Original Article

Comparison of two isolation methods for extracellular vesicles from Faecalibacterium prausnitzii A2-165

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ABSTRACT

Introduction: Extracellular vesicles (EVs) are spherical structures, naturally secreted by Gram-negative and Gram-positive bacteria. EVs play a critical role in the modulation of immune responses, bioactive cargo delivery, and cell-cell communication. The conventional method of EVs preparation involves the use of detergent (ultracentrifugation method). For the first time, we used a polyethylene glycol (PEG)-based method in our study to isolate EVs from prokaryotic cells, namely *Faecalibacterium prausnitzii* A2-165. We then compared various features of this method with those of the ultracentrifugation method. **Methods:** Extraction of EVs was performed via sequential deoxycholate ultracentrifugation and PEG-based methods. The physicochemical properties of the extracted EVs were compared via scanning electron microscopy (SEM), SDS-PAGE, and dynamic light scattering (DLS). **Results:** The protein content of the extracted EVs was 1.6 and 0.5 mg/mL, based on the ultracentrifugation and PEG-based methods, respectively. According to the SDS-PAGE analysis, vesicle-associated proteins were located at 20-150 kDa. The SEM analysis showed that the extracted EVs had a diameter of 50-200 nm in both methods. The results of DLS analysis showed 4 populations of approximately 50-8000 nm in the ultracentrifugation method and approximately 100-2000 nm in the PEG-based method. The EVs extracted by the ultracentrifugation method showed higher negative charge densities in contrast to EVs extracted by the PEG-based method. **Conclusion:** Our result showed that PEG-based extraction is a fast, simple, and cost-effective method and EVs purity was within the acceptable range. Further studies are needed to confirm the safety and the efficacy of EVs in clinical practices, especially as vaccine delivery vehicles.

KEYWORDS: *Faecalibacterium prausnitzii*, isolation methods, outer membrane vesicles, vaccine vehicle.

INTRODUCTION

The human gastrointestinal tract is inhabited by a broad spectrum of microorganisms, including bacteria, fungi, and viruses. According to the previous reports, over 1000 gut bacterial species have been identified so far [1]. These bacteria and their metabolites are the main mediators of the crosstalk between different cell types in the mucosa. They are known to affect the intestinal barrier function through their interaction with epithelial cells [2]. Changes in the gut microbiota are linked to various disorders, including autoimmune diseases, diabetes, and inflammatory bowel disease (IBD) [3, 4]. The association between health and gut microbiota has directed a lot of attention to probiotics which are used to maintain the balance of microbiota and prevent a variety of diseases.

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Faecalibacterium prausnitzii is an obligate anaerobic bacterium from the *Ruminococcaceae* family (*Firmicutes* phylum, *Clostridium* genus), accounting for almost 8% of the total colonic microbiota [5]. According to the previous studies, the relative abundance of *F. prausnitzii* may be an indicator of intestinal health in adults with IBD [6, 7]. *F. prausnitzii* metabolizes lactate in the gastrointestinal tract and produces butyric acid which is the primary source of adenosine triphosphate (ATP) for colonocytes. Therefore, a low *F. prausnitzii* level can lead to a shortage of energy in the gut and reduction in protection against inflammatory responses [8]. Overall, butyrate is speculated to have anti-inflammatory and chemo-preventive activities [9]. In this regard, Martin et al. (2017) have evaluated the use of *F. prausnitzii* as a probiotic. They have confirmed the safety and efficacy of live *F. prausnitzii* cultures and have suggested *F. prausnitzii* as a good candidate for the next generation of probiotics [10].

