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Isolation of Extracellular Vesicles of *Akkermansia muciniphila* as a Potential Therapeutic Platform

Pegah Noori[1], Fattah Sotoodehnejadnematalahi[1], Pooneh Rahimi[2], Seyed Davar Siadat[3]*

[1]Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran. [2]Department of Hepatitis and AIDS, Pasteur Institute of Iran, Tehran, Iran. [3]Department of Mycobacteriology and Pulmonary Research, Pasteur Institute of Iran, Tehran, Iran

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***Corresponding Author**
Seyed Davar Siadat,
Department of Mycobacteriology and Pulmonary Research, Microbiology Research Center (MRC), Pasteur Institute of Iran, Tehran, Iran.
Tel/Fax: +98-2164112823/+98-2164112213
Email: d.siadat@gmail.com

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ABSTRACT

Introduction: Extracellular vesicles (EVs) are the active biological compounds with significant roles in pathogenesis and intercellular interactions in both Gram-negative and Gram-positive bacteria. *Akkermansia muciniphila* secretes EVs and represents 3–5% of the gut microbiota community and has impact on health conditions of individuals with healthy gut through mucin degradation. Recently, several studies have used innovated methods to extract and characterize EVs with potential applications as a part of a vaccine component. **Methods:** EVs extraction from *A. muciniphila* was performed by ultracentrifuge technique. The size, shape and protein patterns of the EVs were investigated by Electron Microscopy and SDS-PAGE. **Results:** The extracted EVs were confirmed by the shape of the vesicles and their sizes ranging from ~ 40 to 150 nm. **Conclusion:** The results of the current study indicated that the shape, size and conformation of *A. muciniphila* EVs were within the accepted range to be considered as a component of a vaccine delivery vehicle in the future studies.

INTRODUCTION

Evidence shows that extracellular vesicles (EVs) are formed and released from most cell types as a part of intercellular communication[1]. Intercellular signaling is a significant feature of EVs by delivering important molecules into the extracellular space[2]. Aside having pathological activities, Evs have other crucial advantageous capabilities such as drug or vaccine delivery vehicles [1, 2]. In recent studies, many methods of characterization, isolation and detection of EVs have been proposed[3, 4]. The component of EVs have surfactant-like properties, amphipathic helical construction and heterogeneous, and usually nano to micro-sized membrane vesicles which are released within the size range of 30 to 10,000 nm in diameter by Gram-negative and Gram-positive bacteria [5, 6]. Exosomes (30-150 nm in diameter), microvesicles (50-1,000 nm in diameter), and apoptotic bodies are the main classifications of EVs [7, 8].

Due to the abundance of outer membrane proteins, phospholipids, and lipopolysaccharides (LPS), and also parts of the cell walls in EVs, they have been proposed as immunoreactive components. Moreover, by removing harmful compounds from EVs, their immunogenicity and safety as parts of putative vaccines has been amplified [9, 6]. The most impressive characteristic of EVs is transferring their cellular content from a donor to a recipient. First, the packaged content reflects the state of the donor cell, which renders them useful as biomarkers. For instance, Glioblastoma microvesicles transport RNA and proteins that promote tumor growth and provide diagnostic biomarkers[3, 10]. Moreover, this intrinsic characteristic makes EVs interesting candidates for intracellular delivery of therapeutics[11]. Additionally, inducing strong immune responses by EVs in human microbiota, including their functional and practical antigen-presenting roles have gained attention in recent decades[2, 12]. EVs have also been explored in different fields of study such as cell biology and biotechnology, as well as pharmaceutical industries as diagnostic markers and therapeutic vehicles [1, 12].

Akkermansia muciniphila is one of the normal flora bacteria in human gut[13] which is also an indicator of a healthy microbiome. Its adherence to the colonic epithelium is known to stabilize the thickness of the intestinal mucosa and causes the upregulation of the tight junction in human and mice[13, 14]. *A. muciniphila* can secrete enzymes into the gut tract which regulate the mucin protein on the mucosa layer[15]. Previous studies have shown that the presence of *A. muciniphila* has roles in regulation of the gut barrier, the metabolic functions and the secretion of bioactive materials, such as microbial anti-inflammatory molecule (MAM) protein. Meanwhile, the lack of abundance of *A. muciniphila* in normal

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flora can cause obesity and type 2 diabetes [16]. Interestingly, Chelakkot et al, have isolated extracellular *A. muciniphila* and indicated that the EVs induce gut permeability and intestinal barrier integrity by in vivo and in vitro experiments[16]. The goal of this study was to extract and characterize EVs from *A. muciniphila* which could potentially be applied as a natural delivery vehicle for vaccines or other therapeutics in the future studies.

