SHORT COMMUNICATION

High-speed centrifugation induces aggregation of extracellular vesicles

Romain Linares¹, Sisareuth Tan¹, Céline Gounou¹, Nicolas Arraud¹ and Alain R. Brisson¹,²*

¹Molecular Imaging and NanoBioTechnology, University of Bordeaux, Pessac, France; ²Institut Universitaire de France, Paris, France

Plasma and other body fluids contain cell-derived extracellular vesicles (EVs), which participate in physiopathological processes and have potential biomedical applications. In order to isolate, concentrate and purify EVs, high-speed centrifugation is often used. We show here, using electron microscopy, receptor-specific gold labelling and flow cytometry, that high-speed centrifugation induces the formation of EV aggregates composed of a mixture of EVs of various phenotypes and morphologies. The presence of aggregates made of EVs of different phenotypes may lead to erroneous interpretation concerning the existence of EVs harbouring surface antigens from different cell origins.

Keywords: extracellular vesicles; blood plasma; cryo-electron microscopy; immuno-gold electron microscopy; flow cytometry; vesicle aggregation; centrifugation

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*Correspondence to: Alain R. Brisson, UMR-5248-CBMN, Bat. B14, Allée Geoffroy Saint-Hilaire, FR-33600 Pessac, France, Email: a.brisson@cbmn.u-bordeaux.fr

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Blood and other body fluids contain membrane vesicles that are released by cells upon activation or apoptosis (1–3). According to current hypotheses, cell-derived vesicles either form by blebbing and shedding of plasma membrane pieces or they are secreted by cells after fusion of multivesicular endosomes with the plasma membrane. Here, the term extracellular vesicle (EV) will refer to all types of vesicles present in blood plasma (4,5). EVs are objects of low abundance suspended in biological fluids that are highly enriched in proteins and other components; thus purification and concentration are often required for EV analysis. Centrifugation is the most popular method of EV isolation, as indicated by its use in a large number of studies (6–20). Centrifugation enables not only separation of EVs from proteins and most other soluble components, but also concentration of EVs or elimination of unbound probes in labelling studies. However, several studies have reported that centrifugation may cause aggregation or morphological changes of EVs (11,21,22), which could cause artefacts and lead to erroneous conclusions about EV composition or phenotype.

In order to further address this question, we decided to use cryo-electron microscopy (EM) combined with immuno-gold labelling. Using this approach, we were recently able to reveal in detail the diversity of EVs in pure plasma, showing in particular that EVs from the plasma of healthy subjects are isolated, with a total absence of aggregates (23). In addition, we used flow cytometry (FCM), which is the main method of EV characterization (24–26), to compare plasma samples before and after high-speed centrifugation.

Materials and methods

Reagents

Anti-CD235a (glycophorin-A) and CD41 (αIIb chain of αIIbβ3 integrin) monoclonal antibodies (mAb) either unlabelled or conjugated to PE were from Beckman Coulter (Villepinte, France). Phe-Pro-Arg chloromethyl ketone (PPACK) was from Haematologic Technologies (Cryopep, Montpellier, France). SPHERO Ultra Rainbow beads (1 μm) were from Spherotech (Interchim, Montluçon, France). F-XC100 (1 μm) and F-XC040 (400 nm) were from ESTAPOR (Merck Chimie SAS, Fontenay-sous-Bois, France).
Preparation of plasma samples
Blood was collected after written informed consent from 4 healthy donors who had fasted for at least 12 h. Blood was drawn in 4.5 mL BD Vacutainer® tubes containing 0.5 mL of 129 mM sodium citrate (BD, Le Pont de Clai, France). The preparation of platelet-free plasma (PFP) was started within less than 1 h after blood collection and consisted of 2 consecutive cycles of centrifugation at 2,500 g for 15 min (27).

High-speed centrifugation of PFP samples
Fresh PFP (1.5 mL) was mixed with 3 mL HEPES-buffered saline (HBS) containing 10 mM HEPES pH 7.4, 150 mM NaCl and 2 mM Na23 as a preservative. The mixture was centrifuged at 100,000 g for 1 h at 20°C with a low brake in a Beckman Coulter Optima Max-E ultracentrifuge using a MLS 50 rotor and polyallomer tubes. After centrifugation, 3.8 mL of supernatant were discarded and the pellet, which is not visible, was homogenized at least 10 times by gentle pipetting with a 200-μL pipette. The volume of the pelleted suspension was then adjusted to 1.5 mL with HBS containing 0.1% BSA and 10 μM PPACK as anticoagulant (28) (HBS-BSA). This resuspended pellet is referred to hereafter as 100k-PFP.