It has been reported that many bacteria secrete Extracellular vesicles (EVs) naturally which are responsible for the delivery

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of bioactive molecules to the host cells and regulation of immune responses. EVs are produced in various environments during all growth stages [11] and are 10-300 nm in diameter [12]. EVs are spherical in shape and have a bilayer membrane, composed of the outer membrane (OM) components (i.e., proteins, phospholipids, and lipopolysaccharides) which protect the EVs content against proteases and nucleases. Nevertheless, the cargo of EVs may involve the inner membrane or cytosolic proteins and nucleic acids [13]. In fact, EVs provide a means for the secretion of these components to the environment [14]. In addition, EVs interact with both eukaryotic and prokaryotic cells. They carry microbe-associated molecular patterns (MAMPs) which can affect the host responses to the infection [12]. EVs play a critical role in the modulation of host immune responses, nutrient acquisition, delivery of bioactive cargos, and cell-cell communication. Recently, their role in immune homeostasis has also been proposed. In addition, EVs has gained intense attention due to their potential roles in medical research [15]. Therefore, extraction and characterization of EVs can be a step forward in their application as new biotechnological tools such as vaccine manufacturing, adjuvants and drug delivery domains.

Despite the great importance of EVs, their isolation at an adequate amount and purity remains challenging for most bacteria. The typical method of EVs purification depends on the properties of EVs, such as small size and buoyant density, allowing them to be separated from the bacterial cells via centrifugation and/or ultracentrifugation [16]. The first stage of isolation involves the removal of particles with buoyant density where the majority of intact bacteria and cell debris are removed by low-speed centrifugation [17]. Then, apoptotic bodies, aggregates of biopolymers, and other structures with a higher buoyant density than EVs are sedimented via centrifugation at 10000 x g. Finally, EVs in the resulting supernatant are sedimented by ultracentrifugation at > 100000 x g. The EVs preparations are further purified according to the size through filtration, using filters with a pore diameter of 0.22 µm [18].

In the ultracentrifugation method, EVs are extracted from the bacterial biomass by sodium deoxycholate in the presence of EDTA which is a divalent ion chelating agent that destabilizes the outer membrane and enhances EVs release. These EVs are detergent-derived and are prepared artificially [19]. Another method involves the precipitation of EVs with hydrophilic polymers, such as polyethylene glycol (PEG), protamine, and sodium acetate. According to the literature, the method based on the precipitation of EVs in PEG solution is the second most popular method following ultracentrifugation. Unlike ultracentrifugation, the PEG-based method is detergent-free; also, the naturally secreted EVs are highly similar to the native vesicles *in vivo* [20]. PEGs with different molecular weights have been used for precipitation of the small particles, such as proteins, nucleic acids, and viruses. The PEG-based methods decrease the solubility of compounds in PEG solutions and form a mesh-like polymeric web which captures EVs of a certain size (60-180 nm). This procedure reduces the mixing of the culture medium and the PEG solution, as well as the incubation and sedimentation of EVs via low-speed centrifugation. The pellet is then suspended in phosphate-buffered solution (PBS) for further analysis.

New technologies used for the isolation of EVs are based on the specific interactions with molecules on the EVs surface. Each of these methods has particular advantages and disadvantages which should be considered in the design of experiments involving EVs [18]. The purpose of the present study was to evaluate two different isolation methods, namely ultracentrifugation (detergent-extracted EVs) and PEG-based method (detergent-free extraction), by comparing the physiochemical properties of the isolated EVs as a first step for development of EVs-based vaccines.

MATERIALS and METHODS

Bacterial culture

F. prausnitzii strain A2-165 (DSMZ, Braunschweig, Germany) was grown in brain-heart infusion medium (BHI), supplemented with hemin and vitamin K in an anaerobic chamber (N_2 85%, CO_2 10%, and H_2 5%) at 37± 0.5°C [21]. The fresh liquid broth culture of F. prusnitzii (500 mL) was incubated until an optical density of 0.6 was achieved which corresponded to an early stationary phase culture.

