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RESEARCH PAPER

Facile and simple purification method for small extracellular vesicles obtained from a culture medium through cationic particle capture

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Abstract

Although small extracellular vesicles (sEVs) carry DNA, miRNA, and proteins, and they play an important role in long-distance intercellular communication, their generation and circulation mechanisms are unclear. sEVs can be used as biomarkers for the early diagnosis of diseases (e.g., cancer, Alzheimer's disease, melanoma, and cardiovascular diseases) and as drug delivery carriers to the target tissues. Hence, sEVs are attracting considerable attention from scientists and medical professionals. In the present study, we investigated four different commercially available cationic particles (two silica particles modified with diethylaminopropyl or trimethylaminopropyl groups, and two agarose particles modified with diethylaminopropyl or trimethylaminopropyl groups) for the purification of sEVs obtained from a cell culture medium. All the cationic particles captured the sEVs well. The NaCl concentrations required for elution of the captured sEVs differed for the different cationic particles. sEVs were most efficiently captured by silica particles modified with diethylaminopropyl groups, and they were eluted from these particles using 200 mM NaCl as the elution solution. Because the developed method can be used to easily purify sEVs obtained from a culture medium, it is expected to facilitate the functional analysis of sEVs, as well as early diagnosis and treatment of diseases using sEVs.

Keywords Small extracellular vesicles · Purification · Culture medium · Cationic particles

Introduction

Small extracellular vesicles (sEVs) are 30–200 nm sized phospholipid-bilayer membranous structures secreted from various cells (e.g., mammalian, invertebrate, and plant cells) [1–3]. They are also present in many biological fluids such as blood, urine, saliva, and milk [4, 5]. sEVs carry many biological substances, including DNA, miRNA, mRNA, and proteins, and are responsible for long-distance intercellular communication [2, 6]. sEVs are delivered to the target cell by the circulatory system of the body (i.e., the blood stream). Because the sEVs secreted from diseased cells contain various disease biomarkers, they can be used for the early diagnosis of many diseases, thereby increasing the possibility of a complete cure [7–9]. Furthermore, because sEVs can deliver inclusion substances (miRNA and proteins) to target cells deep inside the body, they can be used as excellent drug carriers [10, 11]. Although sEVs play a crucial role in vivo and are expected to be used for diagnostics and therapy, they are not yet very well understood; for example, the mechanisms of their generation and circulation have yet to be determined. Furthermore, large amounts of sEVs are required for their application in diagnosis and therapy. Hence, it is necessary to develop a purification technique for sEVs that can provide large amounts of sEVs quickly and easily.

The first technique developed for the purification of sEVs is ultracentrifugation [12]. However, this is a time-consuming method because it requires multiple centrifugation and ultracentrifugation steps. Size exclusion chromatography, polymer precipitation, and immunoaffinity capture have also been used as purification techniques [13–15]. Recently, a purification technique using agarose gel electrophoresis has been developed [16]. However, these techniques are only applicable to small amounts of material, so facile purification techniques to obtain sEVs in large amounts are still needed.

Functional particles are an efficient tool for isolating target compounds from large volumes of solution [17, 18]. The target in the solution is captured by the particles through mixing,

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and the particles are then recovered using gravity or a magnetic force [19–21]. The purified target is obtained by detaching the particles by changing the components of the elution solution. To capture sEVs, magnetic beads labeled with T-cell immunoglobulin and mucin domain-containing protein 4, which show an affinity for phosphatidylserine present on the sEV surface, have been developed and are commercially available [22, 23].

Because the sEVs have a negative zeta potential, it is believed that the cationic particles capture sEVs by electrostatic interactions. The captured sEVs are detached from the particles by adjusting the salt concentration of the elution solution. The salt concentration required for detaching the target sEVs depends on the strength of the affinity between the sEVs and the particle. Hence, a purification technique for sEVs obtained from a biological solution can be developed by considering the cationic particles and ionic concentration of the elution solution together. The preparation of cationic particles is inexpensive. They can also be stored for a long period of time and are suitable for application in purification techniques. Furthermore, because different types of cationic particles (based on functional groups, base materials, and size) are commercially available, they can be used for the purification of sEVs. Hence, in this study, we attempted to develop a purification technique for sEVs obtained from a cell culture medium using commercially available cationic particles.

