Scientific contribution/Original research

Morphological Parameters of Erythrocyte Extracellular Vesicles at Hypoosmotic and Isoosmotic Conditions

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Abstract: Extracellular vesicles (EVs) are membrane-enclosed structures of nanometer dimensions. They are formed by cells and can be found in isolates from body fluids. It is indicated that they play important role in intercellular communication in health and disease. In this work we observed the morpholoical parameters of EVs isolated by differential centrifugation from washed erythrocytes, in which vesiculation was accelerated by addition of detergent (sodium dodecyl sulfate). Aliquots of the isolate were suspended medium of two different osmolarities. Isolates were imaged with a scanning electron microscope (SEM) and the images were analyzed by using the contours of EVs from which the volume V, surface area S, and relative volume $v = (36\pi S^3/V^2)^{1/2}$ were assessed by a geometrical model. EVs were considered as spheres or spheroids. The isolates were rich in erythrocyte EVs so that representative sets (86 vesicles at osmolarity of 50 mOsmol/L and 109 vesicles at osmolarity of 300 mOsmol/L) could be outlined. The EVs at osmolarity of 50 mOsmol/L had shapes close to a sphere, while at osmolarity of 300 mOsmol/L they had elongated shapes. The shapes were approximated by prolate spheroids. The average volume/surface area of EVs at osmolarity 50 mOsmol/L were 3.18×10^5 nm^3/2.20×10^4 nm^2, and at osmolarity 300 mOsmol/L they were 4.3×10^5 nm^3/2.79×10^4 nm^2. The respective differences in favor of the values at 300 mOsmol/L were statistically significant and of sufficient power. The relative volume of EVs at 50 mOsmol/L and 300 mOsmol/L were 1 and 0.96, respectively. While the difference in v suggests that, similarly to erythrocytes, water enters EVs in order to attain the Donnan equilibrium, the differences in V and A suggest that selective popping of (larger) EVs in the hypoosmolar sample took place.

Keywords: Extracellular vesicles; Erythrocytes; Osmolarity; Differential centrifugation; Scanning electron microscopy (SEM); Morphology; Interdisciplinary connection; Medicine

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1. Introduction

Extracellular vesicles (EVs) are heterogenic group of nano-/micrometres particles, enclosed with a membrane, and found in samples of biological origin. They work as means of communication between cells and are involved in many physiological and pathological processes like embryogenesis, neuron communication, blood coagulation, inflammation, tumorigenesis, and horizontal gene transfer (Yáñez-Mó et al., 2015, van Niel et al., 2018). Procedures have been developed to harvest EVs from body fluids, blood being the one of particular interest. Extensive research has been put forward because of EVs' their potential implications as biomarkers for the observation the of development of different diseases, as medication, and as vectors for delivering active substances.

It is acknowledged that the erythrocyte shape depends on the composition of the intra and extracellular solution. The membrane of the erythrocyte is selectively permeable. The tendency of the species that can cross the membrane to equalize the (electro)chemical potential and osmotic pressure together with requirement that the outer and the inner solutions remain free or electric field constitutes the base of the Donnan equilibrium that determines the water content of the erythrocyte and therefore its volume (Loeb, 1921). It was of interest to investigate whether similar mechanisms are taking place also in sub-micron sized EVs that were shed off from erythrocyte plasma membrane and present from a physical view essentially similar membrane-enclosed system without internal structure.

In this work, we considered EVs isolated from samples of washed erythrocytes. Vesiculation was accelerated by addition of detergent. The isolate that we found rich in EVs was divided into two aliquots; one was suspended in a physiological isotonic solution (300 mOsm/L) and the other was suspended in a hypotonic solution (50 mOsm/L). The samples were imaged with scanning electron microscope, revealing the shape and size of the EVs. We approximated the contours of the EVs by known geometrical objects (sphere and prolate ellipsoids), for which the expressions for the three-dimensional volume and the surface area are available in an analytical form. As the micrographs contained very many images of EVs, statistical analysis could be made, and two populations pertaining to different osmolarities could be compared. To the best of our knowledge, this is the first report on the volume and surface area of erythrocyte extracellular vesicles, measured in populations of EVs of different osmolarities.

