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Development of an Effective Isolation Method for Plasma Extracellular Vesicles Excluding Lipoproteins by Precipitation With Polyethylene Glycol

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ABSTRACT

Various molecules in plasma extracellular vesicles (EVs) are expected to be applied to minimally invasive diagnosis; however, the high concentration of lipoproteins in plasma, which are similar in size, density and content to EVs, hampers analysis on plasma EVs. To overcome this, we explored an effective isolation method for plasma EVs that excludes lipoproteins by applying precipitation methods that are conventionally used to separate lipoproteins. Human plasma was mixed with heparin and $MnCl_2$, phosphotungstic acid and $MgCl_2$, or polyethylene glycol (PEG), and the expression level of CD9, Apo B and Apo A-I in both the supernatant and pellet was measured by enzyme-linked immunosorbent assay. Morphology was observed by transmission electron microscopy to assess EV yield and lipoprotein contamination. The combination of heparin and $MnCl_2$, or phosphotungstic acid and $MgCl_2$, could not separate plasma EVs and lipoproteins. PEG precipitated EVs and lipoproteins differently, and EVs were specifically precipitated by PEG (3%) to some extent. In comparison with differential ultracentrifugation (UC), size-exclusion chromatography, density gradient centrifugation and precipitation with PEG (8%) followed by UC, PEG (3%) was not inferior in efficiency but was superior in terms of time and cost. The precipitation method using PEG (3%) may contribute to the application of plasma EVs in disease diagnosis.

1 | Introduction

Various substances play crucial roles in facilitating the exchange of information and nutrients between cells and tissues within the animal body. Erythrocytes deliver oxygen to systemic organs. Cells secrete hormones and chemokines, which influence cellular function via signal transduction. This intercellular interaction is essential for the maintenance of homeostasis. Recent studies have revealed that extracellular vesicles (EVs), spherical particles enclosed by a phospholipid bilayer with a diameter of 50–1000 nm, mediate cell-to-cell communication under both physiological and pathological conditions (Martínez and Andriantsitohaina 2017; van Niel et al. 2018; Yáñez-Mó et al. 2015; Yuyama and Igarashi 2016). This is attributed to the fact that EVs secreted by cells contain numerous and various molecules such as DNA, RNA, microRNA (miRNA), long noncoding RNA (lncRNA), proteins, and lipids, protect them from degenerative mechanisms in the body, and transfer them to cells (Li et al. 2020; Lo Cicero et al. 2015; Skotland et al. 2017). For instance, cancer cells release a large number of EVs, whose composition differs from that of normal cells (Abhange et al. 2021; Bebelman et al. 2018; Cheng and Hill 2022). This suggests

that EVs in body fluids, such as plasma, serum and saliva, could serve as potential early stage biomarkers and facilitate the development of minimally invasive diagnoses for various cancers.

Plasma, a common biological sample, contains a substantial quantity of EVs and has been explored for its potential use in diagnosis and therapeutics (Abhange et al. 2021; Cheng and Hill 2022; Nieuwland and Siljander 2024). However, plasma also comprises soluble proteins and lipoproteins. Similar to EVs, lipoproteins contain various molecules, such as miRNA and proteins, are enclosed by a lipid layer, and mediate intercellular communication (Michell and Vickers 2016; Ramasamy 2014). Lipoproteins and EVs exhibit overlapping diameters and densities, with the concentration of lipoproteins notably higher than that of EVs in plasma (Beazer et al. 2020; Johnsen et al. 2019; Menard et al. 2018). These difficulties in distinguishing plasma EVs from lipoproteins prevent the high-precision analysis of plasma EVs; therefore, isolation methods for plasma EVs at high purity excluding lipoproteins are essential. To isolate EVs from culture cell media, conventional methods such as differential ultracentrifugation (UC), density gradient centrifugation (DG), size-exclusion chromatography (SEC) and immunoprecipitation have been widely used (Coumans et al. 2017; Nieuwland and Siljander 2024). However, achieving high yield and purity for the isolation of EVs from plasma, which contains diverse and abundant contaminants, is challenging (Johnsen et al. 2019; Simonsen 2017). Despite efforts to assess the combination of these methods to enhance the purity and efficiency of EV isolation, their time-consuming and labor-intensive nature presents a significant obstacle (Takov et al. 2019; Zhang et al. 2020). These methods are difficult to apply to the purification of plasma EVs for diagnosis because the increase in procedural steps leads to decreased reproducibility and increased time and cost.

It is well established that lipoproteins can be separated into high-density lipoproteins (HDLs) from other classes of lipoproteins, such as low-density lipoproteins (LDLs), very low-density lipoproteins (VLDLs) and chylomicrons, by using polyanion and divalent cation (Demacker et al. 1997; Warnick et al. 1985). This process, known as the precipitation method, comprises straightforward procedures: mixing with a specific concentration of polyanion and divalent cation, followed by centrifugation to separate the mixture into HDL in supernatant and other lipoproteins in a pellet. This method is based on the differences between the surface charge and membrane structure among the various classes. Similar differences may exist between lipoproteins and EVs, because EVs are composed of a phospholipid bilayer (Martínez and Andriantsitohaina 2017; van Niel et al. 2018), whereas lipoproteins are composed of a phospholipid monolayer (Hevonoja et al. 2000; Prassl and Laggner 2009; Wang and Briggs 2004). These characteristics could be attributed to the development of novel isolation methods for plasma EVs. Therefore, we aimed to develop an effective and accessible isolation method for plasma EVs that excludes lipoproteins by applying the precipitation methods conventionally used for lipoproteins.

2 | Methods

2.1 | Reagents and Antibodies

For experiments, we used reagents and antibody as follows: sodium heparin; polyethylene glycol (PEG) (molecular weight: 6000); biotin-conjugated rat monoclonal anti-CD9 antibody (30B) (1.2 mg/mL); sodium dodecyl sulphate (SDS); bromophenol blue (Fujifilm Wako Chemicals, Osaka, Japan); NaCl; manganese chloride tetrahydrate; phosphotungstic acid; 4% paraformaldehyde; dithiothreitol (Nacalai Tesque, Kyoto, Japan); phosphate-buffered saline (PBS) (Sigma–Aldrich, St. Louis, MO, USA); magnesium chloride hexahydrate; trichloroacetic acid; acetone; tris(hydroxymethyl)aminomethane (Tris); HCl; glycerol; NaOH (Kanto Chemical, Tokyo, Japan); polystyrene beads (CPC100) (IZON, Christchurch, New Zealand); EM stainer (Nisshin-EM, Tokyo, Japan); bovine albumin solution (2 mg/mL); fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA); HEPES (Dojindo, Kumamoto, Japan) and Optiprep (Abbott Diagnostics Technologies, Chicago, IL, USA).

The primary antibodies for Western blotting were as follows: Alix (12422-1-AP) (0.8 mg/mL), Apo B (20578-1-AP) (0.8 mg/mL) (Proteintech, Rosemont, IL, USA); TSG-101 (ab125011) (0.226 mg/mL) (Abcam, Cambridge, UK); CD63 (SHI-EXO-M02) (1 mg/mL); CD81 (SHI-EXO-M03) (1 mg/mL); CD9 (SHI-EXO-M01) (1 mg/mL) (CosmoBio, Tokyo, Japan); Apo A-I (GTX112692) (1.23 mg/mL) (GeneTex, Irvine, CA, USA) and albumin (sc-271605) (0.2 mg/mL) (Santa Cruz Biotechnology, Dallas, TX, USA).

The horseradish peroxidase (HRP)-conjugated secondary antibodies for Western blotting were as follows: HRP-linked anti-rabbit IgG (#7074) (0.77 mg/mL) and anti-mouse IgG (#7076) (0.184 mg/mL) (Cell Signaling Technology, Danvers, MA, USA).

