Research Paper

Polyamine-based isolation of extracellular vesicles: Examination in suspension cells and mechanistic considerations

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Abstract We previously reported a novel method for isolating extracellular vesicles (EVs) using a polyamine solution through liquid chromatography-tandem mass spectrometry analysis of the culture supernatant of adherent cells, NCI-N87, and demonstrated its efficacy from multiple perspectives. Herein, K562 cells, a suspension cell line, were used to investigate the differences between the adherent cells. Furthermore, we elucidated the mechanism of EV isolation by focusing on the proteins' isoelectric point (pI) on the EV surface.

Samples were prepared using a conditioned medium from the NCI-N87 and K562 cell lines. The conditioned medium was analyzed after replacing the medium with a serum-free medium and culturing for 48 h. The polyamine precipitation (PA) method was compared with the ultracentrifugation (UC) method.

Using K562 cells, the recovery of EVs using the PA method allowed the detection of common EV marker proteins. The gene ontology analysis also indicated that EV-related terms ranked the highest, similar to the UC method. These results demonstrate that the PA method is also effective for recovering EVs from suspended cells, such as K562 cells.

When the average pI values were calculated with the abundance and the pI values for the surface proteins in the collected EVs, the values with the PA method were lower than those with the UC method. These results suggest that electrostatic interactions bring the easy recovery of EVs with polyamines.

Key words: extracellular vesicle, liquid biopsy, liquid chromatography-tandem mass spectrometry (LC-MS/MS), isoelectric point (pI), polyamine precipitation method

1. Introduction

1.1. *Current status and issues surrounding the extracellular vesicles*

Extracellular vesicles (EVs) are membrane-bound vesicles secreted by cells, including exosomes, microvesicles, and apoptotic bodies[1)]. These vesicles play a crucial role in intercellular communication and have been reported to be useful diagnostic tools in various medical fields, including liquid biopsy[2)]. Because of their small size and low immunogenicity, EVs are also being explored for drug delivery and therapeutic applications, with great promise for practical applications in cancer, regenerative medicine, and gene therapy[3)].

In addition to their medical applications, EVs are gaining attention in agriculture, environmental science, the food industry, and cosmetics. In the food industry, there is ongoing research into developing foods that deliver nutritional

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and functional components to target areas in the body[4,5]. In the cosmetics field, the application of EVs for skin care and anti-aging is being explored, with studies examining their effectiveness in promoting skin regeneration and preventing aging, focusing on the efficacy of promoting skin regeneration and preventing aging[6,7].

Several methods have been reported and implemented to isolate EVs. Accurate isolation and characterization of EVs are critical in their analysis, and each method has its characteristics, with the choice depending on the intended purpose[8]. The ultracentrifugation (UC) method is the most frequently used EV isolation method and is considered the gold standard[9]. Although it can be used with various samples, it requires specialized equipment, and there are concerns about the purity of the isolated EVs[9].

Other methods include polymer precipitation, size-exclusion chromatography (SEC), and immunoaffinity capture (IC). Polymer precipitation is easy to perform and allows for quick recovery, but it has low purity owing to residual polymers and protein contamination[10]. SEC and IC methods produce purer EVs with fewer impurities, but they are not suitable for large sample volumes and are costly[10]. Microfluidic devices represent a novel method for the efficient and rapid isolation of EVs. These devices allow for the quick and high-precision separation of EVs through microscopic channels, yielding high-purity EVs even from small sample volumes. However, they require specialized devices, have high installation costs, and are not yet widely standardized commercially[11].

Another method involves antibodies against specific markers or lipid components on the EV surface. Magnetic bead separation is a common technique in this category, offering high specificity and the ability to isolate the target EVs selectively. However, this method is challenging in terms of cost and sample volume and cannot recover EVs that do not have specific markers[12].

Each EV isolation method has advantages and disadvantages. Obtaining sufficient amounts of EVs may be challenging, particularly when limited clinical samples are used. When isolating EVs, considering various aspects such as the sample volume, simplicity of the procedure, level of purity in the extracted EVs, and associated expenses is crucial. Additionally, employing standardized techniques is essential to guarantee consistent isolation results.

Our research focuses on a novel approach for EVs utilizing a polyamine solution called the PA method. We examined this innovative technique for EV isolation. We presented the findings from liquid chromatography-mass spectrometry (LC-MS/MS) analysis conducted on a conditioned medium obtained from the NCI-N87 adherent cell line[13].