Experimental

Chemicals

Cationic silica particles (Wakogel® 50DEA and 50SAX), sodium chloride, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sulfuric acid, 3,3′,5,5′-tetramethylbenzidine (TMB) solution (for microwell plate), albumin from bovine serum (BSA), and cohn fraction V pH 7.0 were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). DEAE sephacel® and Q sepharose® fast flow were obtained from Cytiva (Tokyo, Japan). Anti-mouse IgG antibody and horseradish peroxidase (HRP) were obtained from Vector Laboratories, Inc. (Burlingame, CA, USA). Anti CD9 and Anti CD63 were obtained from Cosmo Bio Co., Ltd. (Tokyo, Japan). Gibco DMEM (Dulbecco's modified Eagle medium) and Gibco FBS (fetal bovine serum) were obtained from Thermo Fisher Scientific K.K. (Waltham, MA, USA).

sEV purification from the culture medium using cationic particles

A culture medium comprised of DMEM medium containing 10% FBS was used to culture Hela cells (American Type Culture Collection, Manassas, VA, USA) for 3 days. No further purification, freezing, or long-term storage of the culture medium was done. Then, 100 mg of cationic particles and 300 μL of the culture medium were added to an Eppendorf tube and mixed using a vortex mixer. A mixture of 100 mg of cationic particles and 300 μL of 15 mg/mL albumin solution was prepared for examination of the elution fraction of albumin. As a washing step, the following procedures were performed four times. The particles were washed using 100 μL of water and centrifuged in a low speed centrifuge, and the resulting supernatant was discarded. The sediment was eluted with a NaCl solution (100 μL). The NaCl concentration in the elution solution was increased in the order 25, 50, 100, 200, 1000, and 5000 mM to determine the concentration required for eluting sEVs from each particle. The washing time for each solution was approximately 15 s. The supernatant was used for further analysis.

sEVs purification from the culture medium using a commercially available kit

Purification of the culture medium was performed according to the manufacturer's protocol. Briefly, magnetic beads for exosome purification were prepared by mixing 60 μL of streptavidin-modified magnetic beads and 10 μL of biotin-labeled exosome capture reagent. The modified beads were added to the cell culture medium and mixed for 3 h with a rotator at 4 °C. Then, the beads were washed three times by 1 mL of washing buffer and the captured sEVs were detached by using 50 μL of exosome elution buffer.

Measurement of loading index (LI) and size distribution using dynamic light scattering (DLS)

The Nanotrac Wave DLS instrument (Microtrac BEL Corp., Osaka, Japan) employed in this study was previously described elsewhere [24]. All measurements were carried out at room temperature (approximately 20 °C) using a 780-nm laser beam. A minimum of three replicate measurements was performed for each sample, and sample sizes of 20 μL were employed throughout. We used LI, which is an instrument-specific parameter based on the scattering intensity, to determine the nanoparticle concentration [24]. The logarithm of LI is proportional to the logarithm of the nanoparticle concentration.

Protein measurement by the BCA method

The sample solution (30 μL) and BCA reagent (a mixture of Pierce BCA protein assay reagents A and B in a 50:1 ratio, 200 μL) were dispensed into a 96-well plate and mixed thoroughly on a plate shaker for 30 s. The plate was incubated at 37 °C for 30 min and cooled to room temperature

(approximately 20 °C). The absorbance at 562 nm was measured by a multiplate reader (SH-9000, Corona Electric Co., Ibaraki, Japan).