Sample preparation and observation for cryo-EM experiments
Annexin-A5 conjugated to 4-nm gold nanoparticles (Anx5-gold-NPs) and anti-CD235a or anti-CD41 mAbs conjugated to 10-nm gold-NPs (anti-CD41-, anti-CD235a-gold-NPs) were produced as previously described (23).

Samples were labelled as follows. Either PFP or 100k-PFP (6 μL) was mixed with 1 μL anti-CD235a- or anti-CD41-gold-NPs at 1–3 × 1016 NP/L and incubated for 30 min at ambient temperature. Next, 1 μL 100 μM PPACK, 1 μL Anx5-gold-NPs at 1–3 × 1016 NP/L, and 1 μL 100 mM CaCl2 were added, then samples were further incubated for 15 min and processed for cryo-EM.

A 4-μL sample aliquot was deposited onto an EM grid coated with a perforated carbon film (Ted Pella, Eloīse Company, Eindhoven, Netherlands). Images were recorded with a Tecnai F20 microscope operated at 200 kV (FEI Company, Eindhoven, Netherlands). Images were recorded with a USC1000-SSCCD Gatan camera.

Flow cytometry
Samples were labelled as follows: 50 μL PFP or 100k-PFP were mixed with either 10 μL pure anti-CD41-mAb-PE or 20 μL anti-CD235a-mAb-PE diluted 10 × with HBS-BSA; then samples were incubated for 2 h in the dark prior to dilution to 500 μL with HBS-BSA. Next, 20 ng/mL Anx5-Cy5 and 10 mM CaCl2 were added and samples were incubated for an additional 1 h prior to analysis. Before FCM analysis, 1 μm Ultra Rainbow beads were added to each tube at 5 × 108/mL to measure the acquired volume.

FCM was performed with a Gallios flow cytometer (Beckman Coulter) and data were analysed using Kaluza 1.2. The detection of EVs was triggered on the forward scatter (FS) parameter, and only EVs labelled with either Anx5-Cy5 (FL6) or mAb-PE (FL2) were taken into consideration. FS detection sensitivity was tested daily using a mixture of 1 μm and 400 nm FL1 fluorescent beads, ensuring an FS detection efficiency of 98 ± 2% of the 400 nm beads. Each tube was measured twice and EV concentrations, expressed as mean ± SD, were calculated by taking into account the number of 1 μm Ultra Rainbow beads acquired and the sample dilution.

Results

1) EM analysis of PFP samples after high-speed centrifugation
Cryo-EM analysis shows that pure, unprocessed PFP samples consist of isolated EVs (Fig. 1). On the other hand 100k-PFP samples show EV aggregates (Fig. 2), together with isolated EVs. Figure 2 shows 3 representative examples of EV aggregates, in order to appreciate the variety of structures induced by high-speed sedimentation. Figure 2a presents an EV aggregate from a 100k-PFP sample labelled by both 4-nm Anx5-gold-NPs and 10-nm anti-CD41-gold-NPs. In this aggregate, several EVs are mixed with 10 mM CaCl2 for PFP or 2 mM CaCl2 for 100k-PFP and incubated for 30 min at ambient temperature.

Samples were then diluted 30 times with a buffer containing 100 mM sodium cacodylate (2 mM CaCl2, pH 7.4) and deposited into polyallomer centrifuge tubes containing 4 EM grids coated with a continuous carbon film (Electron Microscopy Sciences, LFG-Distribution, Lyon, France), fixed on a resin support (23). Samples were centrifuged at 100,000 × g for 1 h at 20°C as described above, after which the liquid above the EM grids was carefully removed, and the EM grids were recovered and air-dried.

Grids were observed with an FEI CM120 microscope operated at 120 kV. Images were recorded with a USC1000-SSCCD Gatan camera.

