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The Effect of Growth Stage and Isolation Method on Properties of ClearColi™ Outer Membrane Vesicles (OMVs)

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Abstract

Outer membrane vesicles (OMVs) are nanosized spherical blebs derived from the outer membrane of gram-negative bacteria. Outer membrane vesicles (OMVs) play important roles in various physiological functions of bacteria. They can be applied as native vaccines or vaccine adjuvants. The objective of this study was to determine the appropriate growth phase and isolation method for OMV separation from ClearColi™, an endotoxin-free strain of *E. coli*. It was demonstrated that the yield of OMVs is increased while the bacteria are growing. Herein, although total protein concentration of OMVs isolated from the stationary phase is more than other phases; the pre-stationary phase was selected for OMV isolation due to release of smaller size of OMVs as compared to other phases. In the current study, to obtain OMVs with high yield, proper size, and homogeneity, different concentration methods including protein precipitation by ammonium sulfate (AS) and ultrafiltration (UF) were combined to ultracentrifugation (UC) or precipitation-based exosome isolation kit. Among the examined isolation methods, AS (70%) + UC resulted in the highest yield of OMVs. The TEM results demonstrated bilayer round-shaped OMVs isolated by this method. Although AS (70%) + kit resulted in more heterogeneous in size and larger OMVs as compared to AS (70%) + UC, it is applicable when high yield of OMVs is required and UC is not available. Totally, isolation of ClearColi™ OMVs from pre-stationary phase using AS (70%) + UC with enhanced yield can be applied in vaccine research studies.

Introduction

Outer membrane vesicles (OMVs) are a kind of globular nanostructures that generally bud from the membrane of gram-negative bacteria and are classified as a subcategory of extracellular vesicles (EVs) [1, 2]. These vesicles are composed of many bacterial ingredients, like DNA, RNA, lipopolysaccharide (LPS), enzymes, as well as outer membrane, peri-plasmatic, and cytosolic proteins [1, 3, 4]. OMVs play important roles in various physiological functions of bacteria including stress relief, bacterial pathogenesis, and communication [5, 6]. Due to the presence of lipopolysaccharide (LPS) in OMV structure, endotoxic responses can be triggered [7]. To remove LPS from OMVs, several endotoxin removal methods can be applied. However, the used chemical reagents could diminish the efficacy of OMVs [8]. To overcome this problem, ClearColi™ BL21(DE3), an engineered *Escherichia coli* BL21(DE3), strain with a genetically modified LPS, is applied for OMV preparation. In ClearColi™, the formation of toll-like receptor 4 (TLR4) complex that is responsible for the endotoxin reaction in human is not induced. It was found that the mutations in the ClearColi™ strain do not affect the OMV features such as morphology and size [10]. Enough inherent pathogen-associated molecular patterns (PAMPs) are maintained in ClearColi™ OMVs and, thus, they can be used as a potent adjuvant platform [9].

The growth phase influences the vesiculation procedure of OMVs [11] quantitatively and qualitatively [12]. It was demonstrated that the size and proteome of OMVs extracted from different microorganisms are phase and culture medium dependent [13, 14]. Furthermore, the yield of secreted OMVs varied when they were extracted from different growth phases [15, 16]. Till now, the effect of bacterial growth phase on the size of *Pseudomonas aeruginosa* [17], *Acinetobacter*

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baumannii's [18], and *Helicobacter pylori* OMVs [19] has been investigated. However, to our knowledge, there is no published data on the effect of growth phase on the OMV features extracted from ClearColi™. In this study, to figure out the best growth phase for ClearColi™ OMV preparation, the size and yield of OMVs isolated from different growth phases were studied.

Generally, in the first step to extract OMVs, the intact bacteria are removed by centrifugation. Then, the residual bacteria were withdrawn by filtration. Due to the low amount of OMVs in filtrated supernatant, a pre-concentration step is needed before OMV sedimentation by high-speed centrifugation [12]. Ultrafiltration (UF) and precipitation are the possible concentration methods in OMV separation procedure. UF including normal flow filtration (NFF) and tangential flow filtration (TFF) is a concentration process in which a low pressure applies to membranes to separate materials based on size [20]. The OMVs are concentrated using UF possess natural morphology and uniform size [6]. In NFF, like the stirred cell set, the direction of the fluid stream is vertical to the membrane surface. In TFF, the fluid streams are parallel to a membrane surface and sweep remained particles on the membrane continuously [20].

Another applied method for OMV concentration is precipitation by salt. In salting out procedure, high concentration of salt breaks the balance of protein surface charges and hydrogen bonds and makes proteins insoluble [12]. In many studies, OMVs are precipitated by the addition of ammonium sulfate (AS) which results in efficient and uniform spherical vesicle extraction [21, 22].

In the next step of OMV preparation, ultracentrifugation (UC) was performed to pellet OMVs and eliminate contaminant [12]. UC is the most common method used for isolation of EVs from cell culture media [23, 24]. Another approach for OMV preparation is utilizing OMV isolation kit in which ion-exchange chromatography system [25] is used. However, the mentioned methods (concentration methods and UC) could not isolate OMV from other extracellular components like flagella, pili, or large protein complexes. These materials may interfere with the following analyses and may have unforeseen immunomodulatory effects in vaccine preparation. Therefore, it is recommended to employ purification techniques like density gradient centrifugation or gel filtration to separate the non-OMV-associated materials [12].

Up to now, the comparison between different OMV extraction methods and their influence on OMV yield and features for a specific bacterial strain have not been reported. Herein, to optimize the preparation of endotoxin-free ClearColi™ OMVs, they were separated by different methods and the yield and size of the separated OMVs were compared.

Materials and Methods

Growth Conditions and Growth Curve

Herein, the effects of different growth conditions on the cell growth rate of ClearColi™ were investigated. Accordingly, growth curve of ClearColi™ strain under different growth conditions (Table 1) was plotted. Briefly, the bacterial cells were grown overnight (ON) in 15 mL LB broth Miller (1% w/v NaCl (Merck, Germany), 1% w/v Tryptone (QLab, Canada), and 0.5% w/v Yeast extract (QLab, Canada) at 37 °C with shaking at 180 rpm. On the following day, the calculated volume of the inoculum was added to 50 mL of either culture media to adjust the OD_{600} to the 0.1 value and then, the OD_{600} was recorded. Then the growth rate and doubling time were calculated in the logarithmic phase by the following equations:

$$\text{Growth rate } h^{-1}(\mu) = 2.303\left(\log OD_2 - \log OD_1\right)/(t_2 - t_1)$$

$$\text{Generation rate or doubling time (td)} = \ln_2/\mu$$

Preparation of OMVs

ClearColi™ strain was grown in 15 mL LB broth Miller at 37 °C with shaking at 180 rpm and then subcultured in 500 mL LB broth Miller to a starting OD_{600} of 0.1. After distinct incubation time, bacteria were centrifuged (5000×*g*, 4 °C, 15 min) (Rotina 380R, Hettich, Germany) and the supernatant was passed through a 0.22-µm filter (Membrane Solutions, USA) [10]. In this study, several different concentration and separation methods (Fig. 1) were applied to filtrated supernatant for OMV isolation. Finally, OMV pellets were resuspended in sterile phosphate-buffered saline (PBS) and stored at − 20 °C until use.