Isolation of EVs

EVs were isolated using two different methods. On method involved ultracentrifugation based on previously described protocols [22, 20]. Briefly, after overnight cultivation, the culture medium was centrifuged (6000 rpm, 4°C) to harvest the biomass. The pellets were washed twice with PBS and re-suspended in 9% sodium chloride solution. Then, the suspension was homogenized for 30 min and concentrated by centrifugation (6000 rpm, 1 h, 4°C). The total wet weight of the cell pellets was calculated. They were then re-suspended in 7.5 times the wet weight in 0.1 M Tris and 10 mM EDTA and also, adding 1:20 volume of 0.1 M Tris, 10 mM EDTA sodium deoxycholate buffer (100 g/L). The purified EVs were collected by centrifugation at 10000 rpm and ultracentrifugation at 130000 rpm (90 min, 4°C). Finally, the concentrated EVs were re-suspended in 3% sucrose solution and purified using polyvinylidene difluoride filters (pore size: 0.22 µm). The extracted EVs were stored at -20°C until further use [20, 22].

The other method of EVs isolation involved the use of a PEG solution. In this method, a large volume of bacteria was cultured in BHI broth at 37°C overnight. Centrifugation of the bacterial culture was performed at 6000 x g. (45 min), followed by 10000 x g (30 min), in order to remove the cellular debris at 4°C. In the next step, the supernatant was added to an equal volume of 16% PEG 6000 at 4°C. Afterwards, the samples were mixed and incubated at 4°C overnight for at least 12 h. On the next day, centrifugation was performed (3200 x g, 1 h, 4°C). All the pellets were finally re-suspended in 3% sucrose solution for further use.

Protein quantitation

Quantification of the EVs yield is an important step in understanding vesiculation, based on the protein or lipid measurements. In our study, the protein content of the purified EVs was measured via spectrophotometry using a NanoDrop system (Thermo Scientific, Lite, USA).

SDS-PAGE

The protein pattern of EVs was analyzed by SDS-PAGE. The EVs (15 µl/well) were loaded into wells, containing 12% gradient gel. The gel was stained with Coomassie Brilliant Blue [23].

SEM

Light microscopy was not considered suitable for evaluation of the integrity and stability of EVs after extraction due to the small size of EVs and low resolving power of the method. Therefore, SEM is the method of choice to confirm the

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presence of EVs and describe their size and shape. After the extraction process, the size and morphology of EVs were evaluated by SEM (EM3200, KYKY, China). Briefly, in the first step, the samples were fixed in 2% glutaraldehyde for 1 h at room temperature. They were then washed in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and gradient dehydrate ion (30%, 50%, 70%, 90%, and 100%) with ethanol (5 min each). Finally, the samples were air-dried, coated with gold using sputter coaters, and visualized by SEM [24].

Vesicle size analysis by dynamic light scattering (DLS)

The size distribution of EVs was measured by DLS technique, using a particle size analyzer (Nano ZS, ZEN3600, Malvern Instrument, UK).

Zeta potential measurements

The *F. prausnitzii*-derived EVs were prepared via sonication at 35 kHz for 3 min in a Bandelin ultrasonic bath. The zeta potential of EVs was evaluated using the Malvern Zetasizer (Nano ZEN3600, Malvern Instrument, Malvern, UK) [25].

RESULTS

Protein quantitation

The total protein content of *F. prausnitzii*-derived EVs was determined using the NanoDrop technique. The results indicated that the protein contents were 1.6 and 0.5 mg/mL in preparations from the ultracentrifugation and PEG-based methods, respectively.

SDS-PAGE

SDS-PAGE analysis of EVs regarding the presence of proteins revealed bands in 20-150 kDa regions in both methods (Fig.1). The gel image was representative of 2 independent experiments, each yielding similar results with only a few different bands.

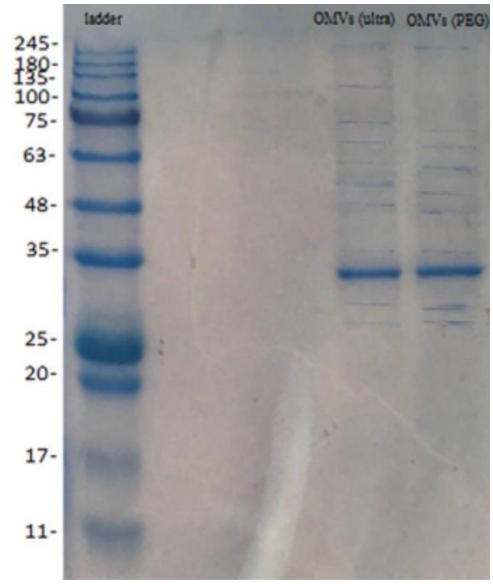

Fig.1. SDS-PAGE of *F. prausnitzii* derived EVs by ultracentrifugation (left lane) and PEG-based method (right lane). The ladder shows the protein bands between 11-245 kDa.