1.2. *Outline of the assumed PA method*

An outline of the proposed PA method is shown in Fig. 1. The method comprises adding the NaCl and polyamine solution, mixing, and allowing the mixture to stand. After centrifugation, the supernatant was removed, and the samples were analyzed.

The PA method is characterized by the fact that it does not require any special equipment and can be performed using an ordinary centrifuge. Furthermore, the polyamine solution used is inexpensive and readily available.

In this study, we present the results of various analyses using K562 cells, which are floating cells, not adherent

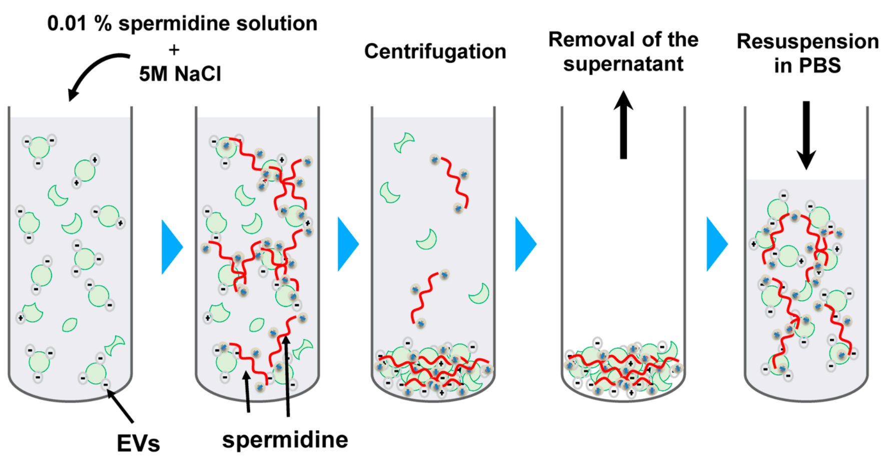

Fig. 1. Overview of the PA method.

cells, and compare them with NCI-N87. Furthermore, we focused on the reaction mechanism of the PA method, specifically examining the protein charge (pI) present in the membrane of EVs, and performed multiple studies using the pI of NCI-N87 (adherent cells) and K562 (suspended cells).

2. Materials and Methods

2.1. Cell culture

Two cell lines were utilized for LC-MS/MS analysis: NCI-N87 (ATCC, Manassas, VA, USA) and K562 (National Institute of Biomedical Innovation, Health and Nutrition, Osaka, Japan). The cells were cultured in RPMI-1640 Medium supplemented with L-Glutamine and Phenol Red (Fujifilm Wako, Tokyo, Japan), along with 10% fetal bovine serum, at 37°C in a 5% CO_2 incubator. Upon reaching confluence, the medium was replaced with an Advanced RPMI 1640 Medium (Thermo Fisher Scientific, Waltham, MA, USA). After 48 h of culture, the media were collected and subjected to initial centrifugation at 1,000 × *g* for 10 min at room temperature (20–25°C) to pellet and remove any cells. All subsequent centrifugation steps were performed at 4°C. The supernatant was then centrifuged at 2,000 × *g* for 20 min. Following centrifugation, the media supernatant was filtered through a 0.22-μm pore filter (HAWACH Scientific Co. LTD, Xian City, China). Each procedure was conducted using a prefiltered culture medium.

2.2. EVs isolation

2.2.1. Ultracentrifugation

To isolate EVs, we removed the cells and subjected the prefiltered culture supernatant_to ultracentrifugation. This procedure was performed utilizing an Optima L-90K ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) at 150,000 × *g* for 100 min with an SW28.1 swinging bucket rotor (Beckman Coulter). After the initial centrifugation, phosphate-buffered saline (PBS) was introduced into the upper portion of the centrifuge tube (17 mL, Open-Top Thin wall_Ultra-Clean Tube (Beckman Coulter), and the ultracentrifugation process was repeated under identical conditions. The supernatant was discarded, and the isolated EVs were resuspended in PBS.