OMV Precipitation by AS

The OMVs were precipitated by the addition of AS (Merck, Germany) at 40% or 70% saturation into filtered supernatant. Briefly, over a period of 1 h, 116.69 and 225.14 g of AS was added gradually to 500 mL of filtered supernatant for

Table 1 Different growth conditions for ClearColi™

Conditions	Culture medium	Shaking speed (rpm)
1	LB-Broth Miller (1×)	180
2	LB-Broth Miller (1×)	250
3	LB-Broth Miller (1.5×) + 0.2% glucose	180
4	LB-Broth Miller (1.5×) + 0.2% glucose	250

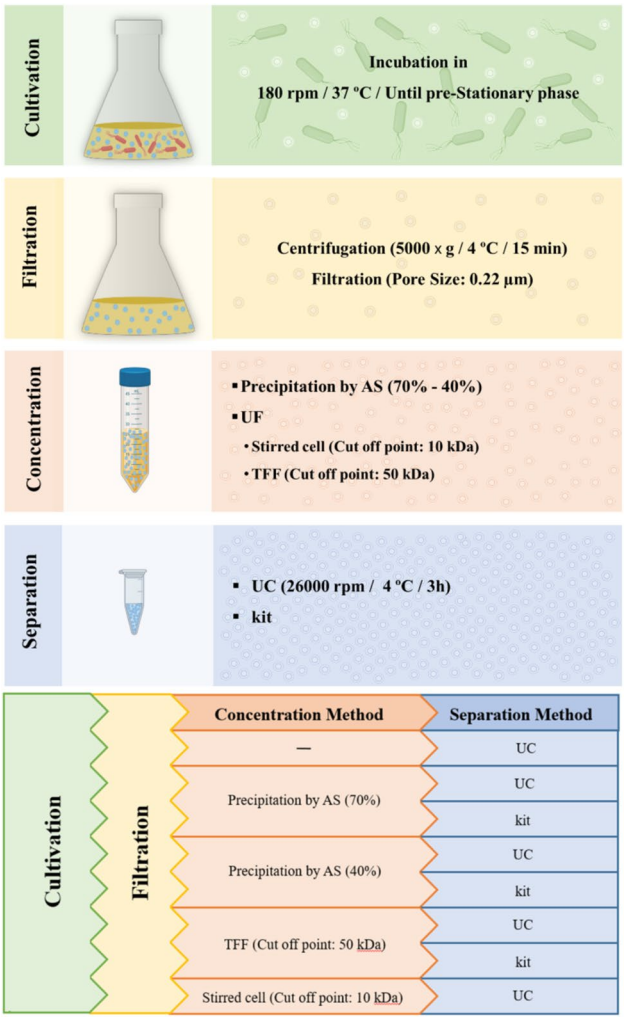

Fig. 1 OMV Concentration and Separation Methods. To isolate ClearColi™ OMVs, the following steps were performed: first, the bacteria were cultivated in liquid media. Then, the bacterial cells were withdrawn by centrifugation. The residual bacteria were further removed by filtration. In the next step, the filtered supernatant was pre-concentrated by ultrafiltration (UF) including stirred cell and tangential flow filtration (TFF) or precipitation by ammonium sulfate (AS). Finally, the OMVs were separated using ultracentrifugation (UC) or exosome isolation kit

40% or 70% saturation, respectively, at room temperature while gently shaking [26]. After ON stirring at 4 °C (on a magnetic stirrer, 1500 rpm), precipitates were collected by centrifugation (10,000×*g*, 4 °C, 30 min) and resuspended in 20 mL PBS. Then, OMVs were separated by UC (Beck Man Coulter, rotor: 70Ti, k-factor: 44, USA) or using Exocib Kit (Cib Biotech Co., Iran) by the methods described below.

OMV Concentration by Tangential Flow Filtration (TFF)

Filtered supernatant containing OMVs was concentrated by passing through a 50 kDa cut-off membrane of TFF (Millipore, DUOBLOC™, USA) device under mild pressure. The sample was introduced and moved parallel to the surface of a porous membrane filter. Then, 50 mL of the concentrated supernatant was collected after 10 times concentration of the filtered supernatant. Then, OMVs were separated using UC or kit as described below.

OMV Concentration by Stirred Cell

500 mL of the filtered supernatant was passed through a 10 kDa molecular weight cut-off regenerated cellulose membrane (Millipore, USA) using 200-mL stirred cell (Amicon model 8400, USA) while applying 5 bar pressure by external compressed N2 gas. Finally, 50 mL of the concentrated sample was collected [27]. Then, OMVs were harvested from the concentrated sample via UC or kit as further described.

OMV Separation by UC

OMVs were separated from the suspension prepared from the previous steps by UC at 26,000 rpm for 3 h at 4 °C. Then, the pellet of OMVs was resuspended in 500 µL of sterile PBS and stored at − 20 °C until use [10].

OMV Separation Using Precipitation by Polymer (Exocib Kit)

According to the Exocib Kit protocol, 5 mL of reagent A was added to 20 mL of concentrated suspension and vortexed for 5 min. Then, the mixture was incubated ON at 4 °C with gentle shaking on a magnetic stirrer (150 rpm). After vortexing the tube of mixture for 1 min, it was centrifuged at 3200×*g* for 40 min at 4 °C. The supernatant was removed and the pellet of OMVs was resuspended in 500 µL of reagent B and stored at − 20 °C until use.

OMV Isolation from Different ClearColi™ Growth Phases

To investigate the effect of bacterial growth stage on size and OMV yield, ClearColi™ was grown for different time periods representing pre-logarithmic, mid-logarithmic, pre-stationary, and stationary growth phases. Then OMVs were isolated from filtrated supernatant using precipitation by AS at 70% saturation and UC as previously described. Finally, OMVs size and yield were analyzed.