2.2 | Preparation of Human Plasma

The human plasma used in this study was frozen within 1 h after collection and anticoagulated with sodium citrate by Continental Blood Bank (Miami, FL, USA), and we purchased them in a frozen state from a distributor (CosmoBio). Hundred millilitres of the frozen plasma was thawed at 37°C for 30 min in a water bath (FS-003) (Tokyo Garasu Kikai, Tokyo, Japan). The thawed plasma was separated into 1 mL in a 1.5 mL sample tube and frozen before EV isolation. For use, it was thawed at 37°C for 5 min in a dry bath (D1301) (Labnet, Edison, NJ, USA) and centrifuged at 10,000 × *g* for 10 min to remove cell debris. The supernatant was filtrated by a 0.22 μm syringe filter (SLPES2522S) (Hawach Scientific, Shaanxi, China). We used the through solution in the following experiment.

2.3 | Isolation of Plasma EVs by Precipitation With Heparin and Manganese Chloride

We explored the isolation conditions of plasma EVs by applying the previously reported fractionation method for lipoprotein

using heparin and manganese chloride (Warnick et al. 1985). Sodium heparin was dissolved in 0.15 M NaCl to achieve a concentration of 35.22 mg/mL. Manganese chloride tetrahydrate was also dissolved in Milli-Q water to achieve a concentration of 0.44–2.50 M. Three-hundred microlitres of human plasma was mixed with 12 μL of heparin solution (final concentration of 1.29 mg/mL) and 15 μL of manganese chloride solution (final concentration of 20.2–114.7 mM) by a vortex mixer (Vortex-genie 2) (Scientific Industries, Bohemia, NY, USA). This mixture was allowed to stand at 4°C for 30 min and centrifuged (1500 × g, 4°C, 30 min) by using a microcentrifuge (model 3740) (Kubota, Tokyo, Japan). The supernatant was collected for the following analysis. The pellet was washed with 300 μL of PBS and centrifuged (1500 × g, 4°C, 30 min) again. The pellet was suspended by 300 μL of PBS and used for the following analysis.

2.4 | Isolation of Plasma EVs by Precipitation With Phosphotungstic Acid and Magnesium Chloride

The precipitation method for isolation of plasma EVs using phosphotungstic acid and magnesium chloride was also performed by referencing the previous report of lipoprotein fractionation method (Warnick et al. 1985). Phosphotungstic acid was dissolved in Milli-Q water to achieve a concentration of 40 mg/mL and the pH was adjusted to 7.4 by adding 1 M NaOH. Magnesium chloride hexahydrate was also dissolved in Milli-Q water to achieve a concentration of 0.5–3.0 M. Three-hundred microlitres of human plasma was mixed with 6 μL of phosphotungstic acid solution (final concentration of 2.91 mg/mL) and 24 μL of magnesium chloride solution (final concentration of 9.1–54.5 mM) by the vortex mixer. This mixture was allowed to stand at room temperature for 5 min and centrifuged (5000 × g, room temperature, 10 min) by using the microcentrifuge. The supernatant was collected for the following analysis. The pellet was washed with 300 μL of PBS and centrifuged (5000 × g, room temperature, 10 min) again. The pellet was suspended by 300 μL of PBS and used for the following analysis.

2.5 | Isolation of Plasma EVs by Precipitation With PEG

The precipitation method for the isolation of plasma EVs using PEG was performed through referencing the previous reports of lipoprotein fractionation (Warnick et al. 1985) and EV isolation (Rider et al. 2016) methods. PEG was dissolved in 0.15 M NaCl to achieve a concentration of 3%–23%. One hundred and fifty microlitres of human plasma was mixed with an equal volume of PEG solution (final concentration of 1.5%–11.5%) by the vortex mixer. This mixture was allowed to stand at room temperature for 15 min and centrifuged (1500 × g, room temperature, 20 min) by using the microcentrifuge. The supernatant was collected for the following analysis. The pellet was washed with 300 μL of PBS and centrifuged (2000 × g, room temperature, 20 min) again. The pellet was suspended by 150 μL of PBS and used for the following analysis.

2.6 | Measurement of CD9 Protein by Enzyme-Linked Immuno-Sorbent Assay (ELISA)

The CD9 protein expression in the supernatant and suspended pellet was measured by using a PS Capture Exosome ELISA Kit (Fujifilm Wako Chemical). Fifteen microlitres of each supernatant and suspended pellet were diluted with 135 μL of Reaction Buffer, and 1.5 μL of Exosome Binding Enhancer was added. This mixture was reacted on a strip plate with shaking (500 rpm, room temperature, 2 h). After washing each well with Washing Buffer added with 1% Exosome Binding Enhancer, hundred microlitres of biotin-conjugated rat monoclonal anti-CD9 antibody (30B) (Fujifilm Wako Chemicals), which diluted at 1:4000 in Reaction Buffer, was reacted on the plate with shaking (500 rpm, room temperature, 1 h). We followed the manufacturer's instructions for the subsequent procedures after this antibody reaction. The absorbance at 450 and 620 nm was measured by using a multimode microplate reader (TriStar LB941) (Berthold, Bad Wildbad, Germany) and the subtracted value [(reading at 450 nm)–(reading at 620 nm)] was calculated. The expression level of CD9 protein in the supernatant and suspended pellet was represented as a proportion to the amount of CD9 protein in the plasma.

2.7 | Measurement of Apo B and Apo A-I Protein by ELISA

The expression of Apo B and Apo A-I protein in the supernatant and suspended pellet was measured by using a Human Apolipoprotein B ELISA kit and Human Apolipoprotein A1 ELISA kit (Mabtech, Nacka Strand, Sweden), respectively, following the manufacturer's instructions. The absorbance at 450 and 620 nm was measured by using the multimode microplate reader and the subtracted value was calculated. Then, the concentration of Apo B and Apo A-I protein was calculated using the standard curve.

2.8 | Morphological Analysis by a Negative Staining

Polystyrene beads, as a spike-in control for negative staining and transmission electron microscopy (TEM) analysis, were added to the supernatant and suspended pellet to achieve a final concentration of 5.0×10^9 particles/mL. They were mixed with an equal volume of 2% paraformaldehyde, which was prepared by diluting 4% paraformaldehyde with PBS, and they were allowed to stand at 4°C for 30 min. Next, 20 μL of the mixtures were applied onto parafilm (Amcor, Zürich, Switzerland), and formvar carbon-coated copper grids (U1007) (EMJapan, Tokyo, Japan) were placed over them at room temperature for 10 min. Before use, the grids were added with a hydrophilic character on the surface by using a PIB-20 Ion-bombarder (Vacuum Devices, Ibaraki, Japan). The excess mixtures on the grids were removed by suction using a filter paper, and the grids were washed twice with Milli-Q water on parafilm. After washing, they are stained with an EM stainer diluted fourfold with Milli-Q water at room temperature for 30 min. After removing the excess solution, the grids were completely dried on air, and the morphology of stained particles was observed by using a TEM (H-7650) (Hitachi,

Tokyo, Japan) at 80 kV acceleration voltage (Rikkert et al. 2019; Ter-Ovanesyan et al. 2023).

2.9 | Bicinchoninic Acid Assay (BCA) Assay

Protein concentration was measured by using a Protein Assay BCA kit (Fujifilm Wako). The standard curve was obtained by diluting bovine serum albumin solution.

2.10 | Protein Purification and Western Blotting

Before performing Western blotting, proteins of the supernatant and suspended pellet were purified. Trichloroacetic acid was added to them to achieve a final concentration of 10%, and the proteins were allowed to precipitate at 4°C for 30 min. After that, the mixture was centrifuged (12,000 × *g*, 4°C, 10 min). The pellet was washed twice with ice-cold acetone and resuspended in an equal volume of SDS sample buffer consisting of 62.5 mM Tris/HCl (pH 6.8), 2% SDS, 50 mM dithiothreitol, 10% glycerol and 0.001% bromophenol blue as in the starting volume of the supernatant and suspended pellet. We isolated EV from an equal volume of plasma, from which we purified EV protein. These protein lysates in the sample buffer were used for the following analysis in Western blotting. Western blotting and data analysis were performed as described previously (Usui et al. 2012). An appropriate amount of protein lysate, adjusted based on the signal intensity of each target protein, was separated by SDS-PAGE (7.5%–14%) and transfected to a nitrocellulose membrane (Pall Corporation, Ann Arbor, MI, USA). After blocked with 0.5% skim milk, the membranes were incubated with primary antibodies (1:500 dilution) at 4°C overnight. They were visualised using HRP-conjugated secondary antibodies (1:10,000 dilution, 1 h) and an EZ-ECL kit (Biological Industries, Kibbutz, Israel) or Trident femto Western HRP Substrate (GeneTex). The results were analysed using CS analyser 3.0 software (ATTO, Tokyo, Japan).