2.2.2. Polyamine precipitation (PA) Method

For the PA method, the treated cell culture supernatant was transferred into a 15 mL conical centrifuge tube (Nunc, Thermo Fisher Scientific, USA). A 1/10 volume of 5 M NaCl solution and a 1/10 volume containing 0.01% spermidine (191-13831, FUJIFILM Wako Chemicals, Osaka, Japan) were added to the supernatant. The mixture was subsequently incubated for 10 min at room temperature before centrifugation at 27,210 × *g* (13,000 rpm) for 60 min in a refrigerated microcentrifuge (Model 3700; KUBOTA CORPORATION, Tokyo, Japan). The supernatant was discarded following centrifugation, and the EVs were resuspended to PBS for subsequent analysis.

2.3. Mass spectrometry

2.3.1. Sample preparation

Samples containing EVs collected using the PA or UC method were treated with single-pot solid-phase-enhanced sample preparation (SP3) technology. The protein concentration in the sample was adjusted to 1–200 μg/mL, and dithiothreitol was added to achieve a final concentration of 25 mM. The sample was subsequently incubated at room temperature for 30 min, followed by adding 1/10 volume of 500 mM 3-methyl-1-butanol and further incubation for 30 min. Subsequently, Sera-Mag™ SpeedBead Carboxylate-Modified [E3] Magnetic Particles (Global Life Sciences Technologies Japan K.K., Tokyo, Japan) were introduced into the samples at a volume of 10 μL for every 10 μg of protein. Then, ethanol was added three times to the sample volume, and the mixture was agitated at 1,000 rpm for 10 min at room temperature. The sample was washed thrice with 80% ethanol (500 μL) using a magnetic stand. In conjunction with 50 mM Tris buffer, lysyl endopeptidase was introduced (100 μL) into the washed sample, which was then sonicated and incubated at 37°C for 3 h. Trypsin was subsequently added, and the sample was mixed at 1,500 rpm at 37°C for 20 h. Following digestion, 20% trifluoroacetic acid was added to achieve a final concentration of 1/20 of the volume, and the magnetic particles were separated. The supernatant was collected, desalted using GL-Tip SDB (GL Sciences Inc., Tokyo, Japan), vacuum-dried, and resuspended in 0.1% trifluoroacetic acid with 2% acetonitrile to facilitate peptide extraction.

2.3.2. LC-MS/MS analysis

LC-MS/MS analysis was conducted using a PAL System autosampler (CTC Analytics AG, Zwingen, Switzerland) and a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific). The peptides were concentrated on a C18 trap column (5-μm particle size, 300 μm inner diameter, 5 mm length; Chemical Evaluation and Research Institute, Tokyo, Japan) and subsequently separated on a C18 analytical column (3-μm particle size, 100-μm inner diameter, 125 mm length; Nikkyo Technos, Tokyo, Japan) at a flow rate of 0.5 μL/min, with an injection volume of 7.5 μL. The mobile phases were solvent A (0.5% acetic acid) and solvent B (0.5% acetic acid in 80% acetonitrile). The gradient elution for solvent B commenced at 5%, increased to 40% over 100 min, increased to 95% over 1 min, maintained at 95% for 3 min, returned to 5% over 1 min, and equilibrated at 5% for 10 min. Electrospray ionization was performed in positive ion mode. The mass spectrometer was operated in data-dependent acquisition (DDA) mode. The peptide spectra were acquired using Xcalibur 4.1.50 software (Thermo Fisher Scientific). Full scans were recorded from 350 to 1,800 *m/z* at a resolution of 17,500, with an automatic gain control (AGC) target of 3×10^6 and a maximum injection time of 60 ms. MS/MS scans were performed at a resolution of 35,000 with an AGC target of 1×10^5 and a maximum injection time of 60 ms. The 10 most intense precursor ions were selected for MS/MS analysis in the quadrupole with an isolation window of 2.0 *m/z* and fragmented via higher-energy collisional dissociation (HCD) using a normalized collision energy of 27%. Multiply-charged peptides were prioritized for MS/MS with a dynamic exclusion set to 20 s.