Protein Analyses of OMVs

Proteins of the OMVs were separated by adding ice-cold Triton X-100 (Merck, Germany) (1% final concentration) and incubated at 4 °C for 1 h with shaking. Lysates were heated at 63 °C for 10 min and two (aqueous/detergent) phases were separated by centrifugation (13,000 × g, 10 min). After protein extraction by standard chloroform/methanol protocol in both phases, the precipitated proteins of both phases were collected and dissolved in 100 µL PBS [28]. Finally, total protein in OMVs was quantified by bicinchoninic acid (BCA) protein assay (Takara, Japan) according to the manufacturer's instruction. The average concentration of the isolated OMVs was calculated according to bovine serum albumin (BSA, Takara, Japan) standard curve. To determine the protein distribution pattern in the separated OMVs, these vesicles were suspended in 500 µL PBS and then 25 µL of the suspension was mixed with 5 µL 6× loading buffer. After 5 min boiling, proteins of OMVs were separated by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and the gels were stained with Coomassie Blue G-250. Furthermore, SDS-PAGE analysis using the same amount of OMVs separated from different growth phases or using various methods was performed.

Dynamic Light Scattering

The separated OMVs were diluted in PBS (1:10). Then, the size distribution of diluted OMVs was measured by dynamic light scattering (DLS) at 25 °C using nano-zetasizer ZEN3600 (Malvern Instrument, UK) and Zetasizer Software (version 7.03). The size of the vesicle was determined as the average hydrodynamic diameter measurement. The average size and homogeneity of the size distribution were reported by cumulant mean (Z-Average) diameter (d.nm) and polydispersity index (PdI), respectively.

Electron Microscopy

To observe OMVs using transmission electron microscopy (TEM), a 400-mesh formvar carbon-coated nickel grid was floated on the OMV sample. After adsorption of the OMV sample, the grid was washed by 0.01 M PBS containing 0.5 M BSA and 0.1 M gelatin. The sample was fixed for 1 h at 4 °C by 0.01 M PBS containing 1% glutaraldehyde. After washing by PBS (1×), the OMV sample was negatively stained with potassium phosphotungstate and dried. Then, the images were obtained using TEM (PHILIPS, EM, Netherlands) [29].

The OMV samples were fixed in 2% paraformaldehyde for 1 h at room temperature, laced on silicon chips, and then dried using freeze-drying. Scanning electron microscopy (SEM) micrographs were taken at low beam energy (15 kV) by SEM system (MIRA3 FEG-SEM, Tescan, Czech Republic) [30]. The SEM images were analyzed using Image J (National Institutes of Health, USA).

Statistical Analysis

Experiments were repeated two or three times independently. Statistical analyses were performed using one-way ANOVA with Tukey's post hoc test in GraphPad Prism software (version 6). Differences with P values less than 0.05 were considered significant.

Results

Growth Rate of ClearColi™ under Different Growth Conditions

The linear and semi-log growth curves of ClearColi™ under different growth conditions were plotted and their growth rate was calculated. Our results demonstrated that glucose addition and richer medium did not improve the growth rate of ClearColi™. Furthermore, increased aeration did not have a large influence on the growth rate. Therefore, ClearColi™ was cultured in LB Miller (1×) with shaking at 180 rpm (Table 2).

The Effect of Growth Phase on Size and Yield of ClearColi™ OMVs

According to the growth curve of ClearColi™ (Fig. S1), growing for 2, 4, 6, and 24 h represents pre-logarithmic, mid-logarithmic, pre-stationary, and stationary phases, respectively. OMV separation was carried out by AS (70%) precipitation and UC at these time points. The results of this study showed that the average particle size of ClearColi™ OMVs isolated from the pre-stationary phase is significantly lower than those isolated from pre-log, log, and stationary phases (Fig. 2a and S2). ClearColi™ OMVs isolated from mid-logarithmic,

Table 2 The growth rate of ClearColi™ in different growth conditions

Growth condition (media-shaking rate)	Growth rate (h^{-1}) (μ)
ClearColi™ – LB Miller (1×) – 180 rpm	$\mu_1 = 0.53$
ClearColi™ – LB Miller (1.5×) + 0.2% glucose – 180 rpm	$\mu_2 = 0.35$
ClearColi™-LB Miller (1×) – 250 rpm	$\mu_3 = 0.61$

