On the surface-to-bulk partition of proteins in extracellular vesicles

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Abstract

Nanomaterials are characterized by extremely large surface-to-volume ratio. Extracellular Vesicles (EVs) – which have been recently recognized as the universal agent of intercellular communication, being involved in many physiological and pathological processes and in interkingdom biochemical communication – are nanoparticles, but this key aspect has never been rationally addressed. Here we report the first attempt to quantify the membrane-to-lumen partition of proteins in EVs. A semi-quantitative model based on available well-established compositional and microstructural data is formulated. The model allows for estimation of the overall protein content of an EV as well as of the partition between membrane (surface) associated and lumen (bulk) contained proteins as a function of the EV size and shape. It further identifies 180 nm as a switch diameter, below which EVs result composed by more membrane than luminal proteins. At larger diameters the partition is reversed, reaching predominance of luminal proteins (> 80 %) in large EVs (diameter > 800 nm). The model is successfully tested to analyze and describe a real preparation composed of subpopulations of small EVs (diameter < 200 nm), including exosomes and ectosomes, and large EVs including large oncosomes (diameter > 1000 nm) from human prostate cancer cells. These findings provide basis for better colloidal description of EV samples, might help to understand the stoichiometry of proteins in distinct EV sub-populations and will improve design and interpretation of experiments, including EV engineering and dosing *in-vitro* and *in-vivo*.

Keywords

Extracellular Vesicles, Large Oncosome, Protein, Membrane, Nanoparticles, Bio-nano Interfaces

1. Introduction

One of the most popular properties of nanomaterials is the extremely large surface-to-mass ratio, which in some cases can hit more than 1000 m^2 per gram. At the microscopic level, to cut a bulk material into nanoparticles means to force half or more of the atoms to pass from the bulk to the surface. From a thermodynamic perspective, the increase of surface-to-bulk ratio is mirrored by an overall, marked increase of the surface energy and, ultimately, of the nanomaterial activity [1].

Despite a few studies on nanomaterials, this aspect has not been explored in EVs, although it is fundamental in determining the colloidal (nanoscale) and interfacial properties of EVs and EV-containing fluids, which in turn contribute to defining EV biological properties and function – including the EV corona [2,3] and EV adhesion, fusion, and cargo delivery to cells [4].

Here we focus on a particular aspect of the colloidal and interfacial properties of EVs: the partition of the proteins between the surface and the lumen. In fact, these soft biogenic nanoparticles carry soluble proteins enclosed in the lumen and surface proteins, either adsorbed, associated, or embedded in the membrane. Lumen proteins are part of the active cargo of EVs and largely depend on the cellular source and the biogenesis pathway of EVs, while surface proteins determine EV natural targeting and circulation properties [5].

Despite its capital importance, this is the first time such an analysis is proposed, and the few attempts only have considered the overall biomolecular content. Worth to mention the very first attempt made by Sverdlov [6] to estimate the protein content of EVs and the more recent calculations performed by Murphy *et al.* [7] and Baldwin *et al*. [8] to analyze the miRNA cargo.

Since proteins are physical entities and occupy space, the number of proteins that compose an EV must be finite, and strictly linked to physical parameters that define the EV surface area and bulk volume, such as the EV diameter. By applying a simple geometrical model to well-established data referred to whole cell obtained through different techniques (mass spectrometry, genomic analysis, or simple stoichiometry) available in the literature, we could estimate the overall number of moles of proteins and the surface-to-bulk molar partition of proteins in function of the EV size. In the following, a semi-quantitative model is presented, and its predictive value discussed. Then, its efficacy is shown in a working example on the analysis of a real sample of key biomedical interest, made of mixed populations of large (L-EVs) and small (S-EVs) EVs produced by the human prostate cancer PC3 cell line. Among the large EV sub-types produced by prostate cancer cells, large oncosomes are atypically large EVs (diameter > 1000 nm) that are generated by particularly aggressive and metastatic cancer cells during amoeboid motility [9]. Comparative studies using large oncosomes and ectosomes from the same cancer cell source have demonstrated that large oncosomes

are enriched with tumorigenic cargo, metabolic enzymes, and carry active kinases that elicit remarkable functional effects in cells that uptake them by phagocytosis [10].