2.11 | UC

Three hundred microlitres of human plasma was ultracentrifuged (164,071 × *g*, 2 h, 4°C) by using an Optima XL-80K ultracentrifuge with a swing rotor SW 55 Ti (Beckman Coulter, Miami, FL, USA). The pellet was resuspended in PBS and ultracentrifuged again. Then, the pellet was resuspended in 300 μL of PBS and used for the comparative analysis.

2.12 | Size-Exclusion Chromatography (SEC)

SEC for the isolation of plasma EVs was performed by using an EVSecond L70 (GL Sciences, Tokyo, Japan) following the manufacturer's instructions. Fillers were blocked with FBS and were washed with PBS three times before use. After that, 300 μL of human plasma was loaded into a column, and 1200 μL PBS was added. Hundred microlitres of PBS was 12 times loaded, and the through solution (Fractions 1–12) was collected. Fractions 6–10, in which the existence of EVs by Western blotting and negative staining was confirmed, were used for the comparative analysis.

2.13 | Density Gradient Centrifugation (DG)

The density gradient was made by the step addition of 5%, 10%, 20% and 40% Optiprep diluted with HEPES solution. HEPES solution consists of 10 mM HEPES and 0.85% NaCl, and the pH was adjusted to 7.4 by adding 1 M NaOH. Three hundred microlitres of human plasma was loaded on the top of the gradient and ultracentrifuged (164,071 × *g*, 18 h, 4°C). Then, fractions (500 μL) were collected from the top of the gradient. The density of the fractions diluted at 1:2000 in Milli-Q water was determined by the absorbance at 244 nm using a BioSpectrometer (Eppendorf, Hamburg, Germany). These fractions, whose density at 1.060–1.200 was the same as that of EVs (Brennan et al. 2020; Liangsupree et al. 2021), were gathered and ultracentrifuged (164,071 × *g*, 2 h, 4°C). The pellet was resuspended with 300 μL of PBS and used for the comparative analysis.

2.14 | Precipitation With PEG (8%) Followed by Ultracentrifugation (PEGUC)

The isolation of plasma EVs by PEGUC was performed as previously reported (Rider et al. 2016). Briefly, PEG was dissolved in 0.15 M NaCl to achieve a concentration of 16%. One hundred and fifty microlitres of human plasma was mixed with an equal volume of 16% PEG solution by rotation using a tube rotator (RotoFlex Plus) (Argos, Vernon Hills, IL, USA) for 18 h at 4°C. This mixture was centrifuged (2500 × *g*, 4°C, 15 min). The pellet was resuspended with PBS and ultracentrifuged (164,071 × *g*, 2 h, 4°C). The pellet was resuspended by 150 μL of PBS and used for comparative analysis.

2.15 | Particle Size Distribution of Plasma EVs

The concentration and size of isolated EVs were measured using NanoSight LM10 (Malvern Panalytical, Malvern, UK) equipped with a 405 nm laser. The software used to capture and analyse the data was NTA 3.4. The isolated EVs diluted 5- or 100-fold with PBS were visualised by light scattering at 26°C, camera level 13 and detection threshold 7. We analysed 1499 frames taken per minute (FPS: 25).

2.16 | Measurement of miRNA Concentration

miRNA was purified from the isolated plasma EVs by using a miRNeasy kit (Qiagen, Venlo, the Netherlands), and its concentration was measured by using a Qubit miRNA assay kit (Thermo Fisher Scientific), following the manufacturer's instructions.

2.17 | Statistic Analysis

Data were shown as mean ± standard error of the mean (SEM). Statistical evaluations were performed by one-way ANOVA followed by Bonferroni's post hoc test. Values of $p < 0.05$ were considered statistically significant.

3 | Results

3.1 | Optimisation of the Condition for the Isolation of Plasma EVs by Precipitation With Heparin and Manganese Chloride

We first evaluated the optimal condition for the isolation of plasma EVs by precipitation with heparin and manganese chloride. As the concentration of manganese chloride increases, the expression level of EV marker protein, CD9 (Figure 1a) and chylomicrons/VLDL/LDL marker protein, Apo B (Figure 1b, c) was increased in the pellet but decreased in the supernatant. The expression of HDL marker protein, Apo A-I, was not detected in the pellet (Figure 1d, e). Next, we confirmed the morphology of isolated plasma EVs. The negative stained EVs were observed as cap-shaped particles in TEM images (Rikkert et al. 2019; Ter-Ovanesyan et al. 2023). Lipoproteins (chylomicrons/LDL/VLDL) were identified as circled particles (Zhang et al. 2013), while HDL was not observed possibly due to our technical limitation. EVs and chylomicrons/LDL/VLDL were observed simultaneously in the pellet precipitated with heparin (1.29 mg/mL) and manganese chloride (68.8 and 114.7 mM) (Figure 1f). These results suggested that heparin (1.29 mg/mL) and manganese chloride (20.2–114.7 mM) precipitated EVs and chylomicrons/LDL/VLDL in a similar manner, whereas HDL was hardly precipitated. EVs and lipoproteins in plasma would not be able to be separated by this combination.

3.2 | Optimisation of the Condition for the Isolation of Plasma EVs by Precipitation With Phosphotungstic Acid and Magnesium Chloride

Next, we utilised the solution consisting of phosphotungstic acid and magnesium chloride for the isolation of plasma EVs. As the concentration of magnesium chloride increases, the expression level of CD9 (Figure 2a) and Apo B (Figure 2b, c) was increased in the pellet but decreased in the supernatant, while the Apo B expression in the pellet was high at the low concentration of magnesium chloride compared with CD9. Apo A-I was hardly detected in the pellet (Figure 2d, e). In TEM images, many chylomicrons/LDL/VLDL were observed in the pellet precipitated with phosphotungstic acid (2.91 mg/mL) and magnesium chloride (18.2 mM), while there were a few EVs (Figure 2f). Phosphotungstic (2.91 mg/mL) acid and magnesium chloride (9.1–54.5 mM) would not be able to separate plasma EVs from lipoproteins.

3.3 | Optimisation of the Condition for the Isolation of Plasma EVs by Precipitation With PEG

We further explored the optimal condition for the isolation of plasma EVs by precipitation with PEG. As the concentration of PEG increases, the expression level of CD9 (Figure 3a), Apo B (Figure 3b, c) and Apo A-I (Figure 3d, e) was increased in the pellet but decreased in the supernatant. Noteworthy, in the pellet precipitated with PEG (3%), approximately 30% of plasma CD9 was detected, while Apo B and Apo A-I were hardly detected. Supporting this result, in TEM images, many EVs were observed in the pellet precipitated with PEG (3%), while there were a few chylomicrons/LDL/VLDL (Figure 3f). To check the protein contamination in the pellet precipitated with PEG, we performed the BCA assay. As the concentration of PEG increases, the amount of total protein was increased (Figure 3g). In the pellet precipitated with PEG (3%), the expression level of EV marker proteins (TSG-101, CD63, CD81 and CD9) was detectable, while Apo B was almost undetectable and Apo A-1 was lower compared with plasma (Figure 3h). Albumin, the most abundant protein in plasma, was also expressed at a lower level compared with plasma (Figure 3h). These results suggested that the EV isolation method using PEG-3 successfully minimised lipoprotein contamination to around 4% with an isolation of over 30% of the EVs.