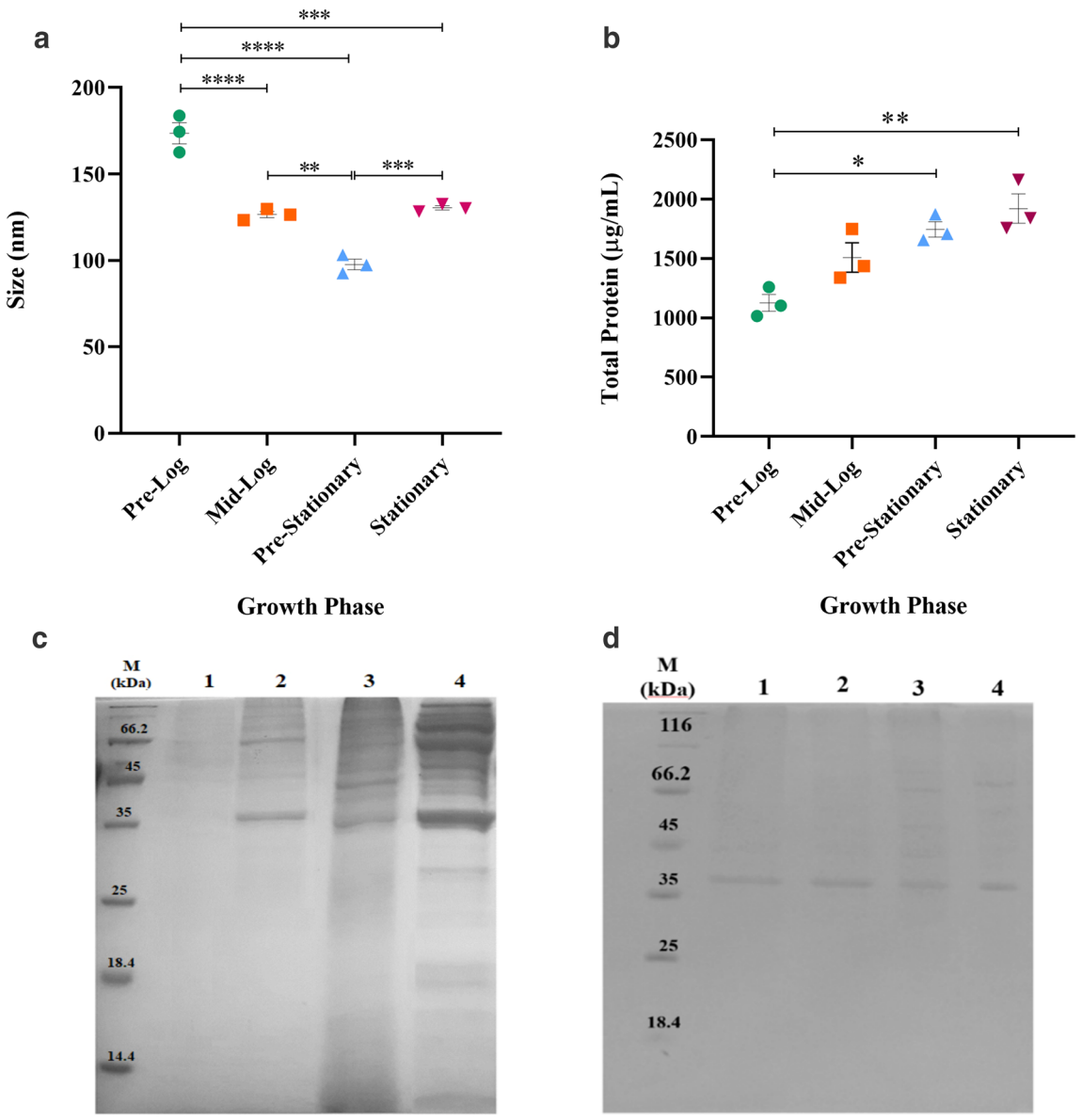

Fig. 2 The Effect of Growth Phase on Size and Yield of ClearColi™ OMVs. **a** The size distribution of ClearColi™ OMVs isolated from different growth phases. **b** Total protein concentration of ClearColi™ OMVs separated from different growth phases. **a** and **b** Error bars represent for SEM of three independent experiments. *, **, ***, **** represent for $P<0.05$, $P<0.01$, $P<0.001$, and $P<0.0001$, respectively. SDS-PAGE analysis of equal c volume (25 µL) and **d** amount (28 µg) of ClearColi™ OMVs separated from different growth phases. (M) Marker (Fermentas, USA), ClearColi™ OMVs separated from (1) pre-logarithmic, (2) mid-logarithmic, (3) pre-stationary, and (4) stationary phase

pre-stationary, and stationary formed a near-homogeneous population with an acceptable level of PdI (< 0.3). However, OMVs isolated from the pre-logarithmic phase formed more heterogeneous population, with higher PdI (0.32 ± 0.02) (Table S1). Furthermore, total protein concentration of ClearColi™ OMVs isolated from the pre-stationary and stationary phases was significantly higher than those separated from pre-logarithmic phase (Fig. 2b). Additionally, ClearColi™ OMVs isolated from different growth phases were separated by 12.5% (w/v) SDS-PAGE. The bands migrating at ~ 35 and 38/42 kDa positions were detected in SDS-PAGE analysis of OMVs separated from mid-logarithmic, pre-stationary, and stationary phase. These bands may represent outer membrane protein A (OmpA) and outer membrane protein F/C (OmpF/C) proteins, respectively [29, 31, 32]. The pattern of protein band in SDS-PAGE analysis of ClearColi™ OMVs isolated from pre-stationary was relatively similar to that isolated from the stationary phase (Fig. 2c). However, relatively stronger protein bands were observed in

ClearColi™ OMVs isolated from stationary phase comparing to those isolated from the pre-stationary phase (Fig. 2c). Furthermore, SDS-PAGE analysis of equal amount of OMVs separated from different growth phases of ClearColi™ confirmed the similarity of protein pattern in all phases (Fig. 2d). Additionally, the same volume of ClearColi™ OMVs, which were released in pre-stationary and pre-logarithmic phases and were related to the same volume of ClearColi™ culture (200 mL), was analyzed by SEM. Qualitative analysis of SEM images revealed that much more spherical structures representing OMVs were observed in the pre-stationary phase as compared to the pre-logarithmic phase (Fig. 3). Therefore, higher protein concentration of OMVs released from the pre-stationary phase can be attributed to higher number of OMVs. Totally, the result of BCA assay and SEM revealed that the release of OMVs from ClearColi™ in the pre-stationary phase is more than in the pre-logarithmic phase. In this study, although total protein concentration of OMVs isolated from the stationary phase is more than other phases (Fig. 2b), the pre-stationary phase was chosen for OMV separation due to their smaller size and consequently better penetration into deep tumors [33].

The Size Distribution of OMVs Separated by Various Methods

The size distribution of OMVs separated by different methods was evaluated by DLS. The separated OMVs using all methods had a normal size distribution. ClearColi™ OMVs isolated using AS (70%) + UC, stirred cell + UC, and UC alone were found to produce OMVs with relatively similar size (Table S2 and Fig. S3). OMV isolation using AS precipitation and kit resulted in relatively higher PdI value (AS (70%) + kit: 0.38 ± 0.02 and AS (40%) + kit: 0.36 ± 0.02) (Table S2). In Fig. 4a, the average size of OMVs separated by different methods was compared. Significantly smaller OMVs were isolated using AS (70%) + UC as compared to those separated by AS (70%) + kit method ($P < 0.01$). Combined with the analysis of SEM images by Image J (Fig. S4), it can be concluded that OMV separation using the kit as final step produces OMVs possessing larger diameter and higher polydispersity, compared to UC as final step.

Protein Analysis of ClearColi™ OMVs Produced by Different Separation Methods

According to our preliminary results (Table S3), among the examined OMVs separation methods, those causing the most protein concentration of OMVs were selected (AS (70%) + kit and AS (70%) + UC) for further analysis. In the next step, statistical analysis revealed that total protein concentration of OMVs separated by AS (70%) + kit and AS (70%) + UC is significantly higher than that isolated by UC (Fig. 4b). Therefore, these two methods were considered as efficient procedures for ClearColi™ OMV separation.

