Application of AFM, TEM, and NTA for characterization of exosomes produced by placenta-derived mesenchymal cells

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Abstract. Exosomes and microvesicles usually require characterization at the single-particle level. The most commonly used methods for this purpose are transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA). However, atomic force microscopy (AFM) is also a promising method for the characterization of exosomes, and its opportunities seem underestimated. Here we describe the characterization of exosomes isolated from the conditioned media of human placenta cells using AFM, TEM, and NTA; we focus on the technical aspects of AFM imaging and data processing.

1. Introduction
Extracellular vesicles (EVs) are cell-derived particles surrounded by a lipid membrane. Their size is in the range from 50 nm to several micrometers, and size is the most straightforward basis for their classification. The smallest EVs are called exosomes (d≤200 nm); they are accumulated in multivesicular bodies (MVBs) and released into the extracellular space by fusion of MVBs with the plasma membrane [1]. Exosomes attract the interest of the scientific community due to their role in maintaining homeostasis and their diagnostic potential [2], [3].

The International Society for Extracellular Vesicles (ISEV) proposed that samples of exosomes and other EVs should be characterized using two different but complementary methods [4]. The most popular pair of methods is the combination of transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA) [5]. TEM can be used to distinguish the EVs from the other particles with similar size but different morphology, and thus to assess the purity of the sample. On the contrary, NTA can be used to measure the absolute concentration of the particles in a suspension. The combination of these two methods has been successfully used to characterize EVs of different origins [5], [6], [7].

Instead of TEM, one can use atomic-force microscopy (AFM); however, it is less popular, and the AFM-based exosome imaging procedures are less established than the TEM-based ones. Imaging exosomes using AFM can be conducted both in air and in liquid, with or without chemical fixation [8], [9], [10]. The substrate for sample deposition is usually mica - either bare or chemically modified. The
most detailed AFM-based exosome imaging procedure is described in [9]. It exploits Ni-modified mica as the substrate for sample deposition; aldehyde fixation is not needed; imaging is carried out in the air. Special precautions are taken to ensure the uniform adsorption of the particles on the substrate. However, it requires prolonged sample incubation on the substrate (12 hours at 4C).

In the current work, we show that the sample preparation and imaging procedure described in [9] can be modified to decrease the sample preparation time. We analyzed the same exosome sample using AFM, TEM, and NTA to compare the sizes measured with these three methods.

2. Materials and methods

2.1. Isolation of exosomes

The exosomes were isolated by differential centrifugation from conditioned media of human placenta-derived mesenchymal cells after their 72-hour incubation in DMEM supplemented with exosome depleted serum, as described in [6].

2.2. AFM imaging

Freshly cleaved mica sheets were treated with 5mM NiCl₂, rinsed with water for 2 minutes, and dried with a steam of air. The suspension of exosomes was diluted by PBS and deposited onto the modified mica with NiCl₂ for 5 minutes. Then the samples were rinsed with water for 15 minutes and dried.

Images were acquired using a Solver PRO microscope (NT-MDT, Russia) operating in semicontact mode. We used NSG10 silicon cantilevers (NT-MDT, Russia) with a typical tip curvature radius of 6 nm according to the manufacturer's specification. Scanning was carried out with 512x512 resolution at 1 Hz. Images were processed using SPMImageMagic (https://spm-image-magic.software.informer.com/), FemtoScan Online [11], and ImageAnalysis (NT-MDT, Russia).

2.3. Nanoparticle tracking analysis

Particle size distribution and concentration of isolated EVs were determined by nanoparticle tracking analysis (NTA) using a NanoSight LM10-HS instrument (Malvern Panalytical Ltd, UK) equipped with a 405 nm, 65 mW laser unit with passive temperature readout and high sensitivity camera of the EMCCD type Andor Luca (Andor, Belfast, UK). All measurements were performed in accordance with ASTM E2834 – 12(2018) with camera and video processing setups optimized for EV measurements for the laser/camera as described in [6, 12]. Briefly, each sample was diluted with particle-free PBS down to a concentration of about 1.5×10⁸ particles/ml. Twelve videos, 60 sec each, were recorded and processed using NTA software 2.3 build 33 (NanoSight Ltd., Amesbury, UK). Results from all measurements (total 4500-6800 tracks) were combined to obtain a particle size histogram and the total particle concentration corrected for the dilution factor using the NTA software feature (Malvern Panalytical Ltd, UK).