MATERIALS AND METHODS

Bacterial Culture Conditions and EV extraction

A. muciniphila strain ATCCBAA-835, was provided by German Collection of Microorganisms and Cell Cultures (DSMZ) Institute. Brain Heart Infusion (BHI) broth and Agar media (Quelab, Canada) were used to activate freeze-dried *A. muciniphila*. The bacterium was cultured in a basal mucin-based medium, supplemented with 0.5% mucin-type III (Sigma-Aldrich, USA), hemin (5 µg/ml), menadione (1 µg/ml)[17] and 0.05% L-cysteine[18] under anaerobic conditions (80% N_2, 10% H_2, and 10% CO_2) at 37 °C for 3–7 days, as described previously[19]. PCR was performed based on 16s ribosomal ribonucleic acid (rRNA) sequence for confirming the bacterium using the primers in Table 1, in addition to macroscopic and microscopic (Gram staining) assays.

After the growth, the bacterium was inoculated into 100 ml BHI broth (Quelab, Canada) with the above-mentioned supplementations incubated in the same condition for 48 h until an optical density (OD) of 1.5 at 600 nm was reached. The bacterial pellets were removed by centrifugation (8000 × g, 5 min) and washed with anaerobic PBS and the cell-free supernatant was obtained for the EVs extraction process. The supernatant was purified by passing through 2.22 nm filters and was kept at −70 °C until use. Four McFarland of bacteria equal to 12 × 10^8 CFU/ml were inoculated in above-mentioned supplemented BHI broth overnight. When the OD reached 1.5 at 600 nm, the EVs were extracted by centrifugation at 6000×g at 4 °C, and the pellets were then washed twice with PBS, followed by centrifugation at 6000×g at 4 °C, suspended with 9% sodium chloride solution at 6000×g at 4 °C for an hour and then mixed with ethylenediaminetetraacetic acid-sodium deoxycholate (Sigma Aldrich, USA) buffers[20]. In advance, centrifugation was performed at 20,000×g at 4 °C for 1 h and ultracentrifugation at 125,000×g twice for 2 h sequentially, and then it was kept in sucrose 3% at −70 °C[21]. The concentration of the total purified protein was evaluated by NanoDrop Lite machine2000 (Termo Fisher Scientific, USA) and confirmed by Bradford assay.

Table1. The sequence of primers for confirmation of *A.muciniphila*.

Primer Name	Forward Primer	Reverse Primer	Product Size	Annealing Tm
A.muciniphila 16s rRNA	CAGCACGTGAAGGTGGGGAC	CCTTGCGGTTGGCTTCAGAT	316	59°c

EVs characterization by Electron Microscopy and SDS-PAGE

To distinguish between the single EVs from non-EV particles, Transmission Electron Microscopy (TEM) was used to confirm the presence and sizes of the EVs. The sample was prepared by negative staining and then observed by a Philips (Netherlands) EM 208 microscope. The morphology of EVs was studied through Scanning Electron microscopy (SEM) to confirm the integrity, and the stability of vesicles. Also, SEM was used to determine the spatial shape and size of them. Filtered EVs in sucrose as mentioned above, were covered on 400-mesh gold grids and stained with 2% uranyl acetate. SEM image was obtained using a HITACHI S-4160 microscope. The protein pattern of the EVs was evaluated by SDS-PAGE on 12% separating gel using 25 µl of each sample, stained with Coomassie Brilliant Blue R-250.

RESULTS

Isolation and Characterization of EVs Derived from *A. muciniphila*

The morphology and size of the extracted EVs from *A. muciniphila* were confirmed by TEM. Multiple spherical vesicles were seen, the majority of which ranged from ~ 40 to 150 nm (Fig1. A). SEM confirmed the vesicular shape of the EVs, constituted of various sizes ranging from ~ 30 to 300 nm (Fig1. B). Total protein concentrations of the EVs were analyzed by NanoDrop and Bradford assays. The concentration of purified total protein was ~ 5 mg/ml and ~5/5 by using Bradford assay and NanoDrop method, respectively. The protein profile observed after SDS-PAGE showed bands ranging from 11 to 245 kDa (Fig1. C).