SEM analysis

The SEM showed that the extracted vesicles were spherical in shape, with a diameter range of 50-200 nm in both methods (Fig. 2A and 2B). No difference was observed between SEM analysis of ultracentrifugation and PEG-based methods.

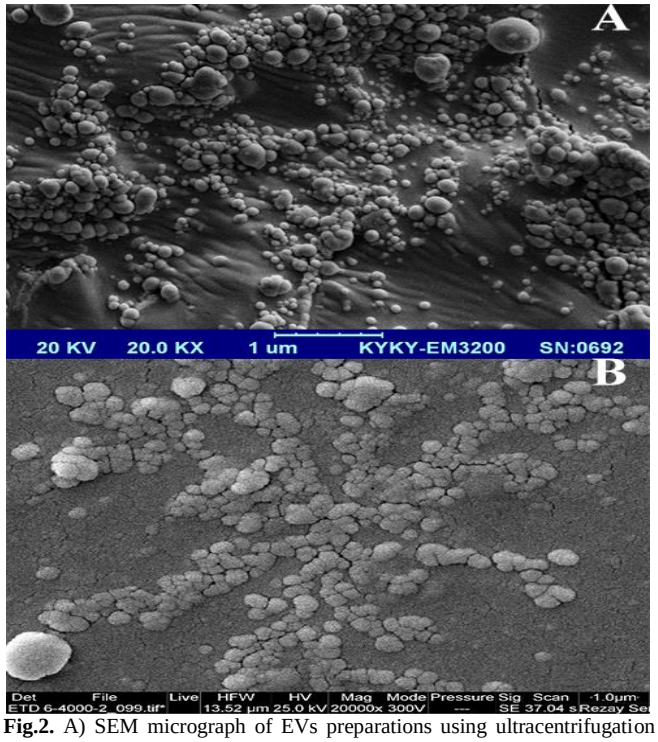

Fig.2. A) SEM micrograph of EVs preparations using ultracentrifugation method at 20Kx magnification. B) SEM micrograph of EVs preparations using PEG-based method at 20Kx magnification.

Size distribution by DLS technique

EVs preparations from *F. prausnitzii* cultures were evaluated by DLS technique to determine the size distribution of EVs. The DLS analysis showed 4 populations of approximately 50-8000 nm in preparations from the ultracentrifugation method and 2 populations of approximately 100-2000 nm in preparations from the PEG-based method (Fig. 3A and 3B).