CD9 and CD63 analyses

First, 100 mg of 50DEA was added to 300 μL of a culture medium to capture the EVs. A mixture of 100 mg of 50DEA and 300 μL of water was used as a control. Each solution was incubated for 30 min at room temperature (approximately 20 °C) after adding 100 μL of 1 mM BSA, and then washed five times using 300 μL of 25 mM phosphate buffer (pH 7). Next, 100 μL of an antibody solution (10 mM each of CD63 and CD9) was added and the mixture was incubated for another 30 min. The obtained mixture was washed five times with the phosphate buffer. The solution was incubated for an additional 30 min after adding 100 μL of an HRP solution (4000× dilution for CD9 and 2000× dilution for CD63) and 300 μL of the phosphate buffer. Then, the solution was washed five times with the phosphate buffer, after which 200 μL of TMB and 100 μL of the phosphate buffer were added to the solution and the mixture was incubated for 30 min. Finally, 100 μL of 500 mM sulfuric acid was added to the sample solution to terminate the reaction. The sample solution (200 μL) was dispensed into the 96-well plate and mixed thoroughly on a plate shaker for 30 min. The absorbance at 450 nm was measured by a multiplate reader.

sEV counting

Counting of the vesicle was performed using the ZetaView (Particle Metrix, Meerbusch, Germany). Each sample was diluted 500 or 1000 times with 50 mM NaCl solution before measurement. The sample solution was loaded into the cell that was adjusted at 25 °C and the instrument measured each sample at 11 different positions throughout the cell.

Results and discussion

Four different particles (Wakogel® 50DEA, Wakogel® 50SAX, DEAE Sephacel®, and Q Sepharose® fast flow) were used for capturing EVs from a culture medium. The captured EVs were then released by sequentially adding different concentrations of NaCl solution (25, 50, 100, 200, 1000, and 5000 mM). DLS is a popular method for measuring the size of particles varying from several tens of nanometers to several micrometers. The presence of particles of these sizes was evaluated by a LI calculated using the scattering intensity [24]. The LI of each NaCl solution was analyzed by DLS; the results are presented in Fig. 1. When 50DEA particles were used, EVs were observed using 25–200 mM NaCl as the elution solution; however, no EVs were observed for elution solutions of 1000 and 5000 mM NaCl. Using 50SAX particles, EVs were observed using 25 mM NaCl as the elution solution. In contrast to the 50DEA particles, EVs were not observed using 50SAX particles when 50 and 100 mM NaCl solutions were used for elution; however, EVs were again observed when 200–5000 mM NaCl was used as the elution solution. With DEAE and Q particles, no EVs were observed when 25–100 mM NaCl was used for elution. In contrast, EVs were observed for these particles when >200 mM NaCl was used as the elution solution. The strongest signal was detected when the EVs were captured by 50SAX particles and released by 5000 mM NaCl. Although the 50DEA and DEAE particles were modified by diethylaminopropyl groups, and the 50SAX and Q particles were modified by trimethylaminopropyl groups, NaCl solutions of the same concentration were required for eluting the EVs captured using DEAE and Q particles. This result indicates that the properties of the particles are also influenced by factors other than the surface group.

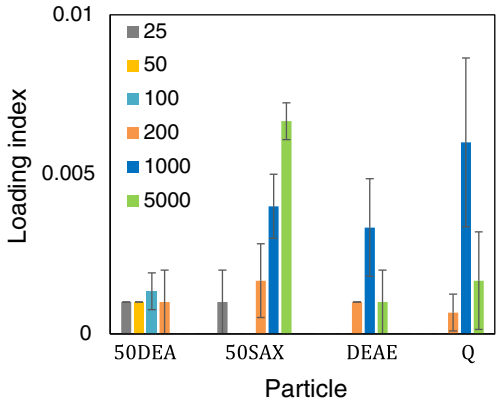

Fig. 1 Loading index of EVs captured by four particles (50DEA, 50SAX, DEAE, and Q) and eluted by six NaCl solutions (25, 50, 100, 200, 1000, and 5000 mM)