3.4 | Comparison of the Five Isolation Methods for Plasma EVs by ELISA and TEM Analysis

UC, SEC and DG were well known as conventional methods for EV isolation. PEGUC was also reported as an effective method (Rider et al. 2016). Then, we next compared the PEG-3 and these conventional isolation methods for plasma EVs in terms of efficiency and purity. The expression level of CD9 in plasma EVs isolated by PEG-3 was similar to that by UC and SEC (Figure 4a). It was the highest in the plasma EVs isolated by PEGUC (Figure 4a, $n = 4$, $p < 0.01$ vs. UC) and the lowest in those by DG ($n = 4$, $p < 0.01$ vs. UC). The expression levels of Apo B and Apo A-I in plasma EVs isolated by all the methods were significantly lower compared with those in plasma (Figure 4b, d, $n = 4$, $p < 0.01$ vs. UC, SEC, DG, PEGUC and PEG-3). In the plasma EVs isolated by SEC, PEGUC and PEG-3, the expression level of Apo B was higher than that by UC (Figure 4c, $n = 4$, SEC: $p < 0.01$; PEGUC and PEG-3: $p < 0.05$). The expression of Apo A-I in plasma EVs isolated by all the methods was hardly detectable (Figure 4d, e). EVs and chylomicrons/LDL/VLDL were observed in plasma EVs isolated by UC, SEC, PEGUC and PEG-3 in TEM images, while, in the plasma EVs isolated by DG, they were hardly observed (Figure 4f).

3.5 | Protein Expression in the Plasma EVs Determined by BCA Assay and Western Blotting

The amount of total protein was low in isolated plasma EVs by UC and SEC, while it was high in isolated plasma EVs by DG, PEG UC and PEG-3 (Figure 4g, $n = 4$, DG, PEGUC and PEG-3: $p < 0.01$ vs. UC; DG and PEG-3: $p < 0.01$ vs. PEGUC; PEG-3: $p < 0.05$ vs. DG). In Western blotting analysis, EV marker proteins (Alix, TSG-101, CD63, CD81 and CD9) were sufficiently detected in plasma EVs isolated by DG, PEGUC and PEG-3, whereas they were barely detected in those by UC and SEC (Figure 4h). The expression level of ApoB and Apo A-I in plasma EVs isolated by all five methods were lower compared with plasma. Apo B was not detected in plasma EVs isolated by UC and DG (Figure 4h), which was consistent with the results of the ELISA assay (Figure 4c). The expression level of albumin (Figure 4h) was high in plasma EVs isolated by DG and PEG-3 as with the case of BCA assay (Figure 4g). The total protein/CD9 ratio was also higher in plasma EVs isolated by PEG-3 and DG than that by UC, SEC and PEG-UC (Figure 4i, $n = 4$, PEG-3: $p < 0.01$ vs. UC and DG).

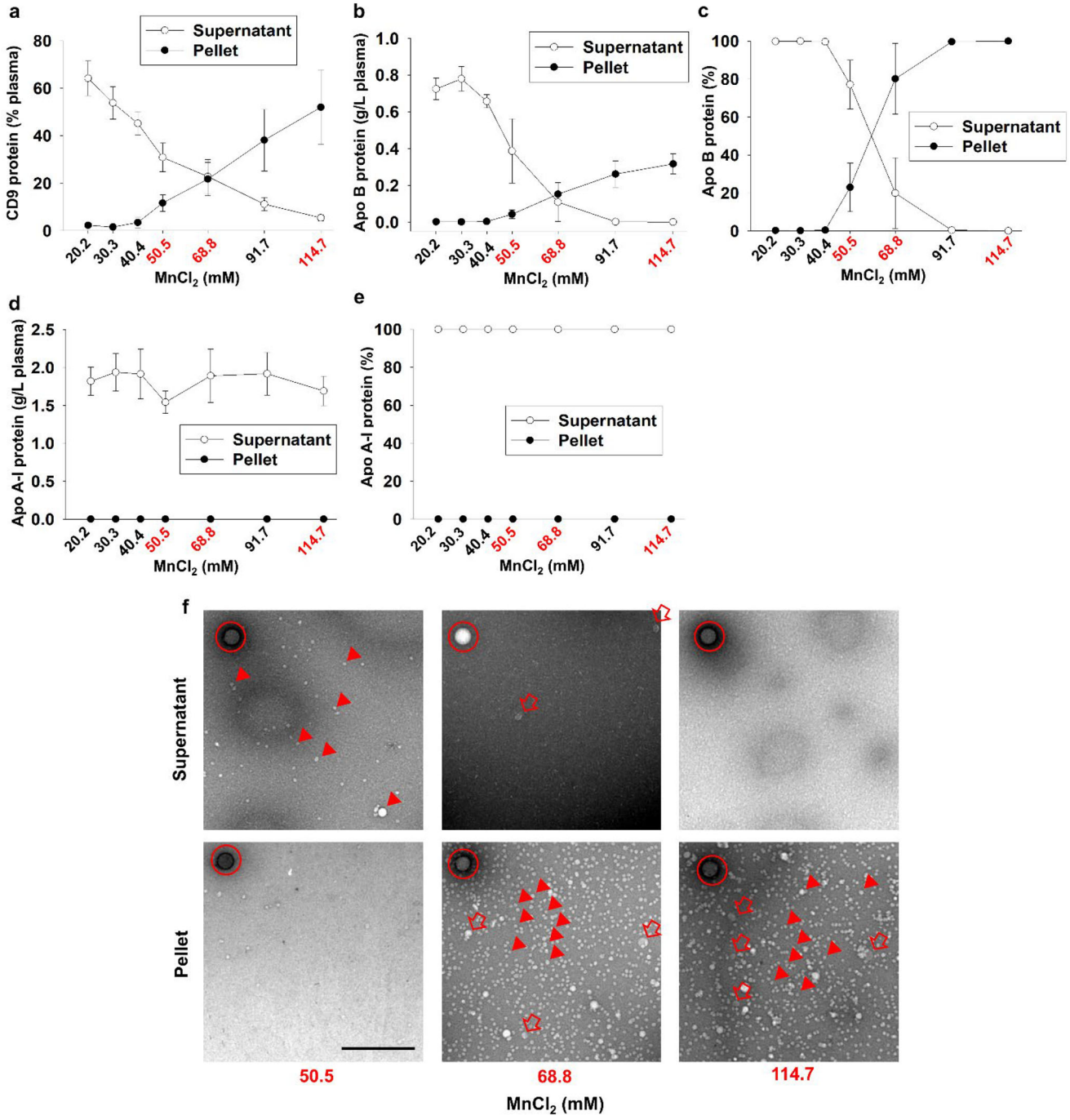

FIGURE 1 | Optimisation of the condition for isolation of plasma extracellular vesicles (EVs) by precipitation with heparin and manganese chloride. Human plasma was mixed with heparin (1.29 mg/mL) and manganese chloride (20.2–114.7 mM). After centrifugation (1500 × g), the supernatant and pellet were separated, and the pellet was suspended in the same volume of phosphate-buffered saline (PBS) as the plasma. The expression of EV and lipoprotein marker proteins was measured by enzyme-linked immuno-sorbent assay (ELISA). (a) Relative amount of CD9 protein, an EV marker protein, in the supernatant and pellet. The data were normalised with the concentration of CD9 protein in human plasma. (b) The concentration of Apo B protein, chylomicron, low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) marker protein, in the supernatant and pellet. The vertical axis shows the Apo B expression level in the fraction isolated from 1 mL of plasma. (c) The proportion of Apo B protein in the supernatant or pellet, relative to the total of the supernatant and pellet in each fraction. (d) The concentration of Apo A-I protein, a high-density lipoprotein marker protein, in the supernatant and pellet. The vertical axis shows the Apo A-I expression level in the fraction isolated from 1 mL of plasma. (e) The proportion of Apo A-I protein in the supernatant or pellet, relative to the total of the supernatant and pellet in each fraction. Results were expressed as means ± standard error of the mean (SEM) in line graphs ($n = 4$). (f) Transmission electron microscopy (TEM) images of the supernatant and pellet with negative staining. Circle: polystyrene beads (known diameter: 100 nm); arrowhead: chylomicrons/VLDL/LDL; arrow: EVs. Scale bar: 500 nm.