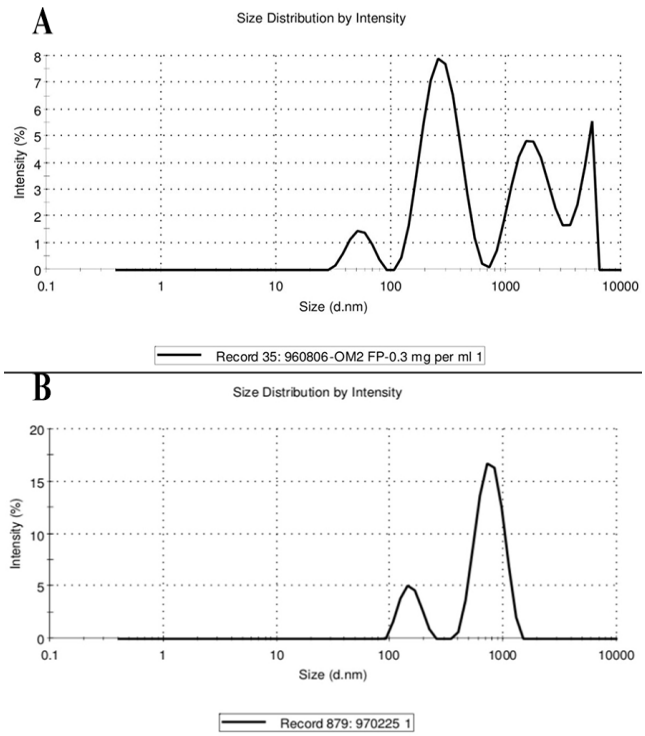

Fig.3. A) Size distribution report by intensity of EVs from ultracentrifugation method. B) Size distribution report by intensity of EVs from PEG-based method.

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Zeta potential measurements

The zeta potential measurements by the DLS technique were negative in both methods. The results showed that the zeta potentials for the extracted EVs were -60.9 and -20.6 mv, respectively in the ultracentrifugation and PEG-based methods (Fig. 4A and 4B).

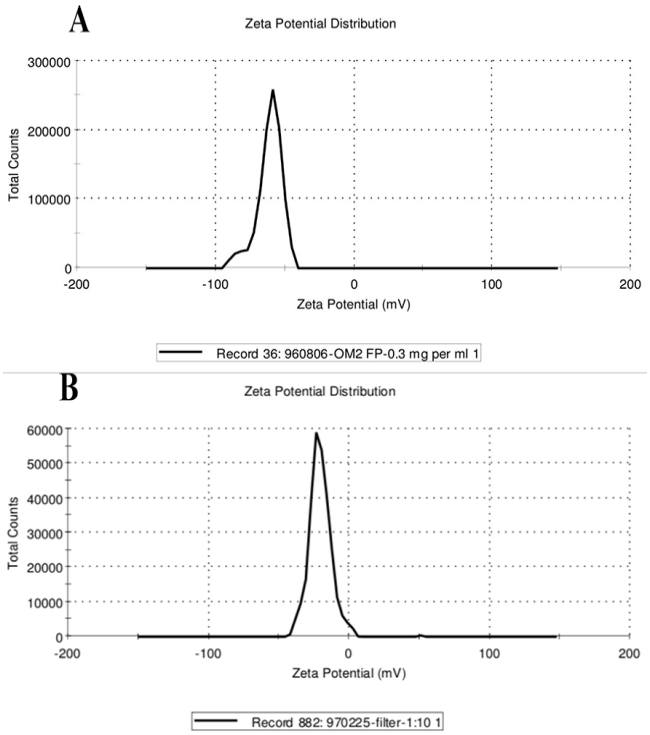

Fig. 4. A) The magnitude of zeta potential for the extracted EVs by ultracentrifugation method. B) The magnitude of zeta potential for the extracted EVs by PEG-based method

DISCUSSION

There are various techniques used for the isolation of EVs. Ultracentrifugation is the most common method for EVs isolation, involving a number of sequential centrifugation steps at different centrifugal forces (*g*). The purpose of this method is to remove unwanted components. Isolation of EVs is performed in the presence of sodium deoxycholate as a detergent. On the other hand, the detergent-free PEG-based precipitation method is an alternative approach, based on the changing solubility of EVs and/or subsequent emergence of aggregates [26]. Each of the proposed methods has advantages and disadvantages. The main disadvantages of ultracentrifugation include the presence of contaminants in EVs preparation, being time-consuming, and requiring expensive equipment. However, this method is suitable for the isolation of EVs from a large volume of cell culture supernatant, requiring a small set of reagents. Because of these advantages, ultracentrifugation is still considered the gold standard method, routinely used for EVs isolation. In contrast to ultracentrifugation, PEG precipitation is a fast, simple, and cost-effective technique which can isolate EVs from a large volume of samples. On the other hand, contamination with non- EVs proteins is a disadvantage of this method [18].