2. Methods

2.1 Blood sampling

Blood was donated by a female adult with no record of disease. The blood collection took place in the morning, the person was fasting for at least 12 hours before the collection. Blood was taken in evacuated Na-citrate tubes (4.5 mL; BD Vacutainers, 367714A, Becton Dickinson, USA.

2.2 Isolation of erythrocyte EVs

Isolation was performed as previously described in Božič, et al. (2021). Blood was centrifuged at 300 × g for 10 min. Plasma was removed and erythrocytes were washed three times with 300 mOsmol/L PBS-citrate. Erythrocytes were then suspended in PBS-citrate with the addition of 10 mM sodium dodecyl sulfate (SDS) (1:10 per sample). The sample was then incubated for 2 hours at room temperature on a rotary stirrer, followed by isolation of EVs by differential centrifugation according to the procedure: 300 × g 10 min, 2,000 × g 10 min, and 4,000 × g 10 min. The 4,000 × g supernatant was filtered through a 0.8-micron filter, then the filtrate was centrifuged at 50,000 × g and 4 °C for 70 min. The resulting pellet was washed with PBS-citrate. To obtain hypoosmolar conditions, one aliquote of the the isolate was suspended in dH$_2$O until a final osmolarity of 50 mOsm/L (for the sample 50 mOsmol/L) while the other aliquote was diluted to the same volume with the base buffer 300 mOsmol/L PBS-citrate.

2.3 Scanning electron microscopy (SEM) of EVs

A sample of EVs isolated from the fresh erythrocyte suspension and suspended in PBS-citrate buffer was diluted with the medium prepared with the appropriate combination of dH$_2$O and 4 M NaCl. Samples of EVs at different osmolarities (50 and 300 mOsmol/L) in PBS-citrate were incubated according to the literature (Božič, et al. (2021)) in 2% aq. solution of OsO$_4$ for 2 hours and applied to a

0.05-micron MCE filter. The filters were taken from the holder and placed into wells of the microtitter plate filled with solutions as described below. After three stages of rinsing in dH$_2$O, the samples were dehydrated in a series of ethanol solutions – from the solution with the lowest ethanol content to the highest: 30%, 50%, 70%, 80%, 90%, and "absolute" ethanol. This was followed by treatment of the samples with 30%, 50% and "absolute" hexamethyldisilazane. The samples were left overnight to dry in air.

To observe the samples under a JSM-6500F scanning electron microscope (JEOL Ltd., Tokyo, Japan), the samples were coated with Au/Pd (PECS Gatan 682).

2.4 Determination of the volume and surface area of EVs

SEM micrographs **(Figures 1** and **2**) were imported into the *CorelDRAW* software program (Corel Corporation version 24.0.0.301, 2022). In each of the micrographs (**Figure 1**), we chose two rectangles of equal sizes within which we assessed the shapes of the contours of all elements that we recognized as EVs.

