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Membrane proteins significantly restrict exosome mobility

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ABSTRACT

Exosomes are membrane nanovesicles implicated in cell-to-cell signaling in which they transfer their molecular cargo from the parent to the recipient cells. This role essentially depends on the exosomes' small size, which is the prerequisite for their rapid migration through the crowded extracellular matrix and into and out of circulation. Here we report much lower exosome mobility than expected from the size of their vesicles, implicate membrane proteins in a substantially impeded rate of migration, and suggest an approach to quantifying the impact. The broadly distributed excess hydrodynamic resistance provided by surface proteins produces a highly heterogeneous and microenvironment-dependent hindrance to exosome mobility. The implications of the findings on exosome-mediated signaling are discussed.

Exosomes are actively secreted by cells and found in all biological fluids, including blood, urine, and saliva. Compared to other extracellular vesicles (EVs), exosomes are distinguished by biomarkers of the late endocytic biogenesis and the smallest size, typically reported to be 30–150 nm in diameter. The uptake of an exosome by a local or distant cell transfers the molecular cargo derived from a secreting parent to a recipient cell. Biologically active molecules transferred in health and disease by this mechanism include surface and luminal proteins, membrane-bound microRNAs, and other compounds. A growing number of studies implicate exosomal signaling in tumor metastasis [1], drug resistance [2], and the modulation of immune response [3].

For signaling to occur it is necessary, but not sufficient, for a secreted EV to migrate from a parent to a recipient cell. In paracrine signaling, the transport barrier is overcome by diffusion through extracellular space, while to reach distal targets an EV may need to enter and exit a biofluid circulation. The smallest among EVs, the exosomes have the highest mobility which favors overcoming transport resistance for exosome-mediated signaling to take place [4]. The mobility of the exosomes may be quantified by their mean squared displacements (MSD) with time, which proportionally depends on their (self-) diffusivity. The oft-reported hydrodynamic diameter of exosomes is the measure of the resistance to the migration and is inversely proportional to their diffusivity.

Transport barriers to exosome dissemination are poorly understood. Here, we examine the impact of surface proteins and macromolecules anchored by them on the mobility of exosomes isolated by dissimilar methods (precipitation and size exclusion) from two biological fluids (growth medium of MCF-7 breast cancer cells and human sera). The excess resistance to the migration imposed by surface proteins is quantified as the thickness of a coronal layer formed by surface-conjugated macromolecules, which we measure as a difference between hydrodynamic and vesicle diameters of the exosomes.

The exosomes released by MCF-7 cells were isolated by precipitation and their enrichment in the isolate was confirmed by positive expression of surface and luminal biomarkers, the globular shape of vesicles with bilayer membrane morphology, and the hydrodynamic diameters in the exosomal range (Fig. 1a and S1-S2 in Supporting Information, SI). The mean and mode hydrodynamic

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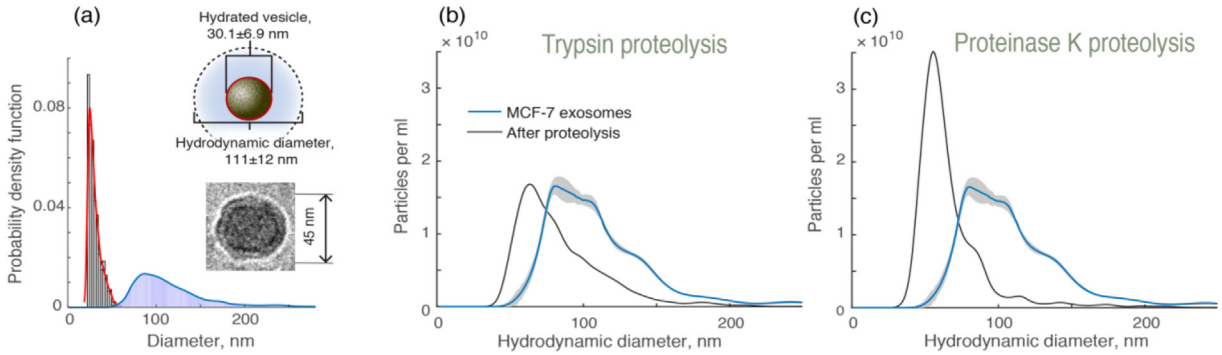

Fig. 1. (a) The size of exosome vesicles in the solution (red curve, grey fill) was obtained from the AFM measurements and confirmed by cryo-TEM imaging. Cryo-TEM shows the expected globular shape and double-layer morphology of vesicles (inset). The vesicles are substantially smaller than their hydrodynamic diameters measured by NTA (blue curve, blue fill). The average thickness of the exosomal corona (inset summary) is estimated as the difference between the average hydrodynamic and vesicle sizes. (b) Trypsinization of surface proteins substantially reduces hydrodynamic diameters. (c) The shift in mobility sizes is even more pronounced after a less specific protease K digestion. PK proteolysis narrows the distribution of hydrodynamic sizes, pointing to the heterogeneity in the macromolecular surface decoration as the source of widely different mobilities of untreated exosomes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