2. Materials and Methods

2.1 Cell culture

PC3 human prostate cancer cells were purchased from ATCC and were grown in DMEM (Invitrogen/Corning), supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Invitrogen), and 1% PenStrep (Invitrogen). All cells were grown at 37°C and 5% CO_2. Cell viability was tested with Trypan Blue (Sigma) exclusion method. The cell line was routinely tested for mycoplasma contamination (MycoAlert PLUS Mycoplasma Detection Kit, Lonza).

2.2 EV isolation and characterization

PC3 EVs were isolated from cell culture supernatants and characterized by western blotting and imaging as previously described [10-13]. Briefly, cells were grown in 18 × 150 mm-cell culture plates (Corning) until 80% confluence, then washed in PBS and serum-starved for 24 hours before the collection of conditioned cell media. The conditioned media was cleared by differential centrifugation of floating cells (3 x 5 min at 300·*g*), of cell debris (2,800·*g* for 10 min), and then spun at 10,000·*g* for 30 min (4°C, k-factor 2547.2) for the collection of L-EVs. 10,000·*g* pellet was then subjected to Optiprep™ (Sigma) density gradient purification (bottom-up flotation). Fresh pelleted L-EVs were resuspended in 0.2 µm-filtered PBS and deposited at the bottom of an ultracentrifuge tube. Next, 30% (4.3 mL, 1.20 g/mL), 25% (3 mL, 1.15 g/mL), 15% (2.5 mL, 1.10 g/mL), and 5% (6 mL, 1.08 g/mL) iodixanol solutions were sequentially layered at decreasing density to form a discontinuous gradient. Separation was performed by ultracentrifugation at 100,000·*g* for 4h (4°C, k-factor 254.7) and EV-enriched fractions were collected at 1.10–1.15 g/mL. Purified EVs were then washed in PBS (100,000·*g*, 60 min, 4°C) and resuspended in PBS. Ultracentrifugation spins were performed in an SW28 rotor (Beckman Coulter).

2.3 EV quantification and size distribution

Resuspended EVs were analyzed by both Bicin Chinonic Acid assay (BCA, Thermo Fischer Scientific) and tunable Resistive Pulse Sensing (tRPS) on a qNano Gold (Izon, Christchurch, New Zealand) to measure protein content and EV size distribution and concentration, respectively. tRPS analysis was performed using an NP2000 nanopore or an NP250 nanopore as previously described [13]. Briefly, a stable current baseline at 110 nA was achieved by stretching nanopores at 47 mm and setting the voltage either at 0.06 V for NP2000 nanopore or 0.5 V for NP250 nanopore. Detection

parameters were calibrated using Izon calibration particles (1:1000 diluted CPC2000 for NP2000, and 1:100 diluted CPC200 for NP250) and a minimum of 500 events were registered for each sample, with a positive pressure of 5 mbar. Three technical replicates were acquired for each sample.

3. Results and Discussion

3.1 Estimation of protein content and surface-to-bulk protein ratio in EVs

In first approximation, the total molar protein content n of a particle carrying proteins on both its surface and in its bulk can be split into two contributions: the surface protein molar content (n^{σ}) and bulk protein molar content (n^{b}):

$$n = n^{\sigma} + n^{b} \quad (1).$$

Eq. 1 can be reformulated as follows:

$$n = (\Gamma \cdot A_s) + (\rho \cdot V) \qquad (2)$$

where Γ and ρ are the surface molar density of proteins and the bulk molar density of proteins, respectively, and A_s and V are particle surface area and particle volume, respectively.