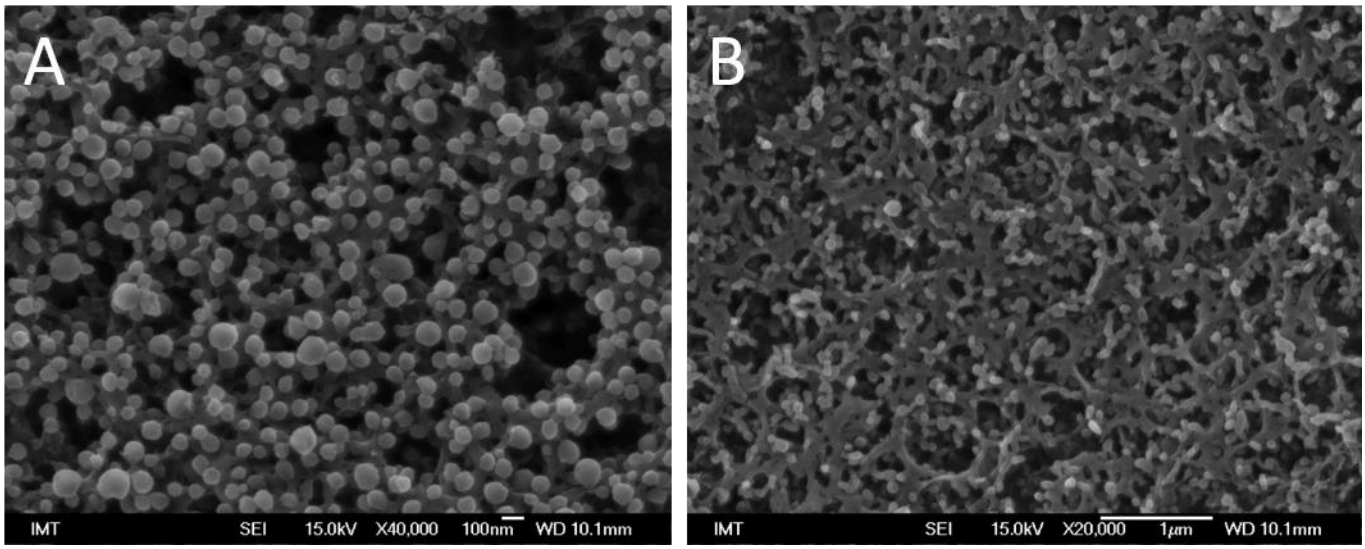

Figure 1. Micrograph of EVs at osmolarity 50 mOsmol/L, (A) and 300 mOsmol/L (B).

We assumed that the EVs had spheroid shapes (shape of rotational prolate ellipsoid, **Figure 2**). The contours of structures were fitted by ellipses with small semiaxes (marked *a* in **Figure 2**) and large semiaxis (marked *b* in **Figure 2**). To facilitate the determination of the semiaxes, the contours were rotated to present the larger semiaxis in the vertical direction (**Figure 2**).

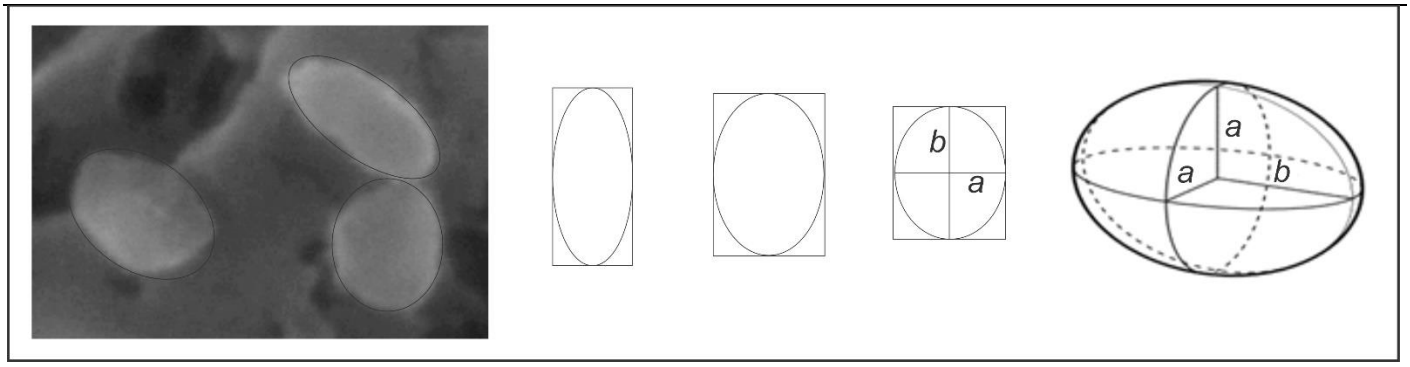

Figure 2. Fitting of the EV contours with prolate spheroid and determination of the semiaxes marked *a* and *b*.