In this study, we used a PEG-based extraction method for the isolation of EVs from prokaryotic cells for the first time and compared various characteristics of the isolated EVs (native vesicles) with the detergent-derived EVs (ultracentrifugation). Based on our findings, the spatial structure of *F. prausnitzii* vesicles conserved their natural form in all stages of the purification process. This finding is compatible with previous studies in terms of size and form of the vesicles [27, 28]. In this regard, Song Gho et al. (2013) have extracted extracellular vesicles from *Akkermansia muciniphila* to investigate their role in the progression of dextran sulfate sodium-induced colitis. The spherical shape of EVs was confirmed by TEM images, and their average size was reported to be 87.76± 198.13 nm [29]. In addition, Li et al. (2017) have extracted EVs from *Lactobacillus plantarum*. The characterization of the vesicles by electron microscopy has shown that the size of the particles was 30-300 nm [30]. Moreover, we (2013) have evaluated the biological and immunological properties of EVs from Neisseria meningitidis. Based on the results of electron microscopy, we found that the size of the EVs ranged from 50 to 150 nm in diameter [31]. The extracted *F. prausnitzii* EVs in this study had similar dimensions to those secreted by other bacteria. In all the mentioned studies, the natural form of the vesicles was conserved in different stages of the purification which is in agreement with our results.

In this study, we indicated that the EVs yield varied in different isolation methods. The ultracentrifugation method recovered higher amounts of EVs, compared to the PEG-based extraction method. This difference is due to the use of the detergent (*i.e.* sodium deoxycholate) in the process of EVs isolation via ultracentrifugation which produces EVs artificially and enhances the EVs yield. On the other hand, in the PEG-based method, natural EVs which are formed spontaneously, are recovered from the bacterial supernatant. The SDS-PAGE analysis of the EVs preparations from these methods revealed similar profiles, with only a few different bands. This shows that the protein composition of the extracted EVs by the ultracentrifugation method was different from that of the PEG-based method. Moreover, analysis of the DLS profile revealed the heterogeneous diameter of the EVs in each method. This finding is consistent with the measurements of SEM images, although larger structures were detected by DLS technique.

The comparison of DLS and SEM results represented that, DLS overestimates and SEM underestimates the size of EVs [32]. Also, the results of DLS analysis indicated differences in the size distribution of the extracted EVs using these isolation methods. The size of the extracted EVs by the ultracentrifugation method was greater than those extracted from the PEG-based method. This finding may be attributed to the production of more vesicles and their aggregation in the ultracentrifugation method. On the other hand, PEG-based method isolates EVs of a certain size, usually 60-180 nm [26]. Therefore, the PEG-based isolation method recovers smaller vesicles, more than the ultracentrifugation method.

In addition, we used a standardized and validated protocol for characterization of the zeta potential of EVs. Generally, the zeta potential analysis is a method to define the surface charge of nanoparticles, such as vesicles in the solution form. This parameter is a major indicator of bacterial stability. The surface charge of these vesicles controls and modifies the behavior of EVs and potentially alters the aggregation state and cellular responses which may affect the fate of EVs. In this study, we measured the zeta potential by DLS technique.

The EVs extracted by the ultracentrifugation method had higher negative charge densities in contrast to EVs extracted by the

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PEG-based method. This significant negatively charged surface implies that the extracted EVs remain without aggregation using the ultracentrifugation method; therefore, they are more stable in the solution, which is helpful for their interactions with other cells. As different methods have certain disadvantages, it is not possible to establish an ideal and universal method for the isolation of EVs. However, we should attempt to overcome certain shortcomings in order to improve the quality of the available standard methods.

In conclusion, different methods for the extraction of EVs can produce various EVs subpopulations. However, ultracentrifugation remains the gold standard method which is routinely used for the isolation of EVs. Also, the PEG-based method has the advantages of being fast, simple, and cost-effective and the EVs extracted by this method had purity and conformation within the acceptable range. Further studies are needed to confirm the safety and efficacy of these bacterial EVs in clinical practices, especially as vaccine delivery vehicles.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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