Eq. 2 can theoretically be used to calculate n for particles of any shape, provided A_s and V are known. EVs are reported to have various sizes and shapes [14] but are often approximated to spheres or spheroids. For a spherical particle, eq. 2 can be reformulated:

$$n = (\Gamma \cdot 4\pi r^2) + (\rho \cdot \tfrac{4}{3}\pi r^3) \quad (3)$$

where r is the particle radius. This equation well evidences the relationship between the total molar protein content n of a particle and its size. Furthermore, after calculating n, it is in principle possible to approximate the total protein mass T_m a particle transport by assuming all the carried proteins have similar molecular weight:

$$T_m = n \cdot a_{MW} \qquad (4)$$

where a_{MW} is the average molecular weight of the single protein.

The application of the proposed equations to EVs may pose a challenge, since Γ and ρ have never been measured at the EV level. A good starting point may be represented by the calculations performed on whole cells. Indeed, the values for Γ and ρ in cells have been reported by others [15-

22] and attested to be ~ $5.0 \cdot 10^{-20}$moles/µm^2 for Γ and between $6.6 \cdot 10^{-19}$ - $6.6 \cdot 10^{-18}$ moles/µm^3 for ρ, depending on the measuring technique. Moreover, in *H. Sapiens* (and in other organisms) [23, 24, 25], a_{MW} has been estimated between ~ 4.0 and $5.5 \cdot 10^4$ Da [26, 27], depending on the technique applied.

As a proof-of-concept, we calculated the total protein content of an ideal spherical EV of d = 100 nm following the proposed approach. For the next calculation, Γ and ρ are assumed to be $5.0 \cdot 10^{-20}$ moles/µm^2 and $1.7 \cdot 10^{-18}$ moles/µm^3 (being an intermediate value among the ones published), respectively. a_{MW} is set to $5.0 \cdot 10^4$ Da, a recurrent value found in the literature. Using the selected parameters, T_m becomes:

$$T_m = n \cdot a_{MW} = \left[\left(5.0 \cdot 10^{-20} \frac{n}{\mu m^2} \cdot 4\pi \cdot 2500\ nm^2\right) + \left(\frac{1.7 \cdot 10^{-18} n}{\mu m^3} \cdot \frac{4}{3}\pi \cdot 125.000\ nm^3\right)\right] \cdot 5 \cdot 10^4 = 1.3 \cdot 10^{-16} g$$

This result is in line with other calculations made on EVs following a different approach [6], and therefore the selected values for Γ, d and a_{MW} were also applied in the working example shown in section 3.2. Moreover, having estimated the total molar protein content n, it is easy to calculate the relative molar protein partition between EV surface ($\%n^\sigma$) and EV bulk ($\%n^b$):

$$\%n^\sigma = \frac{n^\sigma}{n^\sigma + n^b} \quad (5)$$

$$\%n^b = \frac{n^b}{n^\sigma + n^b} \quad (6)$$

For the ideal, spherical EV, Eq. 5 and 6 can be reformulated as follows:

$$\%n^\sigma = \frac{n^\sigma}{n^\sigma + n^b} = \frac{(\Gamma \cdot 4\pi r^2)}{(\Gamma \cdot 4\pi r^2) + (\rho \cdot \frac{4}{3}\pi r^3)} \quad (7)$$

$$\%n^b = \frac{n^b}{n^\sigma + n^b} = \frac{(\rho \cdot \frac{4}{3}\pi r^3)}{(\Gamma \cdot 4\pi r^2) + (\rho \cdot \frac{4}{3}\pi r^3)} \quad (8)$$

Arguably, both $\%n^\sigma$ and $\%n^b$ are strongly influenced by EV diameter. This aspect is visualized in fig.1, where $\%n^\sigma$ and $\%n^b$ (calculated for $\Gamma = 5.0 \cdot 10^{-20}$ moles/µm^2 and $\rho = 1.7 \cdot 10^{-18}$ moles/µm^3) have been plotted as functions of EV diameter. According to the calculation, smaller EVs carry more proteins on their surface (blue dashed line) than in their bulk (red dashed line) and *vice versa*. Protein partition eventually reaches a switch point ($\%n^\sigma = \%n^b$) at d =180 nm (fig.1, black-dotted line),

within the size range typical of S-EVs (50-200 nm). The trend then inverts, with bulk proteins quickly overtaking surface ones, until the ratios stabilize in the size range typical of larger EVs, such as large oncosomes (d > 1000 nm).