SDS-PAGE analysis was performed to compare proteomic differences between OMVs separated by different

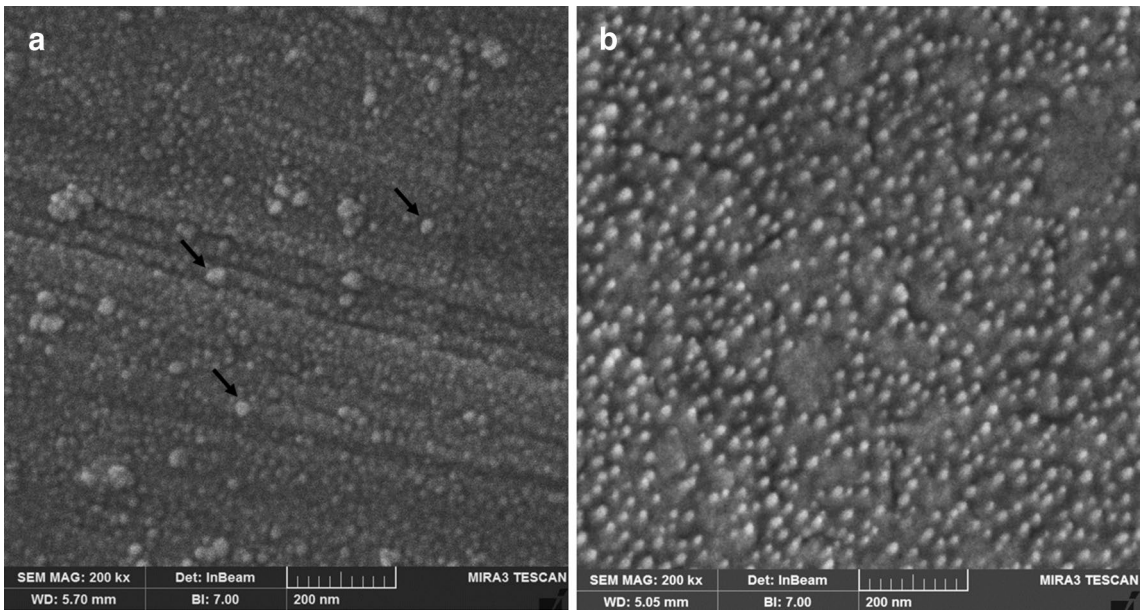

Fig. 3 Scanning Electron Micrograph of ClearColi™ OMVs Isolated from Two Growth Phases. SEM images separated from **a** pre-logarithmic phase and **b** pre-stationary phase of ClearColi™ using AS (70%) + UC separation method

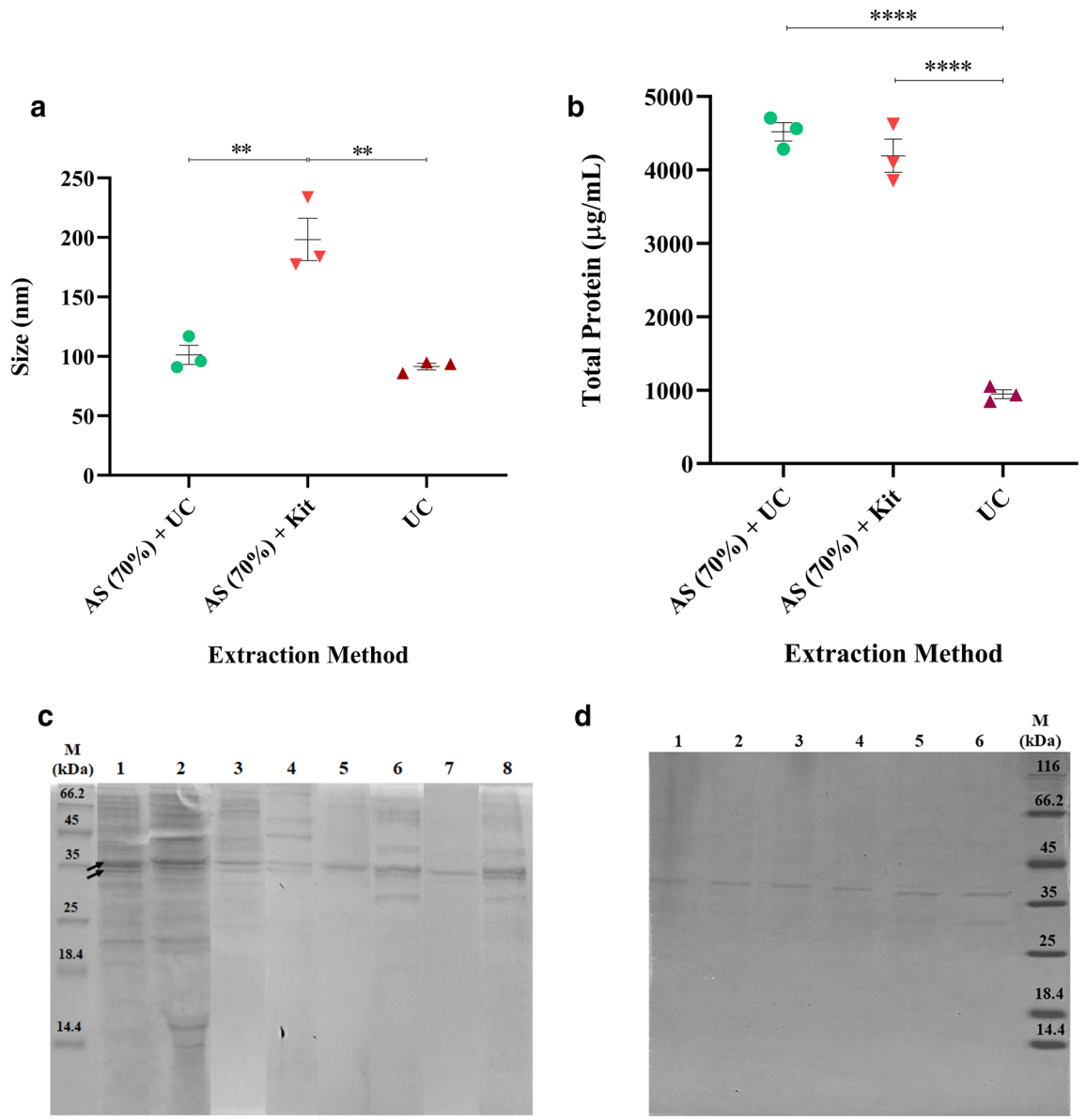

Fig. 4 The Size and Yield of ClearColi™ OMVs Separated by Different Methods. **a** The size distribution of OMVs separated by various methods. **b** Total protein concentration of ClearColi™ OMVs isolated by different methods. **a** and **b** The data are presented as means ± SEM of three independent experiments. $^{**}P < 0.01$ and $^{****}P < 0.0001$. **c** SDS-PAGE analysis of equal volume (25 µL) and **d** amount (14 µg) of the OMVs separated by different methods. (M) Protein marker, OMVs separated using **c** (1) AS (70%) + UC, (2) AS (70%) + kit, (3) AS (40%) + UC, (4) AS (40%) + kit, (5) stirred cell + UC, (6) TFF + UC, (7) TFF + kit, and (8) UC separation methods. Arrows indicate the location of the bands, which may represent OmpA and OmpF/C. **d** (M) Protein marker, OMVs separated using (1) AS (70%) + UC, (2) AS (70%) + kit, (3) AS (40%) + UC, (4) AS (40%) + kit, and (5) stirred cell + UC, (6) UC