Next, the size of the released vesicles in each elution solution was analyzed using DLS (Fig. 2). Figure 2a shows the size distribution of vesicles in the culture medium before the EVs were captured by the particles. Vesicles of different sizes were observed, with ~10 nm being the most common vesicle size. EVs of sizes varying from 100 to 1000 nm were observed in the elution solution from the 50DEA particles. The sizes of the released EVs were almost constant, regardless of the NaCl concentration. The size distribution of the EVs became narrower on increasing the NaCl concentration of the elution solution, and the narrowest size distribution was observed when the NaCl concentration was 200 mM. EVs of sizes varying from 100 to 1000 nm were also present in the elution

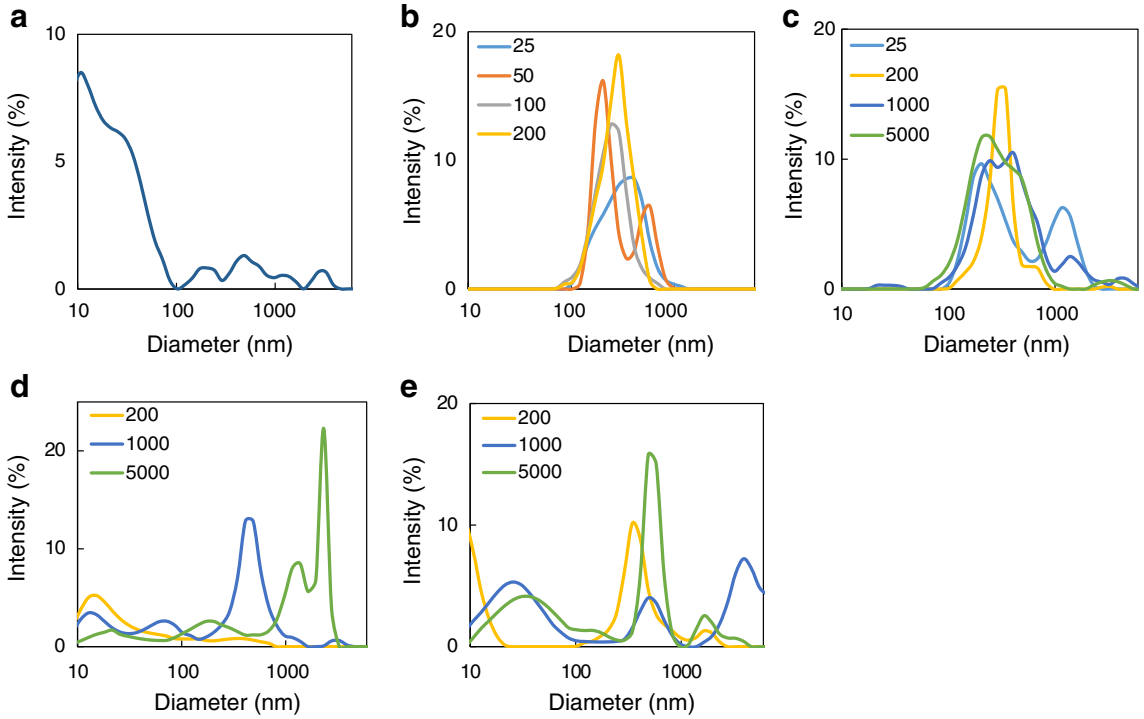

Fig. 2 Size distribution of EVs captured by four particles (50DEA, 50SAX, DEAE, and Q) and eluted by six NaCl solutions (25, 50, 100, 200, 1000, and 5000 mM). EVs captured using **a** no particles, **b** 50DEA, **c** 50SAX, **d** DEAE, and **e** Q particles