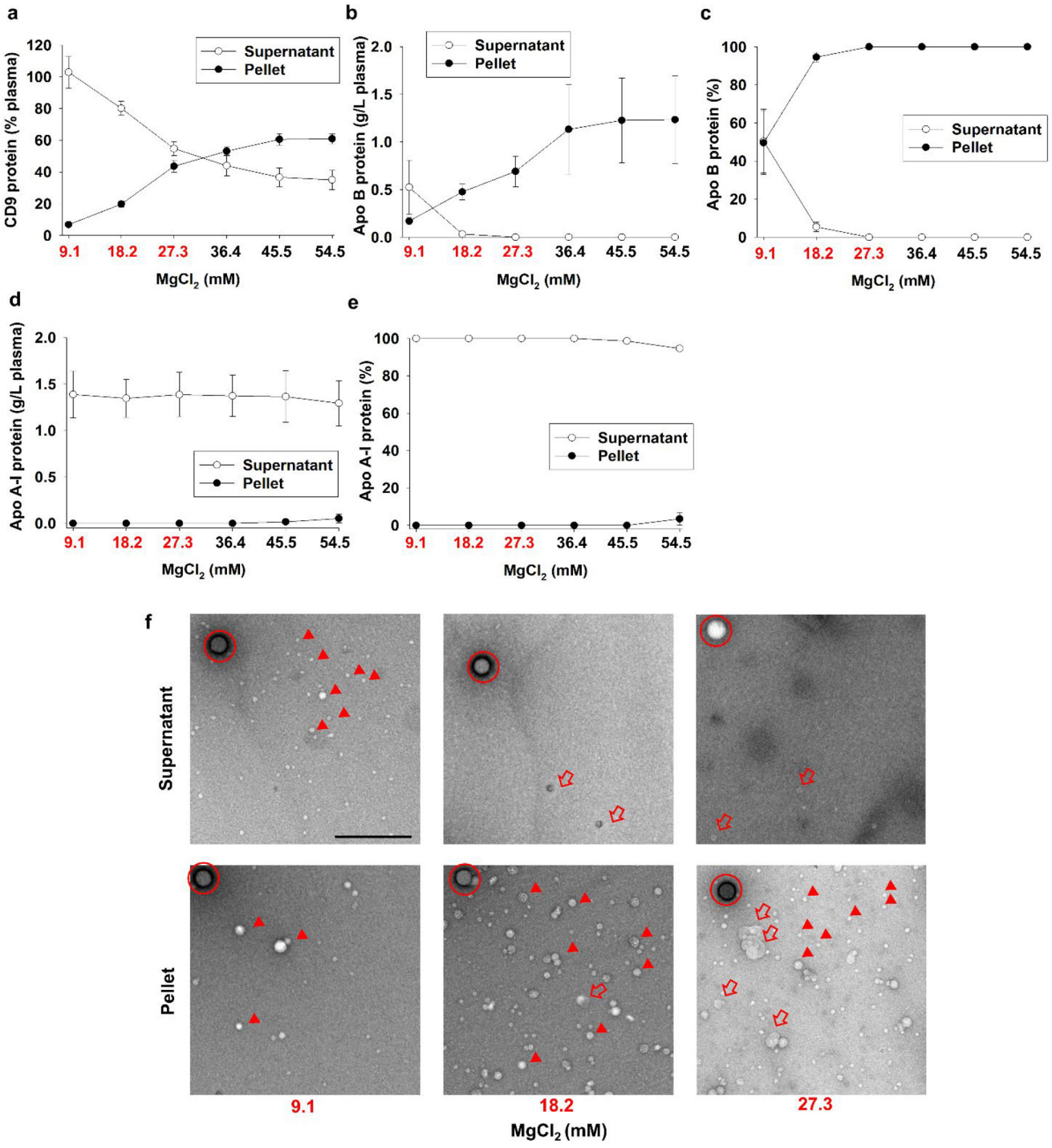

FIGURE 2 | Optimisation of the condition for isolation of plasma EVs by precipitation with phosphotungstic acid and magnesium chloride. Human plasma was mixed with phosphotungstic acid (2.91 mg/mL) and magnesium chloride (9.1–54.5 mM). After centrifugation (5000 × g), the supernatant and pellet were separated, and the pellet was suspended in the same volume of PBS as the plasma. The expression of EV and lipoprotein marker proteins was measured by ELISA. (a) Relative amount of CD9 protein in the supernatant and pellet. The data were normalised with the concentration of CD9 protein in human plasma. (b) The concentration of Apo B protein in the supernatant and pellet. The vertical axis shows the Apo B expression level in the fraction isolated from 1 mL of plasma. (c) The proportion of Apo B protein in the supernatant or pellet, relative to the total of the supernatant and pellet in each fraction. (d) The concentration of Apo A-I protein in the supernatant and pellet. The vertical axis shows the Apo A-I expression level in the fraction isolated from 1 mL of plasma. (e) The proportion of Apo A-I protein in the supernatant or pellet, relative to the total of the supernatant and pellet in each fraction. Results were expressed as means ± SEM in line graphs ($n = 4$). (f) TEM images of the supernatant and pellet with negative staining. Circle: polystyrene beads (known diameter: 100 nm); arrowhead: chylomicrons/VLDL/LDL; arrow: EVs. Scale bar: 500 nm. ELISA, enzyme-linked immuno-sorbent assay; EV, extracellular vesicle; LDL, low-density lipoprotein; SEM, standard error of the mean; TEM, transmission electron microscopy; VLDL, very low-density lipoprotein.