2.4. TEM imaging

The carbon-coated TEM grids (Ted Pella, USA) were treated using a glow discharge device Emitech K100X (Quorum Technologies Ltd., Great Britain) for 45 s. This treatment turned the carbon surface hydrophilic and increased the exosomes' adsorption. The suspension of exosomes was deposited onto the grid for 2-3 minutes and stained with 1% uranyl acetate for 1-2 minutes. Images were obtained using a transmission electron microscope JEM-1400 (Jeol, Japan) operating at 120 kV.

3. Results and discussion

Imaging exosomes with TEM using negative staining is a well-established procedure. Figure 1 (a) shows a typical TEM image of the studied sample. To measure the size distribution of the particles (Figure 1 (b)), we used the ScanEV online tool (bioeng.ru/scanev). The mean size was 75±34 nm (mean ± standard deviation). The particles' cup-shaped morphology is typical for the EVs analyzed using TEM [5, 6, 12].
The mean size of the exosomes measured using NTA was 94±7 nm, which was larger than the diameter measured using TEM (Figure 2). The difference can be explained by the impact of the solvation shell [7, 9].

![Figure 1. TEM image of the studied exosomes (a) and the corresponding size distribution (b).](image1)

For the AFM measurements, we modified mica with NiCl₂, as suggested in [9]. Figure 3 (a) shows the typical images of the control sample (PBS) and two samples prepared from the exosome suspensions with different concentrations (2x10^{12} and 5x10^{11} particles/ml, respectively). The control sample is not perfectly bare; it has some residual debris or salt with a maximum height of less than h=3 nm (b). For the exosome samples, we analyzed the particles with the height exceeding h_{min}=6.5 nm, which is more than two times larger than h. Also, we neglected the particles with an effective diameter of less than 20 nm.

To check the AFM measurement procedure for self-consistency, we acquired images with different scan sizes (either 1.5x1.5 um² or 3x3 um²) and compared the mean surface densities of the particles.
As expected, they perfectly matched when calculated over different scan sizes (Table 1). When the exosome concentration in solution was decreased four-fold, the mean surface density of the adsorbed particles decreased only two-fold. This could be explained by the electrostatic repulsion between the adsorbed exosomes and the ones in suspension or by some uncontrolled variations in the sample deposition procedure.

**Figure 3.** AFM images of the studied exosomes and the control PBS sample (a) and the corresponding height section profiles (b).

**Figure 4.** The size distributions of the exosomes. (a) and (b) were obtained on the concentrated sample \((2 \times 10^{12} \text{ particles/ml})\), (c) and (d) were obtained on the diluted sample \((5 \times 10^{11} \text{ particles/ml})\). (a) and (c) show the height distributions, (b) and (d) show the diameter distributions.
Figure 4 shows the height values and the diameters of the exosomes deposited onto the substrate from solutions with different concentrations (Figure 4 (a) and (b) correspond to the $2 \times 10^{12}$ particles/ml concentration, and Figure 4 (c) and (d) correspond to the $5 \times 10^{11}$ particles/ml concentration). The diameters were calculated as the area-based effective diameters. The mean height values of the two samples were $15\pm6$ nm and $14\pm6$ nm, and the diameters were $76\pm18$ nm and $67\pm16$ nm. The height values matched perfectly, and the difference in diameters could be explained by the tip convolution effect.

When exosomes (or some other EVs) are analyzed using TEM and negative staining, most researchers usually rely on cup-shaped morphology to distinguish between the EVs and the other particles, such as lipoproteins. However, this selection criterion is not applicable to the AFM images. Figure 3 shows only one particle among the globular ones (marked with a green frame). The cup-shaped morphology is believed to be an artifact induced by the particle adsorption onto the surface and sample drying [7, 9]. However, in our experiments, most particles observed on the mica surface retained globular morphology.