This method, referred to as the on-grid sedimentation method, was previously described (see Supplementary Fig. 1 in (23)).
It must be stressed that we did not observe EV aggregates in non-centrifuged PFPs from healthy donors, over more than 100 PFP samples analysed by cryo-EM. This is easily explained by the low concentration of EVs present in normal PFP. Indeed, for a concentration of about 100,000 EVs/µL (29), each EV occupies a cube of about 50 μm edge length. Therefore, the probability of EV collision within a PFP suspension or during the draining process on an EM grid is extremely low. The formation of EV aggregates must therefore result from the fact that, upon high-speed centrifugation, all the EVs contained in a centrifuge tube are concentrated in a small volume, promoting their interaction.

What are the relative amounts of EV aggregates and isolated EVs in 100k-PFPs? This question cannot be addressed by cryo-EM, because an artefact referred to as *fish-net artefact* occurs during the preparation of cryo-EM specimens (23). Indeed, while draining a small droplet of PFP through the perforated carbon net covering cryo-EM grids, the largest objects have a high probability of being retained by the net while isolated EVs, which are smaller, pass freely through the net, as previously reported (see Supplementary Fig. 5 in (23)). Due to this artefact, the proportion of large objects, thus the EV aggregates here, is overestimated. Therefore, in order to determine the relative amounts of isolated EVs and EV aggregates in 100k-PFP samples we used another EM approach, called *on-grid sedimentation*, which has already been applied successfully to the enumeration of EVs in unprocessed PFP samples (29,30). Figure 3 shows representative images of a PFP (A) and a 100k-PFP (B,C) sedimented onto an EM grid after labelling with Anx5-gold-NPs. With the PFP sample isolated EVs are observed, homogeneously distributed, with no EV aggregates (Fig. 3a).

In contrast, the 100k-PFP shows isolated EVs (arrows) together with an EV aggregate (Fig. 3b and c). Two independent experiments were performed, in which we measured the numbers of isolated EVs and EV aggregates before and after high-speed sedimentation. We found that, after high-speed sedimentation, the concentration of isolated EVs decreased from 29,500 ± 500 to 11,500 ± 500 (expressed per microlitre pure PFP), whereas 3,000 ± 1,000 EV aggregates were found in 100k-PFP. These values must be taken as only indicative, because several parameters, principally the conditions used for resuspending the pellets, are likely to affect the aggregate size and concentration.

Similar results of EV aggregation were obtained when PFP centrifugation was performed at 20,000 × g (data not shown). In addition, similar results of EV aggregation were observed when pellets were resuspended in PFP depleted of EVs instead of HBS-BSA (data not shown).

2) FCM analysis of 100k-PFP samples

As FCM is the most common method used for characterizing EVs, we investigated the impact of EV aggregation...
on FCM data. In this study, we used light scattering for triggering detection, because the focus is on the formation of EV aggregates and light scattering is best adapted for detecting large objects (31,32). Figure 4 shows the FCM analysis of a PFP and its counterpart 100k-PFP, after double labelling with Anx5-Cy5/anti-CD41-PE or Anx5-Cy5/anti-CD235a-PE. Concentrations expressed as EVs/μL sample are summarized in Table I. High-speed

**Fig. 2.** Representative cryo-electron microscopy images of EV aggregates in 100k-PFP samples. (a, d–g), double labelling with 10 nm anti-CD41-gold-NPs and 4 nm Anx5-gold-NPs; (b, c), double labelling with 10 nm anti-CD235a-gold-NPs and 4 nm Anx5-gold-NPs. In (a), several A5−/CD41+ EVs and A5+/CD41− EVs are indicated, associated with some amorphous material (white asterisk). (b), Aggregate associating several 10s of EVs, including several A5−/CD235a+ EVs. (c), High magnification view of the dashed box from (b), allowing one to distinguish between A5+/CD235a− EVs and A5−/CD235a− EVs. One Anx5-gold-NP is circled in red. The white asterisk points to amorphous material. (e–g), High magnification views of the EVs from (d) labelled +/+ (for A5+/CD41+), +/− (for A5+/CD41−) and −/− (for A5−/CD41−), respectively. One Anx5-gold-NP is circled in red in (e) and (f). For the sake of clarity, the carbon net has been overlaid in turquoise in (a, b, d). Scale bars: a, b, d: 500 nm; c, e–g: 100 nm.