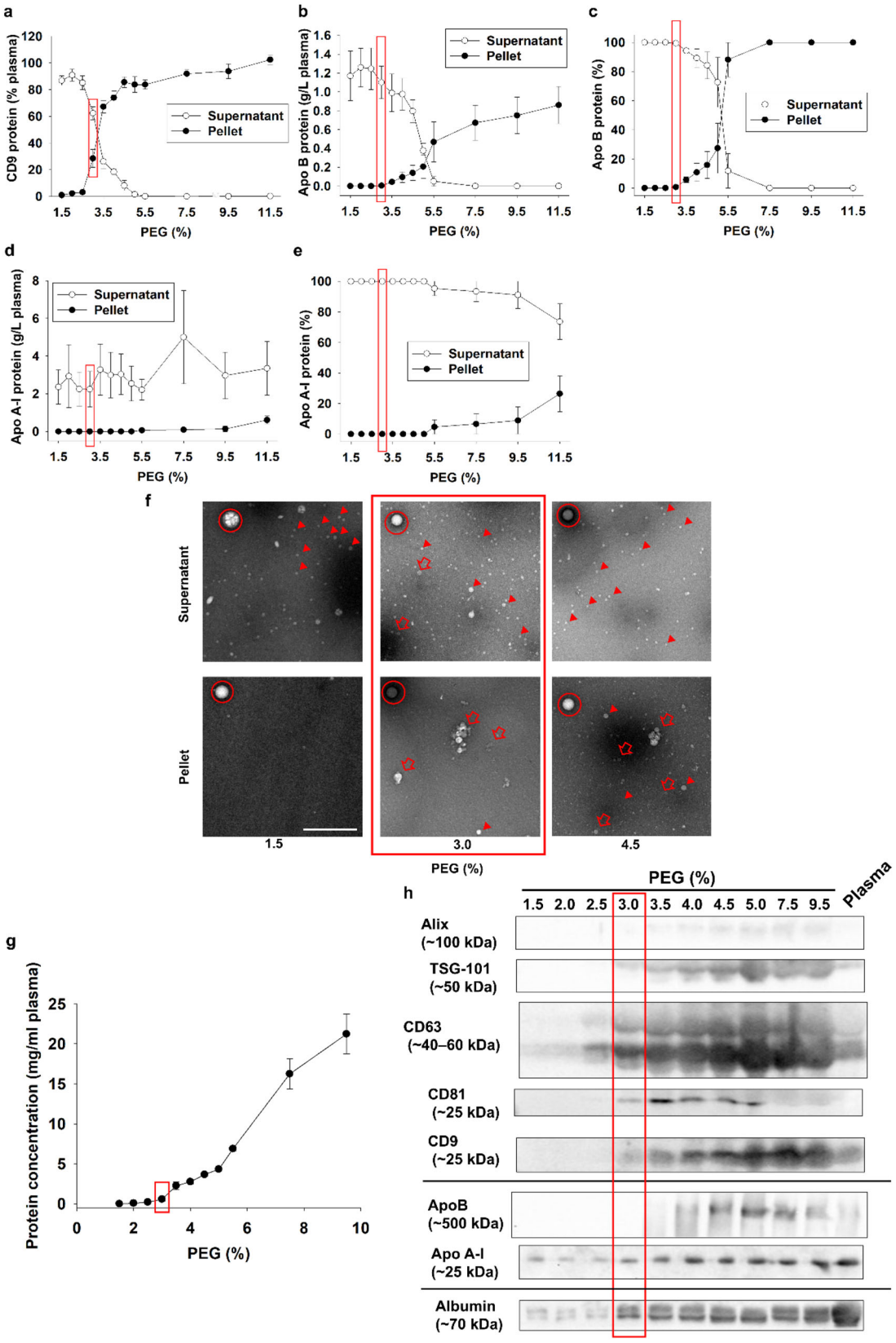

FIGURE 3 | Optimisation of the condition for isolation of plasma EVs by precipitation with polyethylene glycol (PEG). Human plasma was mixed with PEG (1.5%–11.5%). After centrifugation (1500 × g), the supernatant and pellet were separated, and the pellet was suspended in the same volume of PBS as the plasma. The expression of EV and lipoprotein marker proteins was measured by ELISA. (a) Relative amount of CD9 protein in the supernatant and pellet. The data were normalised with the concentration of CD9 protein in human plasma. (b) The concentration of Apo B protein in the supernatant and pellet. The vertical axis shows the Apo B expression level in the fraction isolated from 1 mL of plasma. (c) The proportion of Apo B protein in the supernatant or pellet, relative to the total of the supernatant and pellet in each fraction. (d) The concentration of Apo A-I protein in the

supernatant and pellet. The vertical axis shows the Apo A-I expression level in the fraction isolated from 1 mL of plasma. (e) The proportion of Apo A-I protein in the supernatant or pellet, relative to the total of the supernatant and pellet in each fraction. Results were expressed as means ± SEM in line graphs ($n = 4$). (f) TEM images of the supernatant and pellet with negative staining. Circle: polystyrene beads (known diameter: 100 nm); arrowhead: chylomicrons/VLDL/LDL; arrow: EVs. Scale bar: 500 nm. (g) Total protein concentration was measured by a bicinchoninic acid (BCA) assay in the pellet. Results were expressed as means ± SEM in a line graph ($n = 4$). (h) The protein expression of EV markers (TSG-101, Alix, CD63, CD81 and CD9), lipoprotein markers (Apo B and Apo A-I) and albumin in the pellet. The protein purified from EVs to match the original amount of plasma was loaded, and the protein expression was determined by Western blotting. Plasma: human plasma. ELISA, enzyme-linked immuno-sorbent assay; EV, extracellular vesicle; LDL, low-density lipoprotein; SEM, standard error of the mean; TEM, transmission electron microscopy; VLDL, very low-density lipoprotein.

3.6 | Particle Size Distribution and miRNA Concentration of the Plasma EVs

The mode diameter of plasma EVs isolated by the five methods ranged from 100 to 200 nm (Figure 5a). The concentration of plasma EVs isolated by UC, SEC and DG seemed to be slightly lower, while that of PEGUC and PEG-3 seemed to be slightly higher. Finally, we measured the miRNA concentration in plasma EVs isolated by the five methods. A certain amount of miRNA was obtained from plasma EVs isolated by all five methods, and the miRNA concentration in plasma EVs isolated by SEC was the highest among the five methods (Figure 5b, $n = 4$, $p < 0.01$).

4 | Discussion

In the present study, we explored the effective isolation method for plasma EVs excluding contaminants including lipoproteins. The major findings are as follows: (1) the combination of heparin and manganese chloride (Figure 1) or phosphotungstic acid and magnesium chloride (Figure 2) could not isolate plasma EVs excluding lipoproteins; (2) PEG precipitated EVs and lipoproteins at the different manner, and EVs were specifically precipitated by PEG (3%) to some extent (Figure 3); (3) the efficiency of PEG-3 was not inferior compared with other conventional methods, although some impurities remained (Figures 4 and 5). These results suggested that the precipitation with PEG-3 enabled the isolation of EVs excluding the greater part of lipoproteins from human plasma. This method would be compared in efficiency with other methods.

Numerous studies have reported on the use of PEG precipitation methods to isolate plasma or serum EVs (Andreu et al. 2016; Gámez-Valero et al. 2016; Otani et al. 2019; Rider et al. 2016). However, these reports did not mention the issue of lipoprotein contamination. A limited number of studies have determined the lipoproteins in isolated plasma EVs by using PEG, revealing higher contamination level of lipoproteins compared with SEC (Zhen et al. 2022). However, these studies used 8%–10% PEG, which we demonstrated that abundant lipoproteins, except for HDL, existed in isolated plasma EVs (Figure 3). In healthy individuals, the concentration of HDL is 30,000–35,000 nmol ($1.8–2.1 \times 10^{16}$ particles/mL) (Goff et al. 2005; Mora et al. 2009), and the LDL concentration is 1200–1500 nmol ($7.2–9.0 \times 10^{14}$ particles/mL) (Blake et al. 2002; Kuller et al. 2002). In contrast, the concentration of plasma and serum EVs was lower than the concentration of plasma lipoproteins ($2.0 \times 10^{8}–7.9 \times 10^{11}$ particles/mL) (Dragovic et al. 2011; Momen-Heravi et al. 2012; Mørk et al. 2017; Mustapic et al. 2017; Lobb et al. 2015; Howard et al. 2022; Adamczyk et al. 2023). Based on these data, the influence of lipoproteins cannot be underestimated when analysing plasma EVs. Although PEG-3 removed most of the lipoproteins from plasma EVs (Figure 3), further refinement of this method is required for detailed analysis of plasma EVs. There is a question of whether it is meaningful to distinguish EVs from lipoproteins, as both transport miRNAs and various molecules between cells (Beazer et al. 2020; Menard et al. 2018). However, almost all cells can produce EVs (Flamant and Tamarat 2016; Menard et al. 2018; Samanta et al. 2018), whereas the production of lipoproteins is limited to the cells in liver and intestines (Feingold 2000; Havel 1980). Therefore, when detecting abnormalities in the cells other than those of the liver and intestines, the information contained in EVs secreted by these cells is likely masked by the abundant lipoproteins in the plasma. Thus, the development of techniques to remove lipoproteins is essential.

There was inconsistency with the results in the expression of EV marker proteins between ELISA (Figure 3a) and Western blotting (Figure 3h) among the five isolation methods. The expression level of CD9 in plasma EVs isolated by DG was very low in the ELISA assay, while it was the highest measured in Western blotting. We used a PS Capture Exosome ELISA Kit, which employs a strip plate that binds to phosphatidylserine on the surface of EVs and measures the expression of CD9 protein using an antibody. Previous studies have reported the presence of PS-negative EVs in mouse blood (Matsumoto et al. 2021); therefore, ELISA measurement may overlook these EVs isolated by DG. Conversely, we observed very few particles in the TEM image of plasma EVs isolated by DG (Figure 3f). The particle concentration of the plasma EVs was also low in nanoparticles tracking analysis (NTA) (Figure 4a). These results collectively suggest that the DG method might demonstrate low efficiency for isolating plasma EVs. Given that each method has inherent limitations, it may be challenging to accurately estimate the concentration of EVs using ELISA, Western blotting or NTA alone. A more reliable evaluation could be achieved by integrating results from multiple detection methods (Théry et al. 2018). The PEG-3 method proposed in this study showed consistently higher EV concentration across all analytical methods compared with conventional isolation techniques. This suggested that the PEG-3 method achieved a relatively higher recovery yield of plasma EVs.