2.3.3. Data analysis

The protein sequence database used for the analysis was UniProt Homo sapiens (TaxID=9,606), downloaded on September 24, 2022, in conjunction with the cRAP contaminant database (http://www.thegpm.org/crap/). Proteome Discoverer 2.5.0.400 (Thermo Fisher Scientific) was employed for data analysis, and SEQUEST (Thermo Fisher Scientific) was utilized for database searches. The search parameters were configured to allow for two missed cleavage sites with a peptide mass tolerance of 10 ppm and a fragment mass tolerance of 0.02 Da. Fixed modifications included the carbamidomethylation of cysteine, whereas variable modifications included methionine oxidation and N-terminal acetylation. Peptide identifications were filtered using significant Xcorr values with a false discovery rate of 1% based on the Percolator node in Proteome Discoverer. The mass spectrometry data were deposited in the jPOST[14] repository (project ID: JPST001807, accession ID: PXD PXD035832 for NCI-N87; project ID: JPST003429, accession ID: PXD056938 for K562).

2.4. *Analysis of the K562 cells culture supernatant*

Furthermore, proteins specified in the MISEV2023 guidelines[15] were evaluated for their presence in the sample, and significant peptide spectrum matches (PSMs) were identified. A comparative analysis between the PA and UC methods was carried out using total ion chromatograms, diagrams, and volcano plots to assess the recovered peptides' concordance rates and distinctive characteristics.

Volcano plot analysis was conducted on the proteins obtained using the PA and UC methods. The tetraspanin markers CD9, 63, 81, and Flotillin 1, which are frequently utilized in EV analysis, were examined. Additionally, four markers, actin, EMMPRIN, HSPA8, and HAP90AB1, were selected from the proteins enumerated in MISEV2023 that exhibited PSMs ≥ 4 using both methods and were subsequently analyzed. Gene Ontology (GO) analysis for cellular components was performed on proteins detected using both methods, focusing on PSMs with a mean of 4 or higher. The analysis was conducted using the DAVID program (v2024q1)[16].

Among the proteins detected, the correlation between the two methods was examined for those with PSMs greater than 4.0.

2.5. *Analysis using the pI of EVs membrane proteins*

This study focused on the analysis of proteins localized to the EVs membrane. The target proteins were aggregated and cataloged based on information about the EV membrane surface utilizing GO terms from Metascape[17]. Consequently, 57 proteins were selected for analysis. The cumulative abundance values of these proteins were calculated for pI ranges from 4.5 to 11.5 in increments of 1.0. Furthermore, for proteins detected using both the PA and UC methods with PSMs ≥ 4, the cumulative values of their pI and abundance were calculated using the following formula:

$$\frac{\sum_{pI=4}^{12}(pI \times Abundance_{pI})}{\sum_{pI=4}^{12}(Abundance_{pI})}$$

The sum of the product of pI and the abundance for each detected protein was subsequently indexed by dividing the sum of the pI and the abundance by the sum of the abundance.

3. Results

3.1. *Performance evaluation of the PA method using K562 cells*

Table S1 summarizes the protein data obtained with both methods using the K562 cell content detection data as the protein content-EVs characteristics for PSMs greater than 4. With the PSMs using K562, the correlation coefficient for both methods was R^2=0.646, and the overall correlation was positive, as shown in Fig. 2.

Fig. 3 shows the total ion chromatograms using K562. Each method showed a different profile even though the same samples were treated using the PA and UC methods.

A volcano plot obtained using the K562 cells is shown in Fig. 4. The results for the examined tetraspanin markers and other markers are shown. Table S2 lists the proteins used in the analysis, including the quantitative information.

In the GO analysis, EV markers, including exosomes, accounted for the highest annotation cluster and were enriched; enrichment scores for the PA and UC methods were 171.7 and 176.5, respectively (Table S3).

3.2. *Performance evaluation of the PA method using K562 cells*

The membrane proteins selected for analysis are listed in Table S4. The pI values were 6.30±1.20. The correlation between the cumulative abundance values and pI for NCI-N87 is shown in Fig. 5(a) and for K562 in Fig. 5(b). The comparison of the total abundance was different between NCI-N87 and K562 cells. However, when comparing the average cumulative pI values at pI ≥ 4, K562 cells exhibited 6.05 for the PA method and 6.35 for the UC method, whereas NCI-N87 cells displayed values of 6.18, PA method, and 7.02, respectively. For the UC method, when pI was 4 or higher, the values for K562 cells were 6.08 and 6.37, and for NCI-N87 cells, the values were 5.83 and 7.13. When considering both methods with pI values of 4 or higher, the values for K562 cells were 6.07 for the PA method and 6.35 for the UC method, and for NCI-N87 cells, the values were 5.80 for the PA method and 7.10 for the UC method. In all the cases, the PA method yielded lower values.