methods. SDS-PAGE analysis of partially purified OMVs produced by all separation methods confirmed the presence of 35 kDa and 38/40 kDa protein bands, which may refer to the most common OMV proteins including OmpA and Omp F/C, respectively (Fig. 4c) [29, 31, 32]. Regarding the high concentration of OMVs separated by AS (70%) + UC and AS (70%) + kit, these two samples were initially diluted 5 times by PBS before resolving by SDS-PAGE. As indicated in Fig. 4c, more proteins are enriched in ClearColi™ OMVs isolated when AS precipitation is used as the first step of OMV isolation. Furthermore, ClearColi™ OMVs isolated by AS (70%) + UC contained stronger and more protein bands as compared to AS (40%) + UC. Therefore, AS (70%) precipitation before UC could result in much more protein concentration of OMVs than AS (40%). SDS-PAGE analysis of the equal amount of OMVs protein separated by different isolation methods confirmed the similar protein pattern of OMVs (Fig. 4d). Furthermore, the SEM images

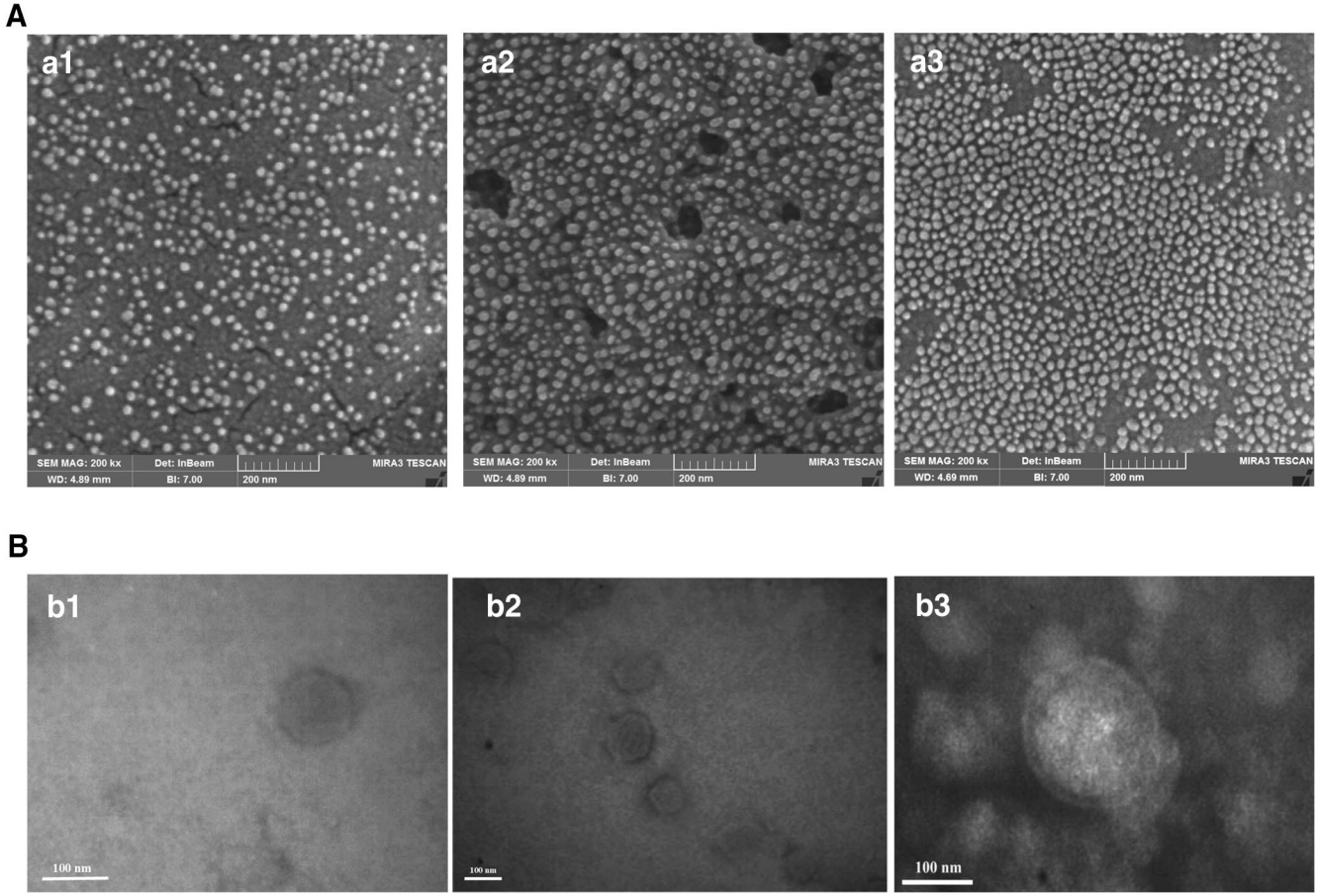

Fig. 5 Electron Micrographs of ClearColi™ OMVs Isolated by Different Methods. **a** scanning electron micrograph (SEM) images of OMVs separated from pre-stationary phase using **a1** AS (40%) + UC and **a2** AS (70%) + UC and **a3** AS (70%) + kit separation method. **b** Transmission electron micrograph (TEM) images of ClearColi™ OMVs isolated by **b1** UC, **b2** AS (70%) + UC, and **b3** AS (70%) + kit. Size bar: 100 nm

were taken from equal volume of OMV separated by AS (40%) + UC, AS (70%) + UC, and AS (70%) + kit isolation methods. The qualitative analysis of SEM images demonstrated that relatively higher number of OMVs is separated by AS (70%) + UC and AS (70%) + kit than by AS (40%) + UC (Fig. 5a). Therefore, according to BCA assay, SDS-PAGE analysis, and SEM images, it can be concluded that the yield of OMV separation using AS (70%) + UC and AS (70%) + kit is higher than AS (40%) + UC.