In the next sub-sections, a working example of the proposed estimations applied to real EVs is shown. The calculations were used to support data interpretation on a preparation of human prostate cancer L-EVs. L-EVs were selected because i) L-EV preparations are usually less contaminated by soluble proteins, thus making total protein quantification more reliable and ii) according to what we showed in fig.1, L-EVs (in particular large oncosomes, of which the preparation is enriched) represent a peculiar "limiting case" for testing our calculations since most of the proteins are expected to locate in the EV bulk.

3.2 A working example on L-EVs

Human L-EVs were separated from cell culture medium and analyzed by tRPS and the BCA assay as described in the M&M section. L-EVs show a size distribution (fig.2A) centered at d = 1200 nm. EV diameter spans from 900 to 4500 nm, with ~ 90.0% of the particles within the 900-1800 nm range. For tRPS 1,48 µL of L-EV stock sample - corresponding to 1,23 µg of total protein mass were measured (fig.2B, cyan bar). However, the estimation indicates that the measured total particle number is too low to contain such an amount of protein. Indeed, the predicted total protein mass, T_m, in a L-EV sample with the given particle number and size distribution should be T_m ~ 0,36 µg, (fig.2B, grey bar), which is ~ 1/3 of the protein mass measured by the BCA assay.

Such discrepancy may origin from two crucial aspects: i) the tested EV preparation contains soluble protein contaminants or ii) the preparation is a mixture of EV populations with a broad size distribution. Hypothesis (i) was excluded thanks to the strict purification protocol used to separate EVs from culture medium, which minimizes protein contaminants in solution. Therefore, the second option was investigated. Indeed, a fraction of S-EVs could co-isolate with L-EVs and would be undetectable by tRPS using the NP2000 nanopore. Hence, the EV preparation was further characterized using the NP250 nanopore which allows quantification of smaller EVs (with d in the range of 120 – 400 nm). As expected, the sample contained a considerable amount of S-EVs (fig.3), thus confirming its mixed composition. The size distribution and the particle number of the S-EVs are reported in fig. 3A and 3B, respectively. S-EVs feature a size distribution (fig.3A) peaked at d = 200 nm. EV diameter spans from 120 to 400 nm, with ~ 90.0% of the particles within the 120 - 300 nm range.

The EV T_m was then estimated again including smaller EVs in the calculation. Considering now the contributions from both L-EVs ($T_{m\ L\text{-}EVs}$ = 0.36 µg, fig.3C table and green bar) and S-EVs ($T_{m\ S\text{-}EVs}$ =

0.79 µg, fig.3C, table and orange bar) to the total protein mass T_m of the analyzed sample, the experimental measurement and the weighted estimation almost overlap (1.23 µg vs. 1.15 µg, fig.3B). Interestingly, the S-EV number surpasses the L-EV number by a ~ 10^2 factor (fig.3C). Whether the S-EVs were co-isolated with L-EVs or generated by the fragmentation of unstable L-EVs during the purification process is unknown. Nevertheless, the presented "rule of thumb" calculations has driven S-EV identification, proofing the key benefit of the model in the estimation of the contribution of the different EV populations to the total protein mass and in turn in data interpretation of real EV preparations.

In addition, the model allows to estimate the specific and overall molar partition of proteins between EV surface ($\%n^{\sigma}$) and EV bulk ($\%n^{b}$) in the whole sample. By using the data reported in Fig. 2A and Fig. 3A the protein molar partition weighted on size distribution and particle number of the L-EVs and S-EVs was calculated. The estimation includes the specific partitions for L-EVs (Fig.4A) and S-EVs (Fig.4B), as well as the overall partition (Fig.4C). Fig. 4A and Fig. 4B highlight a substantial difference of $\%n^{\sigma}$ and $\%n^{b}$ between the two EV populations. It results that L-EVs carry more than 90% of their protein content in their lumen (yellow slice), with only about 10 % of proteins harbored in their membrane (cyan slice). To the contrary, the S-EVs display about 40% of their total protein content in their membranes (lilac slice) and the remaining 60% enclosed in the lumen (green slice), thus showing a more balanced protein partition compared to L-EVs. Finally, the overall data (Fig.4C) describe an EV preparation in which the bulk proteins overwhelm the membrane ones (~ 75% vs. ~ 25%, respectively). Mass spectrometry of L-EV and S-EV samples, together with the interrogation of available proteomic datasets is underway to further validate the estimations showed in Fig.4.