It is well known that the increasing PEG concentration leads to a higher amount of precipitated proteins (Atha and Ingham 1981; Lee and Lee 1981). Among the five methods examined, the plasma EVs isolated by PEG-3 showed higher contamination of plasma proteins (Figure 4h, i). This limitation can be mitigated by implementing additional purification steps, such as UC, to enhance the removal of plasma proteins (Rider et al. 2016). Therefore, the additional purification to remove excess plasma

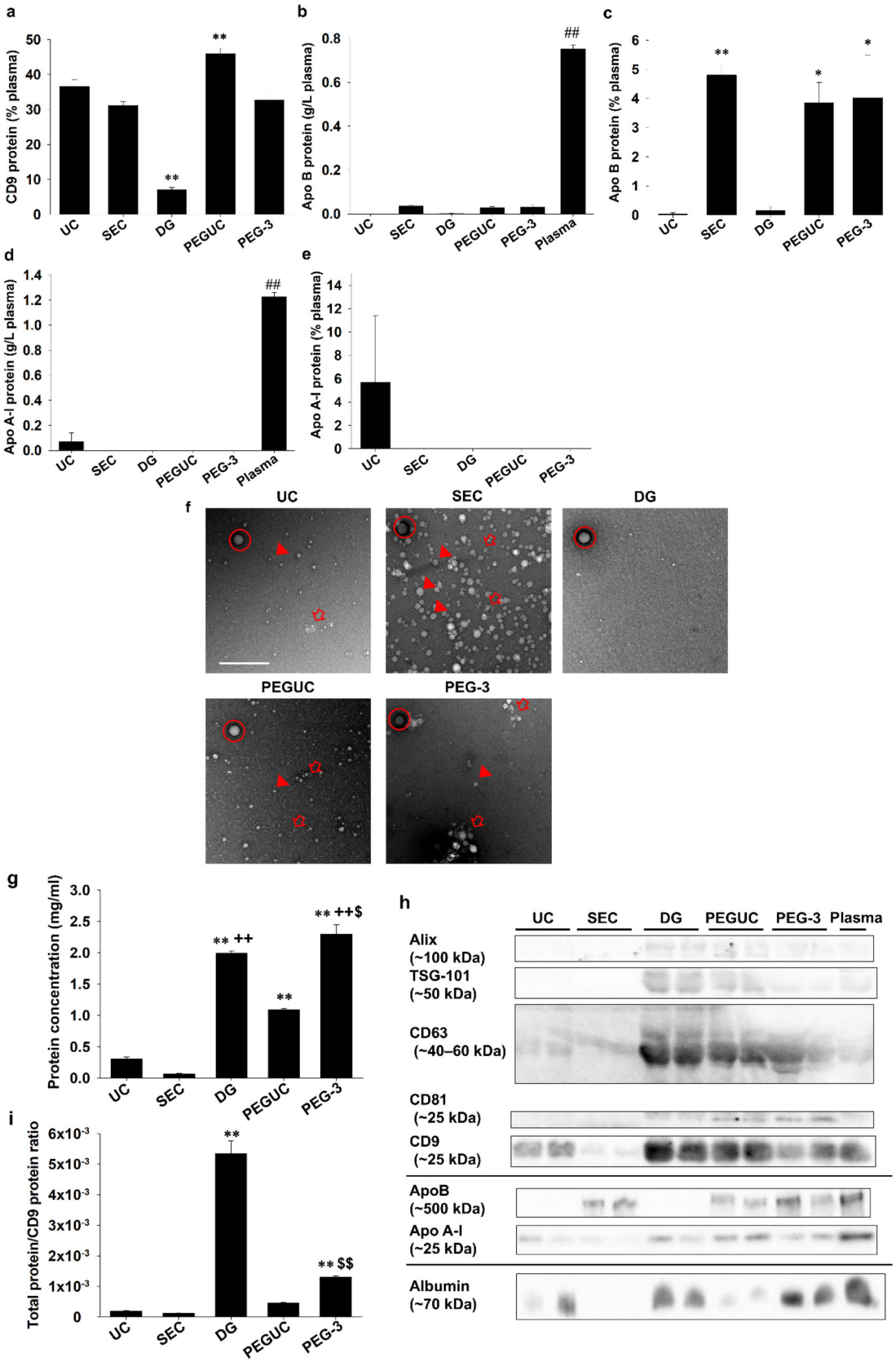

FIGURE 4 | Comparison of five isolation methods for plasma EVs in efficiency and purity. The protein expression of EV and lipoprotein marker was measured by ELISA. (a) Relative amount of CD9 protein in plasma EVs isolated by five methods. The data were normalised with the concentration of CD9 protein expression in human plasma. (b) The concentration of Apo B protein in the plasma EVs isolated by the five methods. The vertical axis shows the Apo B expression level in the fraction isolated from 1 mL of plasma. (c) The proportion of Apo B protein concentration in the plasma EVs isolated by the five methods, relative to the human plasma. (d) The concentration of Apo A-I protein in the plasma EVs isolated by the five methods. The vertical

axis shows the Apo A-I expression level in the fraction isolated from 1 mL of plasma. (e) The proportion of Apo A-I protein concentration in plasma EVs isolated by the five methods, relative to the human plasma. Results were expressed as means ± SEM in bar graphs ($n = 4$). (f) TEM images of the plasma EVs isolated by the five methods with negative staining. Circle: polystyrene beads (known diameter: 100 nm), arrowhead: chylomicrons/VLDL/LDL, arrow: EVs. Scale bar: 500 nm. (g) Total protein concentration measured by a BCA assay in the plasma EVs isolated by five methods. Results were expressed as means ± SEM in a bar graph ($n = 4$). (h) The protein expression of EV marker, lipoprotein marker and albumin in the plasma EVs isolated by five methods. The protein purified from EVs to match the original amount of plasma was loaded, and the protein expression was determined by Western blotting. (i) Total protein/CD9 protein ratio of the plasma EVs. This bar graph shows the ratio of the total protein content in plasma EVs relative to human plasma (50.17 ± 0.25 mg/mL) in relation to the concentration of CD9 protein (Figure 5a). The data were expressed as means ± SEM in a bar graph ($n = 4$). *$p < 0.05$, ** $p < 0.01$ versus UC. ##$p < 0.01$ versus UC, SEC, DG, PEGUC and PEG-3. ++$p < 0.01$ versus PEGUC. \$$p < 0.05$, \$\$$p < 0.01$ versus DG. BCA, bicinchoninic acid assay; DG, density gradient centrifugation; ELISA, enzyme-linked immuno-sorbent assay; EV, extracellular vesicle; LDL, low-density lipoprotein; PEG-3, the precipitation method with PEG (3%); PEGUC, precipitation with PEG (8%) followed by ultracentrifugation; SEC, size-exclusion chromatography; SEM, standard error of the mean; UC, ultracentrifugation; VLDL, very low-density lipoprotein.