solution from the 50SAX particles. Vesicles larger than 1000 nm were observed when 25 or 1000 mM NaCl was used for elution. The size distribution became the narrowest when the NaCl concentration was 200 mM; a similar tendency was observed for the 50DEA particles. EVs of three different sizes were present in the elution solution from the DEAE particles. Most of the released EVs were a few tens of nanometers, 500 nm, and a few micrometers in size when the concentrations of the elution solution used were 200, 1000, and 5000 mM, respectively. Hence, the EV size increases with the increase in NaCl concentration. EVs with three different sizes (a few tens of nm, 500 nm, and more than 1 μm) were present in the elution solution from the Q particles. Although the size of the main peaks changed on varying the NaCl concentration, the average size of the vesicles in each solution remained constant at approximately 600 nm. These results indicate that different EVs can be obtained by changing the type of cationic particles and the concentration of NaCl.

The culture medium contains not only EVs but also proteins and lipids. Hence, it is important to perform purification of sEVs by removing these components from the medium. However, it is very difficult to separate proteins, a major component of the medium. The protein concentration of each elution was measured by the BCA method, a popular protein measurement method. Figure 3 shows the amount of protein in each elution solution. All elution solutions contained protein when 50DEA particles were used to capture the sEVs; however, the amount of protein in each fraction was very low. Similarly, very low amounts of proteins were detected from all elution solutions when 50SAX particles were used. Although a small amount of protein was eluted when DEAE and Q particles were used in the presence of <100 mM NaCl

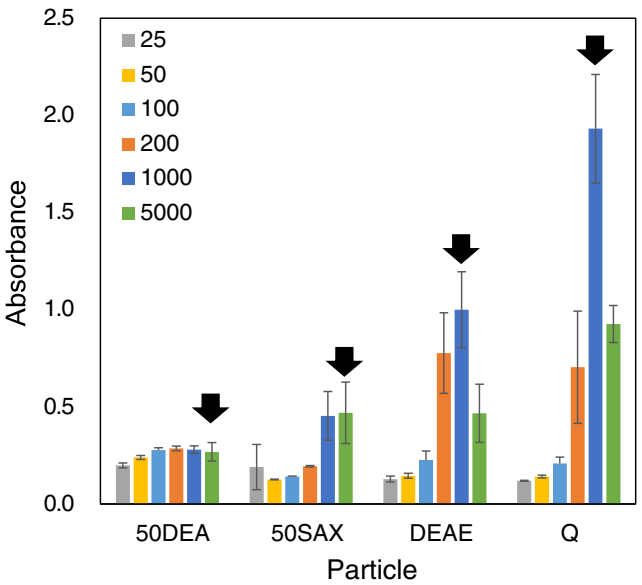

Fig. 3 Amount of protein captured by four particles (50DEA, 50SAX, DEAE, and Q) and eluted by six NaCl solutions (25, 50, 100, 200, 1000, and 5000 mM). The arrows indicate the maximum albumin elution

solution, a large amount of protein was eluted when the solution was >200 mM NaCl. Among the four particle types, the highest amount of the protein was released when Q particles were used.

Albumin is the most abundant protein in the culture medium [25]. Hence, its separation is important for the purification of sEVs. We examined the concentration of NaCl necessary for the elution of the captured albumin from the cationic particles. The 50SAX particles captured hardly any albumin. A small amount of albumin was detected in the elution solution from 50DEA and 50SAX particles when 5000 mM NaCl was used. The captured albumin was also eluted from the Q and DEAE particles when 1000 mM of NaCl was used. The arrows in Fig. 3 indicate the NaCl concentration required for elution of the largest amount of albumin from each particle. The NaCl concentrations required for EV elution and albumin elution were the same when 50SAX, DEAE, and Q particles were used. In contrast, the NaCl concentrations for the elution of EVs (200 mM NaCl) and albumin (5000 mM NaCl) were different when the 50DEA particles were used. Therefore, it is expected that albumin can be easily removed from EVs when 50DEA particles are used.