Determining the Morphology of OMVs Using TEM

TEM was employed to characterize the morphology of OMVs, separated by UC, AS (70%) + kit, and AS (70%) + UC. As demonstrated in Fig. 5b and S5, spherical particles with bilayer membrane and approximately 100, 100, and 200 nm diameter were isolated from ClearColi™ by UC, AS (70%) + UC, and AS (70%) + kit separation methods, respectively.

Discussion

The nanoscale size of OMVs expedites their distribution through various tissues. Moreover, the membrane of OMVs protects their content, and thus, these vesicles can be applied as delivery platforms for medicines to increase drug half-life [34, 35]. OMVs can also be engineered to target tumor cells and deliver anti-cancer drugs without making unwanted cytotoxic effects [34]. Since OMVs can motivate innate and adaptive immune systems, they can be used as native vaccines and vaccine adjuvants. Furthermore, by genetic engineering of parent bacteria, antigen-expressing recombinant OMVs vaccines with desired benefits can be produced [34].

In this study, a genetically modified endotoxin-free strain, ClearColi™, was employed for OMV preparation. We aimed to determine the proper bacterial growth stage and isolation method to prepare OMVs from ClearColi™. The TEM results of our study demonstrated that the ClearColi™ OMVs separated by AS 70% + UC are mostly bilayer round-shaped vesicles (Fig. 5b2). Similarly, globular-shaped efficient rOMVs with size of 50–100 nm were isolated from ClearColi™ [10, 36].

The altered structure of the membrane in ClearColi™ makes several different features for these cells. The growth rate of unmodified BL21(DE3) cells is approximately two-fold of the ClearColi™ cells [9]. These findings were also confirmed in our study by comparing the growth rate of ClearColi™ and BL21(DE3) strains growing in LB Miller (1×) while shaking at 180 rpm (Fig. S1 and Table S4). Due to the LPS glycosylation deficiency, the surface of ClearColi™ is more hydrophobic and permeable, and thus, ClearColi™ becomes osmosensitive. Therefore, it is recommended to speed up shaking while culturing ClearColi™ strain in a liquid medium containing at least 10 g NaCl/L [9]. Accordingly, we studied the growth rate of ClearColi™ in different culture media and aeration conditions. Our data displayed that adding glucose, applying a richer medium or increasing aeration, did not significantly affect the growth rate of ClearColi™ (Table 2). In contrast to the result of our study, Fronczak et al. demonstrated that increased growth rate of ClearColi™ containing kanamycin-resistant plasmid can be obtained by increasing LB concentration up to 1.5× LB [37].

The yield of OMVs can be quantified by various methods, including protein and lipid quantification methods, dry weight measurement, immunodetection methods, and microscopical analyses [12, 38]. Herein, similar to many other studies [15, 39] BCA assay was used to quantify the ClearColi™ OMV yield. Furthermore, to confirm the correlation between the result of BCA assay and the amount of OMVs, SEM images of several samples were analyzed. In SEM images, the extracellular vesicles are distinguishable from other contaminating particles based on the size distribution [40].

Many studies demonstrated that OMV production by different microorganisms is phase dependent [16, 18, 19]. In our study, OMVs from different growth phases of ClearColi™ was isolated using AS (70%) + UC. Based on BCA assay and SDS-PAGE analysis combined with SEM data, it was revealed that the release of OMVs from ClearColi™ in the pre-stationary phase is considerably greater than in the pre-logarithmic phase (Fig. 2b, c and 3). Consistently, in different species of bacteria, the vesicles were produced with their maximum rate at the end of log phase [14]. Bauman and Kuehn demonstrated that the vesicles are produced mainly during the exponential growth phase [16]. McCaig et al. demonstrated that the protein content of OMVs separated from the early stationary phase of *Francisella novicida* was nearly three times more than those separated from the exponential phase [15]. Furthermore, Koning et al. demonstrated that the prevalence of membrane vesicles from *A. baumannii* in early bacterial cultures is low and increased during the stationary phase [18]. The number of OMVs produced by *Helicobacter pylori* after 48 and 72 h of growth was significantly higher than those extracted after 16 h of *H. pylori* growth [19].

Although the OMV yield is critical for selecting appropriate separation phase, purity of the released OMVs is also important. In the late stationary phase, the purity of OMVs is decreased because of bacterial cell lysis and subsequent contamination by damaged membranes and cytoplasmic proteins [12]. Additionally, it was found that the cargo composition of OMVs and the route of OMVs entry into host cells are predetermined by OMV size [36]. Furthermore, it was demonstrated that OMVs less than 100 nm in diameter may enter host epithelial cells and initiate pro-inflammatory responses more

efficiently than larger OMVs [36, 38]. These findings highlight the importance of OMV size especially when they are used as vaccines against bacterial infections in human. Furthermore, longer circulation in blood, longer retention in tumors, and proper drug release were reported for smaller liposome size (≤100 nm) [33]. In the current study, as the size of OMVs isolated from the pre-stationary phase (97.62 ± 3.06) was significantly lower than those isolated from pre-log, mid-log, and stationary phases (Fig. 2a), we selected the pre-stationary phase as the appropriate growth phase for ClearColi™ OMV isolation. In accordance with our study, Orench-Rivera and Kuehn illustrated that the size and proteome of OMVs isolated from different bacterial species are phase- and culture medium-dependent [14]. In the study of Koning et al., the size of *A. baumannii*'s OMVs in the culture of 20 (86 ± 8 nm) and 48 (68 ± 5 nm) h representing stationary phase was smaller than the size of those isolated 6 h after bacterial culture (log phase) (98 ± 29 nm) [18]. In contrast to the result of our study, Tashiro et al. showed that the size of *P. aeruginosa* OMVs did not vary among different growth phases [17].

Despite isolation of globular and hollow spheres OMVs by UC method from different strains of *E. coli* [10], it should be considered that UC of large amounts of filtered supernatant can be difficult and the skill of the operator can affect isolation results. Furthermore, repeated UC steps can destroy vesicles and decrease yields [41]. Thus, it is better to pre-concentrate large volume of supernatant before UC step [42]. Herein, to isolate ClearColi™ OMVs, different concentrating methods such as protein precipitation and UF were compared. AS precipitation at different percent saturation as pre-concentration step and subsequent high-speed centrifugation has been used to isolate OMVs from different bacterial species [21, 22, 29]. Kim et al. demonstrated that the structure of OMVs remains intact during OMV separation by AS precipitation + UC method [29]. To precipitate OMVs, AS could be added to the filtered bacterial supernatant in two ways: 1) adding the saturated AS solution in which extra-large volume is added to samples that complicates the next processing step and 2) adding solid AS to the supernatant [12]. Herein, consistent with some other studies, solid AS [16, 21, 29] was added directly to the supernatant of ClearColi™ culture medium to precipitate OMVs.