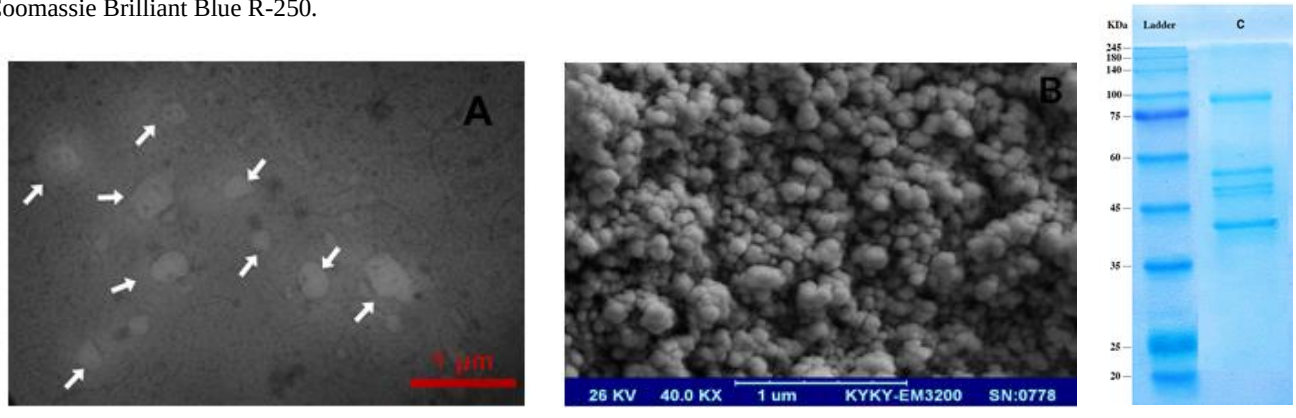

Fig. 1. Morphologic characterization and protein patterns of EVs derived from A. muciniphila. **A)** TEM image×18,000. Arrows indicate EVs in different sizes (30– 300 nm) and vesicle-like structures. Scale bar 1 µm. **B)** SEM image of the EVs at different sizes (ranging 40 to 150 nm). **C)** The SDS-PAGE protein bands from 11 to 245 kDa.

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A. muciniphila is a commensal bacterium of the intestinal tract. In this study, we introduced an improved method for isolation of *A. muciniphila* EVs, among numerous isolation and separation approaches of EVs. Here, we prepared *A. muciniphila*-derived EVs by sequential ultrafiltration at 125,000×g twice for 2 h. According to a previous study by Ashrafian and colleagues, *A. muciniphila* EVs were extracted by ultracentrifugation at 150'000× *g* for 2 hours [22]. For the comparison purposes, EVs of *Streptococcus pneumoniae* could be obtained by centrifugation at 100,000 × *g* (4°C) for 2 hours [23].

Herein, the obtained *A. muciniphila* vesicles had a particle size ranging from ~ 40 to 150 nm, based on TEM image examination. This showed a smaller sizes of *A. muciniphila* EVs, compared to pneumococcal EVs reported by Mehanny et al. with a size range of 130–160 nm[23]. Although *A. muciniphila* EVs reported by Chelakkot et al had a similar spherical shape, their reported sizes were smaller (40-60 nm)[16]. Based on TEM studies by Kameli et al. for *Escherichia coli* and *Staphylococcus aureus*, their monoculture-derived vesicles showed, the sizes of the vesicles were within a 70-200 nm range [24]. There are many reports of of *A. muciniphila*-derived vesicles with varieties in their size range and shape [22, 25, 26]. These varieties in shape and/or size range of EVs could be resulted from differences in bacteria and methods that have directly effects on their downstream analyses[22].

The extracted EVs in this study were confirmed as spherical shapes with a ~40 -150 nm size range by SEM analysis that were in agreement with other study conducted by Ashrafian et al. (21). While, other investigations by using SEM have reported that the shape and size of *A. muciniphila*-derived vesicles in approximately 2.00 µm (24). Our study revealed that the protein and profile pattern, and also the composition of the proteins were different in each EV. We observed by SDS-PAGE that the EVs had the molecular weights of approximately 40 to 245 kDa. In another study by Jafari et al., the molecular weights of *Faecalibacterium prausnitzii* derived-EVs ranged from 11 to 245 kDa[4]. While the *Lactobacillus casei* vesicle pattern obtained from PEG-based, and SDS-PAGE confirmation showed the range from 10 to 200 kDa (25).

The significant role of EVs in intestinal immune responses have been studied for years. Their potential role for induction of immune responses against the foreign pathogens such as enhancing the antigenicity of epitopes in vaccines make the EVs an interesting component for the vaccine design projects. However, there are still many concerns about their safety and possible cross-reactions which require further studies in future.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interests.

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