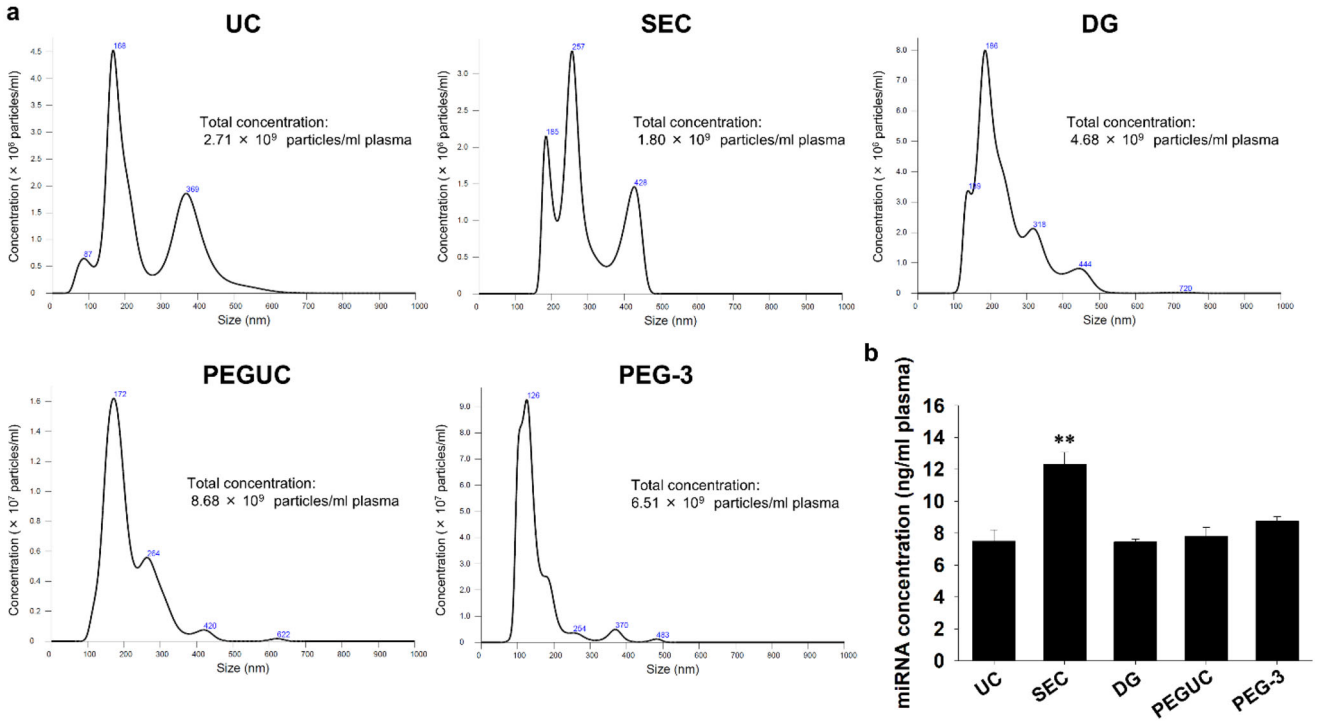

FIGURE 5 | Particle size distribution and microRNA (miRNA) concentration of the plasma EVs isolated by the 5 methods. (a) The concentration and size of plasma EVs isolated by the five methods were measured by nanoparticle tracking analysis. The sample was diluted to fall within the dynamic range of the measurement device (NanoSight LM10). Samples isolated by UC, SEC and DG were measured using a five-fold dilution, while those by PEGUC and PEG-3 were measured using a 100-fold dilution. The concentration in the original sample was calculated based on the dilution factor and presented as the total concentration ($n = 2$). (b) miRNA of the plasma EVs isolated by the five methods was purified by using miRNeasy kit, and the concentration of miRNA was measured by Qubit microRNA assay. Results were expressed as means ± SEM in bar graphs ($n = 4$). **$p < 0.01$ versus UC. DG, density gradient centrifugation; EV, extracellular vesicle; PEG-3, the precipitation method with PEG (3%); PEGUC, precipitation with PEG (8%) followed by ultracentrifugation; SEC, size-exclusion chromatography; UC, ultracentrifugation.

proteins is required after PEG-3. EVs are known to contain a disease-specific subset of miRNAs (Buffolo et al. 2022; Deng et al. 2019; Zhang et al. 2015). Enhancing the analysis of these miRNAs can be achieved by utilising the isolation method that effectively removes lipoproteins, which also contains miRNAs just from EVs (Beazer et al. 2020; Menard et al. 2018). In this regard, PEG-3 is advantageous due to its ability to remove most of the lipoproteins from plasma EVs, as well as its simplicity, brief processing time and cost-effectiveness compared to other methods. Extracellular miRNA also exists in a complex with proteins such as argonaute 2 and nucleophosmin (Arroyo et al. 2011; Wang et al. 2010). Denaturing these complexes using proteinase might eliminate this interference and improve miRNA analysis in plasma EVs.

Approaches other than the precipitation method have been explored for excluding lipoproteins so far. For example, a combination of commonly used EV isolation methods, such as UC following SEC (Karimi et al. 2018), PEG following SEC (Deregibus et al. 2016) and PEG following UC and SEC (Zhang et al. 2020), has been studied. However, these methods consist of several time-consuming procedures, resulting in decreased EV yield with each additional procedure. Another strategy employs cation-

TABLE 1 | Comparison of five isolation methods for plasma extracellular vesicles (EVs) based on required time, operation, equipment, cost, efficiency, purity and concentration.

Method		UC	SEC	DG	PEGUC	PEG-3
Required time		5 h	3 h	24 h	20 h	1.5 h
Operation		Normal	Easy	Difficult	Easy	Easy
Equipment		Ultracentrifuge	Gel and column	Ultracentrifuge	Centrifuge and ultracentrifuge	Centrifuge
Starting cost		High	Low	High	High	Low
Running cost		Low	Middle (polymer beads)	Middle (iodixanol)	Low (PEG)	Low (PEG)
EV yield in each analysis	ELISA	Middle	Middle	Low	High	Middle
	TEM	+	+	—	+	+
	Western blotting	Low	Low	High	High	High
	NTA	Low	Low	Low-middle	High	High
Efficiency (EVs)		Middle	Middle	Low-middle	High	High
Contaminant (lipoproteins)		Low	High	High	High	High
Contaminant (plasma proteins)		Low-middle	Low	High	Middle	High
Concentrate		Possible	Not	Possible	Possible	Possible

Abbreviations: DG, density gradient centrifugation; ELISA, enzyme-linked immunosorbent assay; NTA, nanoparticle tracking analysis; PEG, polyethylene glycol; PEG-3, the precipitation method with PEG (3%); PEGUC, precipitation with PEG (8%) followed by ultracentrifugation; SEC, size-exclusion chromatography; TEM, transmission electron microscopy; UC, ultracentrifugation.

exchanging resins to deplete positively charged lipoproteins from plasma EVs (Ter-Ovanesyan et al. 2023; Van Deun et al. 2020), while it is difficult to fully separate HDL from EVs due to their negative charge. Zhang et al. (2020) evaluated separating EVs and lipoproteins by agarose gel electrophoresis utilising the differences in their size and zeta potential. Nevertheless, this technique alone proved insufficient for isolating plasma EVs, requiring additional SEC using gel eluate after electrophoresis to obtain plasma EVs with minimal contamination of lipoproteins. These various isolation methods of plasma EVs devised so far to remove lipoproteins have shown that purity, yield or cost are not optimal. PEG-3 offers benefits such as simplicity, affordability and short processing time compared to these methods. However, its purity remains a challenge, indicating the need for further refinement.

In conclusion, we developed the isolation method for plasma EVs, namely PEG-3 with adequate efficiency and purity, ease of operation and low cost (Table 1). The widely used 8% PEG method resulted in plasma EVs containing significant amounts of lipoproteins. The contamination of lipoproteins in plasma EVs isolated by PEG-3 was modest, and this isolation method would be superior to the conventional methods in terms of time and cost. Therefore, PEG-3 may contribute to the application of plasma EVs for disease diagnosis if future research enables the development of a more refined method.

Author Contributions

Kosuke Otani: writing—original draft, visualisation, investigation, formal analysis, data curation, conceptualisation, funding acquisition. **Yusei Fujioka**: methodology. **Muneyoshi Okada**: supervision, methodology. **Hideyuki Yamawaki**: writing—review and editing, supervision, resources, project administration.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the authors upon reasonable request.

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