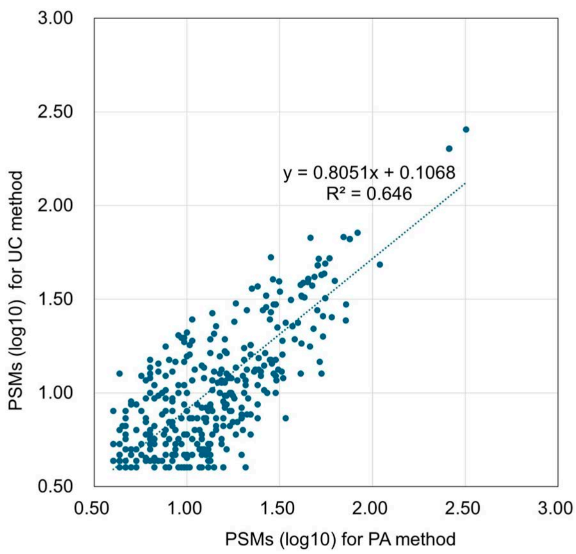

Fig. 2. Relationship between the PA method and the UC method for PSMs using K562.
The x-axis represents the PA method, and the y-axis represents the UC method.

4. Discussion

Herein, we investigated the recovery of EVs using the PA method and their protein composition using LC-MS/MS in the suspension cell line K562. Initially, as observed with NCI-N87, an adherent cell line, the ion chromatograms obtained from K562 cells showed differences between the two methods. Volcano Plot analysis revealed that, unlike NCI-N87, the EV markers with the PA method were less than those with the UC method. This discrepancy may be attributed to the influence of debris generated during the suspension cell culture process, in addition to the presence of a specific factor related to K562 cells, which was the subject of this investigation.

In the GO analysis, analogous to the results obtained using NCI-N87, the terms associated with EVs demonstrated the highest rank, as observed in the UC method. Additionally, the two methods positively correlated with the proteins detected with PSMs ≥ 4.0. These findings suggest that the PA method could be used to recover EVs. On the other hand, the relationship between pI and cumulative abundance for surface proteins in collected EVs from NCI-N87 (Fig. 5(a)) and K562 (Fig. 5(b)) revealed that the averages of pI values with the PA method were lower than those with the UC method. These observations indicate that polyanionic EVs may aggregate through electrostatic interactions with polyamines, which are polycations, and that the PA method can recover a protein population specific to EVs compared with the conventional UC method. Notably, this method is particularly effective for analyzing EV proteins in the low-pI range.

EVs in liquid biopsies can be important information

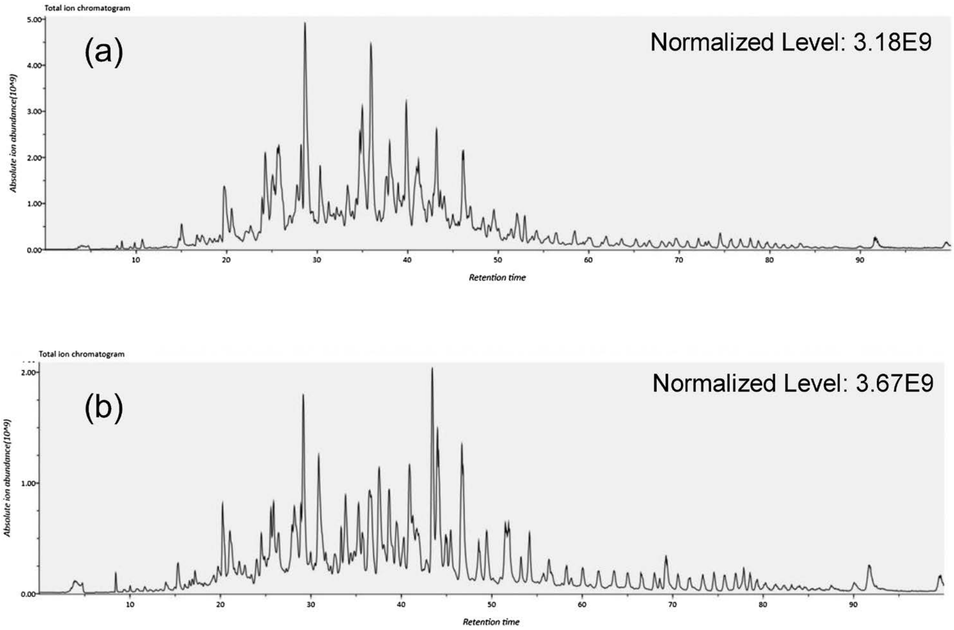

Fig. 3. Comparison of the PA method and UC method using the total ion chromatogram using K562.
(a) PA method, (b) UC method.