In this study, there was not any remarkable difference between the size of ClearColi™ OMVs isolated using UC and AS precipitation + UC (Fig. 4a and Table S2). The SDS-PAGE analysis of OMV proteome isolated via these two methods illustrated OMVs prepared via AS precipitation + UC contained stronger and more protein bands as compared to UC only method. However, OMVs isolated by both methods contained protein bands at 35 kDa and 38/40 kDa, which may represent OmpA and OmpF/ OmpC as the most common outer membrane proteins in OMVs (Fig. 4c and d). Electron microscopy and SDS-PAGE analysis of *P. aeruginosa* OMVs revealed that there is no difference between the vesicles isolated with and without AS precipitation [16].

DLS and similar techniques are not particular for EVs and other particles including lipoproteins and protein aggregates may also be counted, wrongly. Therefore, particle counting by these methods may cause overestimation of EV numbers. Furthermore, exclusive software used for the data analysis of each device may employ unidentified selection and processing of data. Consequently, the certain values obtained by different types of software or various versions of the same software may be different [43]. TEM analysis is based on transparency of the features of EVs and creates 2D images [44]. Therefore, to confirm the size obtained by DLS, the size of OMVs separated by UC, AS (70%) + UC, and AS (70%) + kit was further investigated by TEM images. Our results demonstrated correlation between the size of OMVs obtained by DLS and TEM images. Furthermore, determination of particle size by SEM, which is based on topography of the EV surface, is not as precise as TEM analysis [44]. Nevertheless, the analysis of SEM images (Fig. S4) approximately supported the results of DLS and analysis of TEM images.

In this study, there was not any difference between the size of ClearColi™ OMVs isolated by AS (70%) + UC and AS (40%) + UC (Table S2). However, total protein concentration of OMVs separated by AS (70%) + UC was remarkably more than that isolated by AS (40%) + UC (Fig. 4b and Table S3). In agreement with our study, Bauman and Kuehn found that AS precipitation before the density gradient separation step enhances the recovery of secreted materials and thus the yield of purified OMVs isolated from *P. aeruginosa* is remarkably enhanced [16].

In our study, the filtered supernatant was concentrated 10 times using NFF and TFF methods and then OMVs were separated via either UC or kit methods. Here, by comparing the size of OMVs isolated via UC and UF (NFF or TFF) + UC, it was revealed that UF did not cause any change in OMVs size (Table S2). However, the yield of OMVs separated by UF (NFF or TFF) + UC was lower than those isolated by AS (70%) + UC (Fig. 4b and Table S3). In agreement with the result of our study, OMV morphology isolated from hyperblebbing mutants of *Shigella flexneri* by NFF + UC method [27] and from Enterohemorrhagic *E. coli* (EHEC) by TFF + UC method [45] was confirmed by TEM.

The purification mechanism of exosomes, mammalian cell-derived extracellular nanoscale vesicles [46, 47], in kit (Exocib) is based on capturing the hydrate envelope of particles and subsequently precipitation of the subcellular particles below 100 nm. Therefore, due to the advantages of the exosome isolation kit (Exocib) including requiring less time and centrifuge speed, its accessibility, and price we aimed to examine it for OMV separation. To our knowledge, this is the first report of using an exosome isolation kit for OMV isolation. Herein, it can be assumed that more

heterogeneous in size and larger ClearColi™ OMVs were isolated when the kit was used as the second step of OMV isolation method (Fig. 4a and Table S2). Furthermore, total protein of ClearColi™ OMVs isolated using AS (70%) + kit was nearly similar to that isolated by AS (70%) + UC and more than those isolated by other OMV isolation methods used in this study (Fig. 4b and Table S3). Qualitative analysis of SEM images also confirmed that relatively high number of OMVs are separated by AS (70%) + UC and AS (70%) + kit isolation method (Fig. 5a). Additionally, SEM and TEM images (Fig. 5) demonstrated that relatively pure OMVs are isolated by exosome isolation kit. In contrast to the result of our study, ExoBacteria™ OMV isolation kit which uses precipitation-free gravity column system led to higher yields of *E. coli*-derived OMV isolation with narrower size distribution than UC approach. Furthermore, salmonella-derived extracellular vesicles with size ranging from 117 to 140 nm were harvested by ExoBacteria™ OMV isolation Kit [25].

PdI (polydispersity index) reveals variation in size belonged to a population of particles. The range of PdI is between 0.0 (monodispersed) and 1.0 (entirely heterodispersed). PdI of 0.3 and below is favorable and represents the homogeneity of a suspension of nanoparticles [48]. In our study, PdI of the OMVs separated from all growth phases of ClearColi™ with the exception of the pre-logarithmic phase was the same (approximately 0.2), indicating they were highly homogeneous in size. However, the OMVs separated from the pre-logarithmic phase of ClearColi™ were more heterogeneous in size (PdI: 0.32 ± 0.02) (Table S1). The size distributions of *P. aeruginosa* OMVs were similar in all growth phases [17]. In contrast, in the study of Zavan et al. narrower distribution of OMV size which means less heterogeneous OMVs in size was observed when *H. pylori* growth stage progressed [19].

Herein, the ClearColi™ OMVs obtained via different OMV isolation methods with the exception of AS (40%) + kit and AS (70%) + kit methods showed acceptable PdI values (PdI < 0.3), indicating they were homogeneous in size (Table S2). Similarly, the z-average and PdI of *Klebsiella pneumoniae* OMVs isolated via UC method were 86.54 nm and 0.236, respectively [49]. However, Huang et al. isolated OMVs from *E. coli* DH5α using UF + UC method in the pre-stationary phase and could obtain OMVs with 124 nm in diameter and PdI of 0.394 [50].

Finally, the present study provided an appropriate growth phase (pre-stationary) and an improved method with enhanced yield (AS (70%) + UC) for ClearColi™ OMV isolation. The method of AS (70%) + UC can be applied in vaccine research studies when ClearColi™ OMV as an adjuvant or recombinant ClearColi™ OMV as a vaccine is required. Furthermore, despite isolation of more heterogeneous in size and larger OMVs using AS precipitation + exosome isolation kit as compared to AS precipitation + UC, this method can be applied for OMV isolation when high-yield ClearColi™ OMV isolation is required and high-speed centrifugation is not accessible.

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Declaration

Conflict of interest The authors declare that there are no conflicts of interest.

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