The values of *a* and *b* were introduced into the expressions for volume and surface area of the prolate spheroid (Satterly, 1960),

$$V = \frac{4}{3}\ \pi a^2 \quad , \tag{1}$$

$$S = 2\ \pi a^2 + \left(\frac{2\ \pi\ a\ b^2}{\sqrt{b^2-a^2}}\ (\arcsin\ \frac{\sqrt{b^2-a^2}}{b})\right) \quad . \tag{2}$$

Also we calculated the relative volume *v* representing the ratio of volume to the surface area,

$$v = \sqrt[2]{\frac{36\ \pi\ V^2}{S^3}}\ . \tag{3}$$

2.5 Statistical analysis

Figure 1 shows that the isolates contained a large number of EVs, which allowed statistical analysis of the samples. We captured 86 EVs from the sample at osmolarity 50 mOsm/L and 109 EVs from the sample at osmolarity 300 mOsm/L.

The geometric parameters (*V*, *S* and *v*) of both populations were compared by using descriptive statistical methods. The mean values and the standard deviations of the two populations were calculated. The statistical significance of the differences between the two populations were calculated by the two-tailed *t*-test. The values of the probability *p* smaller than 0.05 were considered as statistically significant. We used Microsoft Excel (Microsoft Corporation, version 2018). Also we determined statistical power of the difference by using free software: Brant R. Inference for means: comparing two independent samples (https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html). The values of *P* larger than 0.8 at alpha 0.05 were considered of sufficient power.

3. Results and Discussion

Statistical analysis showed that the average volume and surface area of EVs of erythrocytes at osmolarity 50 mOsmol/L were substantially and statistically significantly smaller than at osmolarity 300 mOsmol/L. The relative volume, which indicates the ratio between volume and area, was however substantially and statistically significantly larger at higher osmolarity (**Table 1**). This indicates that the population of EVs at higher osmolarity contains larger and more flaccid EVs.

Table 1. Geometrical parameters of EVs at two different osmolarities, the differences between the hypoosmolar and isosomolar samples Δ and statistical parameters of the differences: the probability *p* and statistical power *P*.

Average value (standard deviation)	50 mOsmol/L [86 EVs]	300 mOsmol/L [109 EVs]	Δ	*p*	*P*
V (nm³)	3.18×10^5 (1.49×10^5)	4.32×10^5 (2.66×10^5)	-30%	$<10^{-3}$	0.82
S (nm²)	2.20×10^4 (0.68×10^4)	2.79×10^4 (1.09×10^4)	-24%	$<10^{-6}$	0.94
v	1.00	0.94 (0.07)	6%	$<10^{-12}$	1.00

The difference between the values of both osmolarities, at 50 mOsmol/L and 300 mOsmol/L, was normalized by the mean value at both osmolarities.

It seems reasonable and in line with previous results on erythrocytes (Canham, 1970) that in hypoosmotic medium water enters the EVs to approach the Donnan equilibrium. It is therefore expected that the shapes of EVs would approach spherical shape with decrease of the osmolarity, which was actually observed in our results (**Figures 1,2**). In agreement with this, the relative volume was higher at smaller osmolarity (**Table 1**). However, as the two samples were aliquots deriving from the same isolate, a question can be posed why the average surface areas of EVs of both samples would be different. A possible explanation of this could be that some of EVs (e.g. the larger ones) that approached spherical shape popped, leaving in the population the smaller ones.

At an osmolarity of 300 mOsmol/L, where the shapes are not spherically symmetric, the two dimensional image is limited in revealing the three-dimensional shape of the particle although the brighter parts of the image are closer to the source of the electrons (**Figure 2**). Since we cannot identify a specific reason for the preferred orientation of EVs, it may be that the EV which seems spherical in the image is actually a prolate ellipsoid oriented perpendicular to the plane of imaging. Therefore the volume and the surface area of the EVs at 300 mosm/L is underestimated. This would however further increase the difference in V, S and v of the two samples.

4. Conclusions

Increase of the relative volume of EVs in hypoosmotic medium indicates that EVs are (like erythrocytes) subjected to Donnan equilibrium. The explanation of the difference in average surface areas of EVs pertaining to the two aliquots at different osmolarities remains indecisive.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, blood was donated voluntarily by the authors of the study.

Conflicts of Interest: The authors declare no conflict of interest.

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