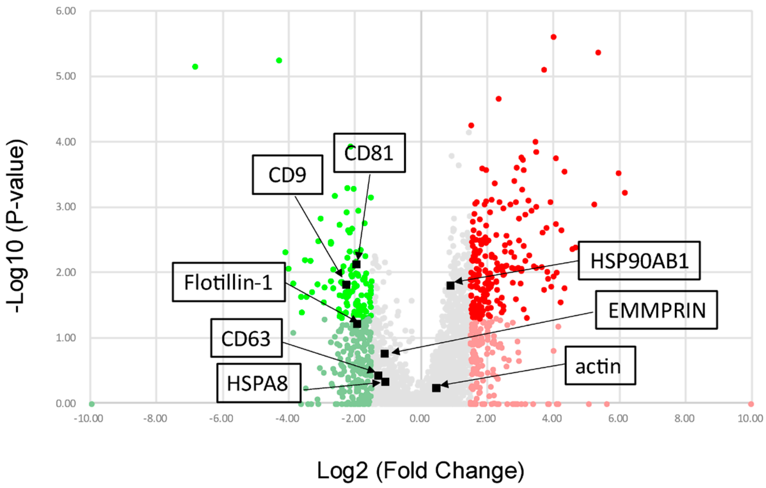

Fig. 4. Volcano plot for each method.
Marked for proteins in EVs at K562. Created using abundance data obtained by LC-MS/MS. The x-axis indicates the log2 fold change for each gene, and the y axis indicates the log10 p-value. Red dots indicate proteins with p-values <0.05, and green dots indicate proteins with p-values ≥ 0.05. The significance of differences was evaluated using t-test, with p-value <0.05 being significant.

sources for cancer diagnosis and treatment monitoring[18]. Tumor-derived EVs often contain tumor-specific biomarkers that aid in tracking cancer progression and treatment response[19]. Furthermore, the stability of EVs as biomarkers is notable because their lipid bilayer protects their contents, making them suitable for analysis[20]. Analysis of the contents of EVs can reveal the genetic characteristics and molecular mechanisms of cancer, thereby contributing to personalized medicine. Thus, EVs are expected to be valuable tools in liquid biopsies for cancer diagnosis and treatment monitoring.

To investigate the reaction mechanism of the PA method, we focused on the pI and found that the PA method recovered proteins with lower pI values than the UC method.

Exosomes in EVs are surrounded by a phospholipid bilayer containing negatively charged phosphate groups[21]. These phosphates provide the exosome surface with a negative charge[22, 23]. In addition, surface proteins and glycans on exosomal membranes contribute to the anionic nature of exosomes[24]. Exosomes play a role in intercellular communication, and their negative charge facilitates interactions with cationic (positively charged) regions on the recipient