This proposed, not trivial, estimation of protein partition in polydisperse preparation of EVs should provide improved design, control and understanding of a wide range of EV experiments. For example, peculiar protein partitioning may contribute, together with other parameters such as membrane stiffness or the enrichment of specific surface proteins, to the different uptake and effects observed for S-EVs and L-EVs of the sample under analysis [10].

Conclusions

The theoretical and experimental outcomes reported in this communication provide a new complementary way to describe EVs. In particular, they identify a switch diameter, located at about 200 nm, below which the EVs are composed by more membrane (surface) than luminal (bulk) proteins, and above which the partition is reversed and become, reaching predominance of luminal proteins (> 80 %) in EVs with a diameter larger than 800 nm. The model is successfully tested to

analyze a real formulation of EVs of relevance to cancer research composed of subpopulations of S-EVs and L-EVs. Further refinements of the model and tests on its limits and its applicability are currently ongoing, including proteomic analyses and *in vitro* functional experiments.

Overall, the proposed advancement in understanding of EV samples widens the perspectives in experimental design and data analysis, including: (i) ways of dosing EVs *in vitro* and *in vivo*, (ii) interpretation of EV biological effects and (iii) drawing protocols for EV engineering. Last, but not least, extension of the description to any biogenic, hybrid or synthetic nanovesicle is straightforward.

Author contributions

Conceptualization: P.B., A.Z. Data curation: G.G., K.S., A.Z. Funding acquisition: P.B., D.D.V. Investigation: G.G., K.S., T.V., A.Z. Methodology: G.G., K.S., T.V., A.Z., P.B. Project administration: P.B., D.D.V. Resources: P.B., D.D.V., T.V. Supervision: P.B., D.D.V. Visualization: A.Z. Writing - original draft: P.B, A.Z. Writing - review & editing: all authors.

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Captions

Figure 1. Representation of the protein molar partition between EV surface ($\%n^{\sigma}$) and EV bulk ($\%n^{b}$) as functions of EV radius. Represented $\%n^{\sigma}$ and $\%n^{b}$ were calculated for $\Gamma = 5.0 \cdot 10^{-20}$ moles/μm^2 and $\rho = 1.7 \cdot 10^{-18}$ moles/μm^3.

Figure 2. L-EV characterization. A) L-EV size distribution and particle number measured through tRPS. B) Experimental measurement (cyan, BCA assay) and mathematical estimation (grey) of L-EV total protein mass T_m. Predicted T_m was calculated for $\Gamma = 5.0 \cdot 10^{-20}$ moles/μm^2 and $\rho = 1.7 \cdot 10^{-18}$ moles/μm^3, respectively, and tailored on the specific particle size distribution and concentration of the tested sample.

Figure 3. Characterization of S-EVs in the EV preparation. A) S-EV size distribution. B) L-EV vs. S-EV particle number in the analyzed EV preparation. S-EV number is considerably higher (~ 300-fold) than L-EV one. C) Comparison between the total protein mass T_m measured through BCA assay (cyan bar) and mathematically estimated (orange + green bar). The experimental and predicted total protein mass are almost identical. Predicted T_m was calculated for $\Gamma = 5.0 \cdot 10^{-20}$ moles/μm^2 and $\rho = 1.7 \cdot 10^{-18}$ moles/μm^3, respectively**.**

Figure 4. Partition of proteins in the EV sample under analysis. A) Specific partition of proteins in L-EVs. B) Specific partition of proteins in S-EVs. C) Overall protein partition (S-EVs + L-EVs).