Table 1. Mean surface density comparison of different scan fields and concentrations.

| Scan size, $\mu$m | Mean surface density of the particles |
|------------------|--------------------------------------|
|                  | $c = 2 \times 10^{12}$ particles/ml in the suspension | $c = 5 \times 10^{11}$ particles/ml in the suspension |
| 3x3              | 60                                    | 31                                    |
| 1,5x1,5          | 64                                    | 28                                    |

The difference between the height and the diameter of the particles is typical for AFM [8–10]. The match between the diameters measured using TEM and AFM is surprising; the AFM-based measurements usually yield higher values than the TEM-based ones due to the tip broadening. However, in our case with TEM, we analyzed only the cup-shaped particles and neglected all the others, especially the small ones. Presumably, the effects of tip broadening in the AFM images and selection of the cup-shaped particles in the TEM images compensated each other, and the diameters coincided.

The key properties of the AFM-based measurements were the following:
1. The mean surface density of the adsorbed particles did not depend on the scan size.
2. The mean surface density of the adsorbed particles increased when the suspension concentration increased.
3. The mean particle size was the same when the exosomes were deposited onto the substrate after different dilutions of the suspension.

These properties were observed not only for the placenta-derived exosomes but also for the ones isolated from the conditioned cell growth media (A549 cell line, data not shown).

4. Conclusion
Imaging exosomes with AFM is less popular than TEM, so it still requires further optimization of the experimental procedures. In comparison with the previously described sample preparation procedure [9], at the exosome adsorption step, we used higher exosome concentration and lower adsorption time. Using a higher sample concentration is preferential not only because of time-saving but also because of the sample purity considerations. According to NTA data, cell culture media and buffers can contain debris at a concentration of up to $\sim 10^{10}$ particles/ml [6]. To minimize their adsorption onto the surface and their presence in the AFM images, we should ensure that the exosome concentration is as high as possible and the sample adsorption time is low. The validity of the AFM measurements can be
assessed by several control experiments, including a comparison of the samples prepared using different concentrations of the exosomes. In the current work, the data on sample size obtained using NTA, TEM and AFM were in good agreement. Here we used exosomes isolated from the conditioned media of human placenta cells; however, we expect that the described imaging procedures should be applicable regardless of the exosome origin.

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**References**

[1] Denzer K, Kleijmeer MJ, Heijnen HFG, Stoorvogel W and Geuze H J 2000 *J. Cell Sci.* 113 3365–3374
[2] Wang Y, Zhang Y, Cai G and Li Q 2020 *Int. J. Nanomedicine* 15 4257–4273
[3] Mashouri L, Yousefi H, Aref A R, Ahadi A M, Molaei F and Alahari S K 2019 *Mol. Cancer* 18 1–14.
[4] Théry C, et al. 2018 *J. Extracell. Vesicles* 7
[5] Bachurski D, et al. 2019 *J. Extracell. Vesicles* 8
[6] Skryabin G O, et al. 2021 *J. Cell Biochem.* 122 100–115
[7] Chernyshev V S, Rachamadugu R, Tseng Y H, Belnap D M, Jia Y, Branch K J, Butterfield A E, Pease L F, Bernard P S and Skliar M 2015 *Anal. Bioanal. Chem.* 407 3285–3301
[8] Woo J R, Sharma S and Gimzewski J 2016 *J. Circ. Biomarkers* 5
[9] Skliar M, Chernyshev V S 2019 Imaging of extracellular vesicles by atomic force microscopy. *J. Vis. Exp.* 151
[10] Beekman P, Enciso-Martinez A, Rho H S, Pujari S P, Lenferink A, Zuilhof H, Terstappen L W M M, Otto C and Le Gac S 2019 *Lab Chip* 19 2526–2536
[11] Yaminsky I, Akhmetova A and Meshkov G 2018 *Nanoindustry Russ.* 6 414–416
[12] Semina S E, Scherbakov A M, Vnukova A A, Bagrov D V, Evtushenko E G, Safronova V M, Golovina D A, Lyubchenko L N, Gudkova M V and Krasil’nikov M A 2018 *Molecules* 23 4–9