diameters measured by nanoparticle tracking analysis (NTA) were 111 ± 12 nm and 80 ± 9 nm, respectively. The volume encapsulated by hydrated vesicles was measured by atomic force microscopy (AFM; Fig. S5-S6 and Table S4, SI). The diameter of hydrated vesicles in their innate globular shape was estimated by mapping the AFM volume measurements, obtained for exosomes electrostatically immobilized on a surface, into volume-equivalent spheres in the solution. Fig. 1a shows the obtained probability density function of vesicle sizes, which has 30.1 ± 6.9 nm ensemble average. A much smaller size of vesicles compared with their hydrodynamic diameters was independently confirmed by cryo-TEM imaging which showed MCF-7 vesicles with an average diameter equal to 34.2 nm (Fig. S7a, SI). The macromolecular corona is indistinguishable in cryo-TEM images because of its low excess density [5,6].

The NTA measurements of hydrodynamic sizes were repeated after enzymatic proteolysis of MCF-7 exosomes. The four digestion protocols (Table S5, SI) differed in enzymes used and the treatment duration. A statistically significant ($p < 0.01$) increase in exosome mobility after the proteolysis by as much as 50% was observed (Fig. 1b and c; Fig. S3 and Table S2, SI). The duration of enzymatic treatments had little effect on hydrodynamic sizes, indicating a sufficiently long incubation to complete the digestion. The following factors were ruled out from contributing to the observed reduction in hydrodynamic sizes: a) Enzymatic treatments did not change the size of vesicles which remained in the same range before and after digestion (Fig. S8, SI and b) The protein concentration in the solution was too low (less than 3.5 mg/mL) to cause changes in the viscosity after the proteolysis [7].

The mobility of exosomes after proteolysis was enzyme dependent (Fig. 1b and c). Trypsin preferentially cleaves at lysine and arginine locations but does not affect surface-anchored segments of membrane proteins void of these α-amino acids. Trypsinization thinned the coronal layer, reducing the hydrodynamic size of exosomes to having the mode diameter equal to 64 nm, down from the original 80 nm (Fig. 1b and Table S2, SI). The reduction was even more significant (down to the 56 nm mode diameter; Fig. 1c) when we used a less specific proteinase K (PK), known to cleave peptide bonds of hydrophobic, aliphatic and aromatic amino acids. Compared to trypsinization, the broader PK activity leaves shorter undigested fragments of surface proteins and, thus, a thinner coronal layer, as reflected by a higher mobility of PK-treated exosomes. The indiscriminate PK proteolysis shifts the hydrodynamic diameters of the exosomes into the range of sizes partially overlapping with vesicle sizes. The remaining difference may be explained by short fragments of surface proteins that survived the digestion and the presence of pericellular coats formed by glycosaminoglycans, and hyaluronan specifically [8], which are unaffected by trypsin and PK.

The described mechanism of enzyme-dependent thinning of exosome corona is summarized in Fig. 2, where we compare the distributions of the vesicle and hydrodynamic diameters before and after the proteolysis. After the proteolysis, the hydrodynamic sizes are distributed more uniformly. The less specific PK treatment, which closely "shaves" the membrane surface, leads to the especially narrow range of hydrodynamic diameters with a closer resemblance to the distribution of vesicle sizes. The broadly distributed hydrodynamic diameters of vesicles with narrowly distributed membrane envelopes reveal the heterogeneity in the macromolecular surface decoration of exosomes and the widely varying thickness of their coronal layer from one exosome to another.

The direct visualization of diverse migration rates of MCF-7 exosomes was obtained by tracking the light scattered by a single exosome as it moves in PBS. The three trajectories in Fig. 3a were

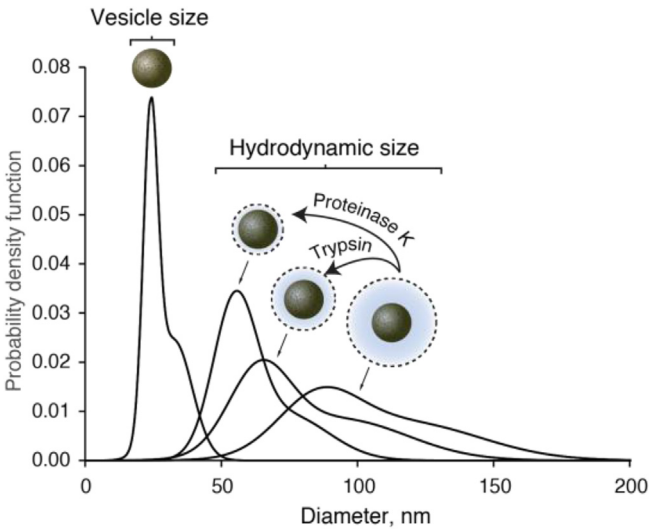

Fig. 2. The reduction in the coronal layers is enzyme dependent. Non-specific protein digestion by proteinase K shifts the hydrodynamic diameters closer to the range of vesicle sizes.

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