Next, the property of EVs captured by 50DEA particles and eluted by 200 mM NaCl was examined. Because the size of the sEVs is approximately 200 nm and is similar to that of an exosome, the presence of marker molecules (CD9 and CD63) for exosomes was examined [26]. The signals of both the marker molecules were compared for particles with and without sEV capturing. Particles with captured sEVs showed a higher signal intensity (Fig. 4).

Finally, we compared the sizes and size distributions of the EVs obtained from the same medium using the 50DEA particles and a commercially available purification kit for exosomes (MagCapture® Exosome Isolation Kit PS). The size distribution of the EVs purified by the cationic particles and that by the commercially available kit overlapped as

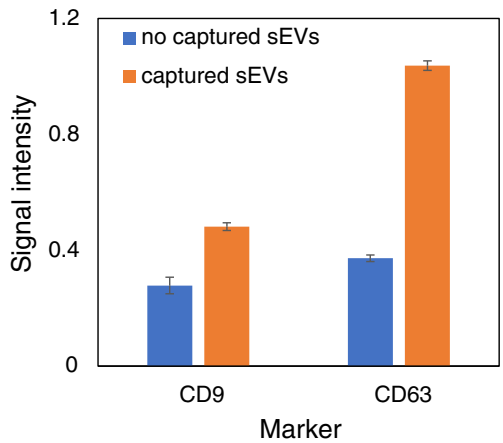

Fig. 4 Detection of marker molecules (CD9 and CD63) from the EVs captured by 50DEA particles. The amounts of marker molecules detected were compared for particles with and without sEV capturing

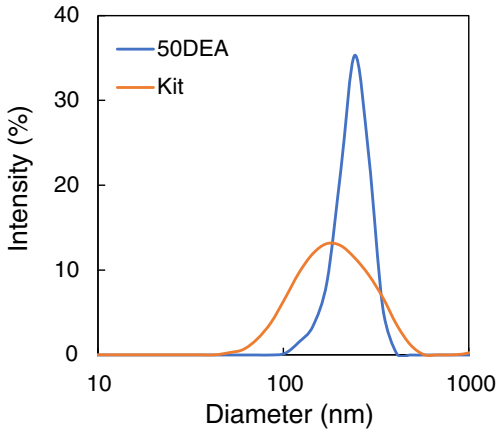

Fig. 5 Size distributions of EVs obtained using 50DEA particles and a commercially available kit (MagCapture® Exosome Isolation Kit PS)

determined by DLS analysis (Fig. 5). The purified EVs obtained using 50DEA particles and 200 mM NaCl solution were thought to be sEVs (exosomes) because the EVs contained marker molecules for exosomes, and the size distribution of the EVs was similar to that of exozomes purified by the kit. Because the size distribution of the EVs purified by the particles was narrower than that obtained by the kit, sEVs with a more homogeneous size distribution were obtained by the particles. It was supposed that the difference in size distribution was derived from the chemical structure difference in the capturing site of the vesicles by the commercial kit (phosphatidylserine) and the developed method (negative charge). Because approximately 4×10^8 particles were obtained from 300 μL of the culture medium, the recoveries of this method were similar to those of other purification methods [22]. Therefore, the developed method that uses 50DEA particles and a 200 mM NaCl elution solution is suitable for the purification of sEVs (exosomes) from a culture medium. Because the 50DEA particles are prepared by modifying silica particles with aminopropyl groups, the particles are fairly inexpensive and remain highly stable for a long period of time. Therefore, the 50DEA particles are suitable for the extraction of sEVs from a culture medium.

Conclusions

A facile and simple purification method for sEVs was developed using a combination of cationic particles and NaCl solution for elution. sEVs were most efficiently captured by silica particles modified with diethylaminopropyl groups, and they were eluted from these particles using 200 mM NaCl as the elution solution. We successfully purified the sEVs obtained from a culture medium using the developed method. This method is expected to aid in the research of sEV functions and the application of sEVs to disease diagnosis and treatment.

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Declarations

Conflict of interest The authors declare no competing interests.

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