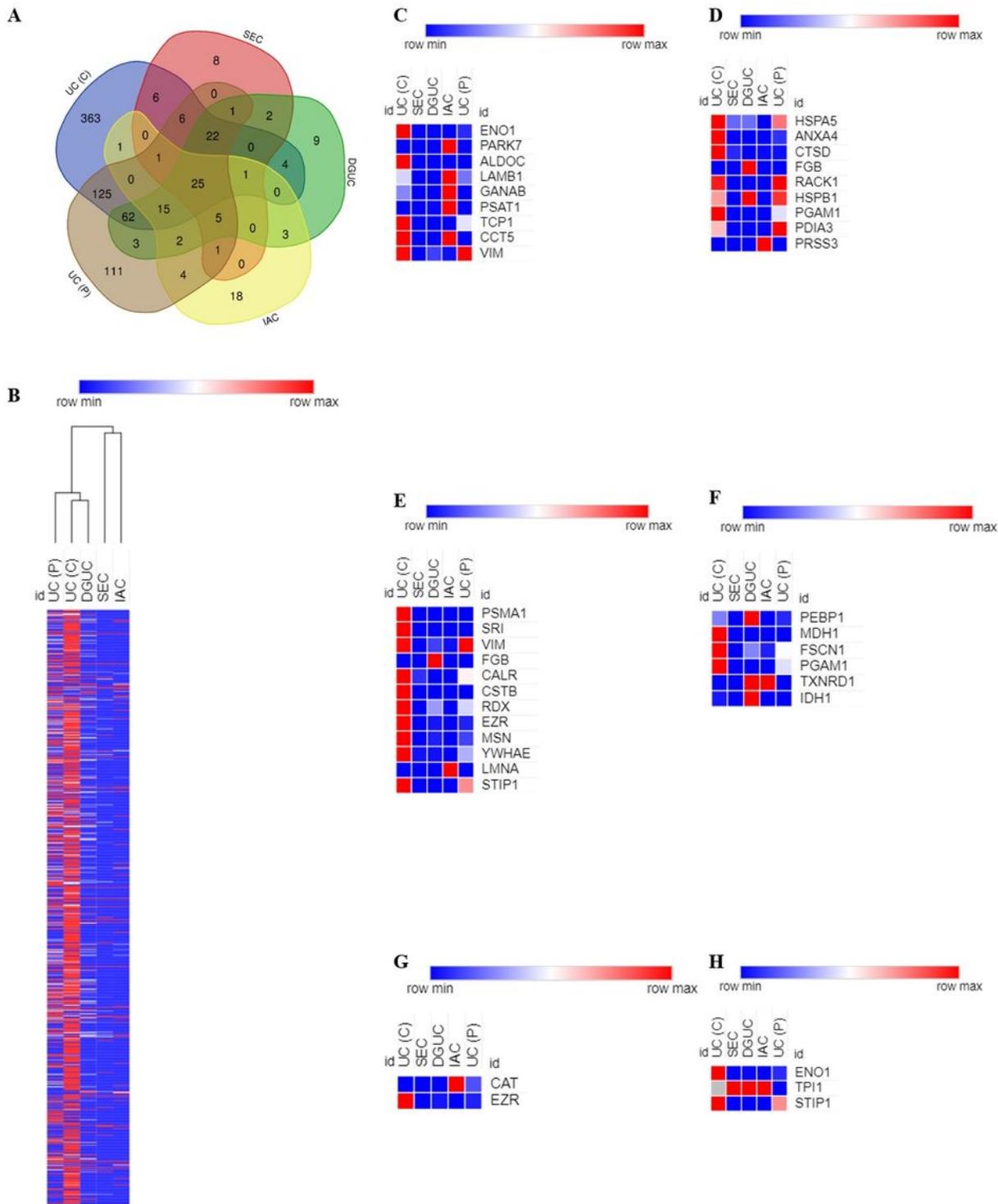


Figure 6

Proteomic analysis of sEVs. (A) Venn diagram for number of proteins in sample. (B) The heat map of all proteins of sEVs (n=3). (C) Analysis of overexpressed proteins (pancreatic cancer tissue compared with normal adjacent pancreatic tissue) in sEVs. (D) Analysis of downexpressed proteins (pancreatic cancer tissue compared with normal adjacent pancreatic tissue) in sEVs. (E) Analysis of overexpressed pancreatic cancer metastasis-associated proteins in sEVs. (F) Analysis of downexpressed pancreatic

normal adjacent pancreatic tissue) in sEVs. (D) Analysis of downexpressed proteins (pancreatic cancer tissue compared with normal adjacent pancreatic tissue) in sEVs. (E) Analysis of overexpressed pancreatic cancer metastasis-associated proteins in sEVs. (F) Analysis of downexpressed pancreatic cancer metastasis-associated proteins in sEVs. (G) Analysis of overexpressed pancreatic cancer drug resistance-associated proteins in sEVs. (H) Analysis of downexpressed pancreatic cancer drug resistance-associated proteins in sEVs.