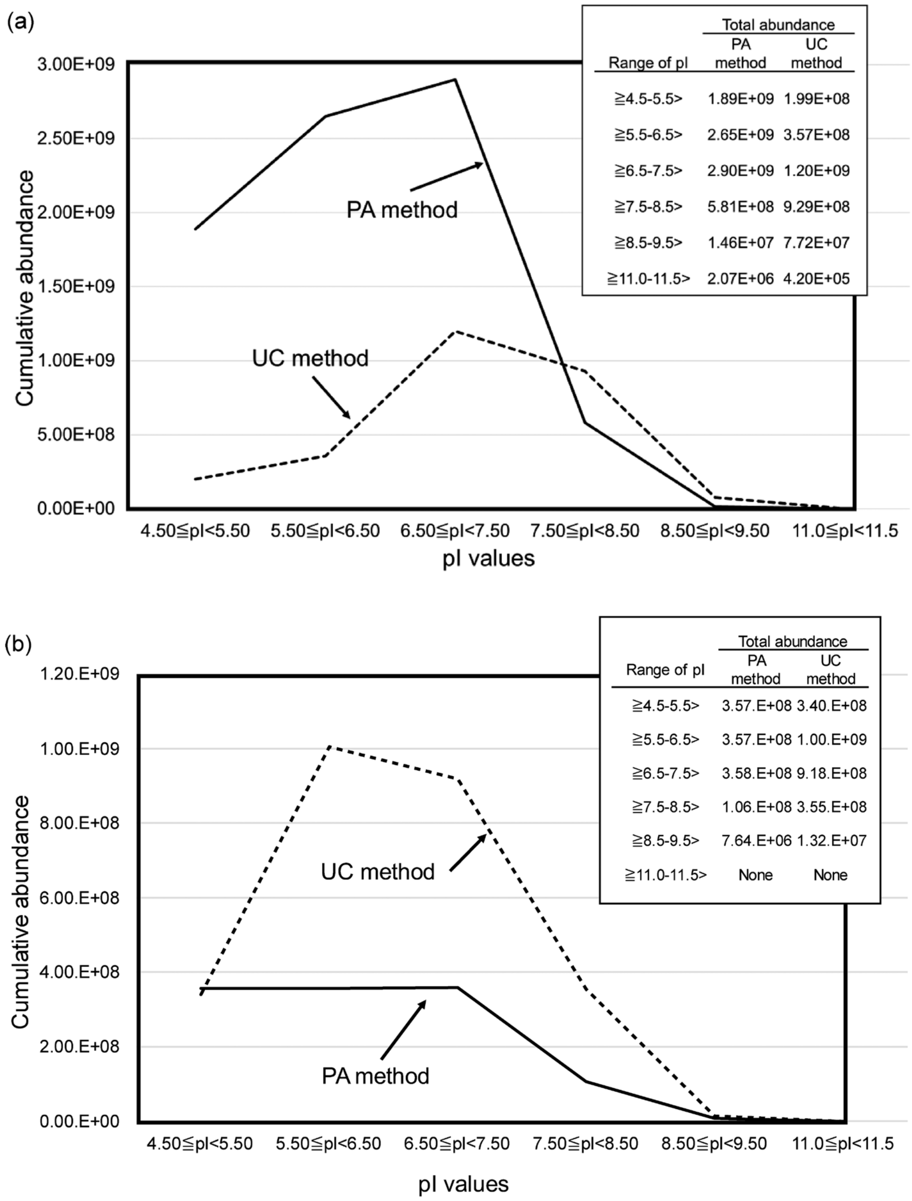

Fig. 5. Relationship between pI and cumulative abundance for surface proteins in collected EVs.
(a) NCI-N87, (b) K562.
The y-axis represents the cumulative abundance, and the x-axis represents the pI value, which was set in the range of 4.5 to 11.5.

cell surfaces[25].

The PA method is based on the electrical properties of the polyamines. In other words, an ion complex considerably forms between EVs and polyamines to form a coacervate[26]. Various free proteins in the culture medium are also assumed to form ion complexes with polyamines because they exist in a random coil state and have a negative charge. We consider that concerning the proteins detected by the UC method, a high concentration of NaCl in the reaction solution prevents coacervate formation in those with small particle sizes and low charge densities. However, further studies are required to confirm these findings.

While this study was a basic examination using culture supernatants, we aimed to conduct further research using clinical specimens to explore the potential for the clinical application of the PA method.

Conclusions

A study using the culture supernatant from the suspension cell line K562 and analyzed by LC-MS/MS demonstrated that the PA method is a practical approach for EV recovery. Analysis of the pI suggests that the interaction occurs because of the charge properties of the polyamines and EVs.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Supplementary material: additional data and analysis

Table S1. Protein content using the PA and UC methods analyzed by LC-MS/MS

PA method (a) and (b) UC method. The analysis was conducted in triplicate (N=3), and the list was composed of PSMs (peptide spectrum matches) with an average value of 4 or higher across any of the methods. Data are presented as means with standard deviations.

Table S2. List of proteins used in the volcano plot analysis

A. All proteins
B. Down regulated proteins
C. Up regulated proteins

Table S3. gene ontology (GO) classification

A. PA method
B. UC method

Table S4. Proteins on the membrane of the selected EVs

Gene symbol, accession number, and pI value are shown.

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