**Fig. 3.** Representative images of EVs from (a) PFP and (b, c) 100k-PFP sedimented onto electron microscopy grids after Anx5-gold labelling. (a) Isolated Anx5-positive EVs are observed, with no EV aggregates. (b) An EV aggregate, about 800 nm in overall size, is observed, together with isolated EVs (arrows). (c) High magnification view of the dashed box from b; the EV aggregate contains Anx5-positive and Anx5-negative EVs. Scale bars: 500 nm.
centrifugation was found to induce an increase in the total amount of EVs, in the number of Anx5-positive EVs and in the number of double labelled EVs. The increase of the amount of double labelled EVs, both Anx5+/CD41+ and Anx5+/CD235a+, strongly suggests the formation of EV aggregates. However, it is not possible to distinguish genuine double-positive isolated EVs from EV aggregates within these EV populations.

As indicated in the Materials and methods section, the 100k-PFP was obtained after repeated gentle homogenization with a 200-µL pipette. As the pellet itself is too small to be visible, we questioned the efficiency and reproducibility of this procedure. When the same treatment of centrifugation and resuspension was applied in triplicate to a PFP sample, the results from FCM were found to be reproducible, with CVs <10%.

**Discussion**

Altogether, the results presented here lead to the conclusion that high-speed centrifugation induces the formation of EV aggregates composed of a mixture of EVs of various phenotypes. EV aggregates are highly heterogeneous in size and in the number of EVs associated.

**Fig. 4.** Flow cytometry analysis of a PFP (left column) and the corresponding 100k-PFP (right column) after double labelling with Anx5 −/Cy5/CD41 −/PE (a, b) or Anx5 −/Cy5/CD235a −/PE (c, d). EV concentrations (expressed as EV/µL sample) are indicated for each EV population. Colour code for the dot plots: Anx5+/CD41 − or Anx5+/CD235a −, blue; Anx5−/CD41 +, green; Anx5+/CD41 +, orange; Anx5+/CD235a +, red; Anx5+/CD235a +, purple; background noise, grey.
Most importantly, the formation of EV aggregates of various phenotypes may lead to erroneous interpretation concerning the existence of EVs harbouring surface antigens from different cell origins. By FCM and also by immunofluorescence, EVs are considered to present a double or multiple positive phenotypes simply based on the presence of 2 or more markers on a given object. Yet FCM, like most optical methods, does not allow distinguishing between a single large EV and an aggregate of several small EVs. One may therefore question the validity of conclusions from previous studies, mainly by FCM, concerning the unexpected co-localization of CD markers from different cell origin on EVs. Such conclusions include, for example, TF-positive EVs harbouring platelet (CD61) and granulocyte (CD66e) markers in patients with type 2 diabetes (33), the presence of Anx5-positive EVs co-stained with platelet (CD41) and endothelial (CD62e) markers in essential thrombocytopenia (34) or the presence by immuno-fluorescence microscopy of objects co-stained by platelet antigen CD61 and MUC1, a protein originating from breast cancer epithelial cells (35,36).

Table 1. Extracellular vesicle (EV) concentrations measured by flow cytometry in platelet-free plasma (PFP) and 100k-PFP after double labelling with Anxx/CD41 or Anxx/CD235a

| Labelling | EV Phenotype | PFP | 100k-PFP |
|-----------|--------------|-----|----------|
| Anxx/CD41 | Anxx+/CD41-  | 239±23 | 373±18   |
|           | Anxx-/CD41+  | 45±24  | 13±21    |
|           | Anxx+/CD41+  | 84±17  | 166±18   |
| Anxx/CD235a | Anxx+/CD235a- | 233±17 | 379±30   |
|           | Anxx-/CD235a+ | 319±23 | 297±35   |
|           | Anxx+/CD235a+ | 108±13 | 187±29   |

EV concentrations per microlitre are expressed as mean values ± SD for 2 independent experiments and 6 phenotypes: Anx5-/CD1+; Anx5-/CD141-; Anx5+/CD41+; Anx5-/CD235a+; Anx5+/CD235a- and Anx5+/CD235a+.

Conflicts of interests and funding
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