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NanoBioAnalytical characterization of extracellular vesicles in 75-nm nanofiltered human plasma for transfusion: a tool to improve transfusion safety

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Abstract

Plasma transfusion induces some transfusion related acute lung injury (TRALI) mediated through neutrophil extracellular traps (NETs). We investigated whether extracellular vesicles (EVs) present in plasma or obtained from resting (N-PEVs) or thrombin activated platelets (T-PEVs) can trigger NETs, and whether 75 nm-nanofiltration, to partially remove EVs, prohibits NETs formation. EVs size and concentration were determined by conventional biophysical approaches and by an original NanoBioAnalytical (NBA) platform based on EV immunocapture biochip, combining Surface Plasmon Resonance Imaging (SPRi) and Atomic Force Microscopy (AFM) exploration. EVs effective diameter was in the 25-1000 nm range, with a majority ($\approx 90\%$) ≤ 100 nm. Both T-PEVs in buffer (but not N-PEVs) and non-nanofiltered plasma containing T-PEVs triggered NETs formation. Nanofiltration depleted large EVs (> 70 nm) and decreased NETs formation. The NBA platform was found to be a suitable tool to investigate the safety of plasma for transfusion.

Key words: Extra-cellular vesicles (EV); NanoBioAnalytical platform; SPRi; AFM; plasma; platelets; nanofiltration; NETs

Background

Extra-cellular vesicles (EVs) are a superfamily of lipid bilayer membrane vesicular subsets that comprise exosome, microvesicles (MVs) and apoptotic bodies (1). These naturally occurring EVs are found in all body fluids (blood, urine, tears, CSF, sperm, milk, etc.). Their size range from ca. 30 to 1000 nm, but recent data suggest a major, less characterized, population at 30-200 nm (2). Cells release EVs upon activation by agonists, immune cells or other EVs, or when they undergo apoptosis or are exposed to shear forces in the micro-circulation as well as during *ex vivo* processing as occurs during blood collection (3). EVs are increasingly recognized to exert definite functions in many physiological events by playing an important role as cell-cell communication messengers that can influence homeostasis and the delicate physiological balance existing between health and pathological trends (4,5). EVs act as a cargo of functional molecules that encompass signalling mediators, growth factors, lipids, proteins and miRNA (1). EVs lipid bilayer membrane is decorated by receptors or other biochemical components originating from their parent cells that confer targeting and modulating properties to EVs (6). The impact of these nanovesicles is strengthened by the surrounding membrane, which protects this biological cargo from rapid catabolism and processing by macrophages (7).

EVs circulate at a particularly high concentration ($> 10^{10}$ /ml) in blood and plasma (8,9). Platelet-derived EVs (PEVs) represent a major part of blood-EVs, as these anucleated cells are prone to undergo activation, form pseudopods, degranulate, and release multiple EVs, as a result of these morphological and functional changes (10). Emerging evidence suggests links between some pathological situations, including prothrombotic states, inflammatory disorders, and cancer progression and metastasis, and increased circulating levels of EVs, and particularly PEVs, in blood (3,4,11–15). The functional role of PEVs is, in part, associated to various platelet integrin membranes that can bind coagulation factors (3,16,17) and to the exposure, upon platelet activation, of phosphatidylserine (PS), a pro-coagulant phospholipid

that induces thrombin generation and, eventually, a conversion of fibrinogen into fibrin (16,18–20).

The presence and function of EVs in cellular blood components and plasma for transfusion is becoming a major concern in transfusion medicine as these specific EVs are increasingly recognized as not being “innocent by-standers” in the clinical effect of blood components (14,21–24). Shear stresses, contact with surfaces, leucoreduction, pathogen inactivation, and storage during blood collection may all affect, and typically increase, the presence of PEVs in transfused blood products. Processing of blood and storage have therefore the potential to lead to a PEV accumulation. Such accumulation has understandably been claimed to support a beneficial functional haemostatic effect (25) but is also increasingly believed, when reaching high levels, to trigger thromboembolic events or inflammation when transfused to critically ill patients (22,26–28).

There is yet no licensed technology to remove EVs from cellular blood components or plasma for transfusion. Recently, we have provided evidence that a 75-nm nanofiltration process on dedicated hollow fiber filtration device, typically used to remove viruses, could partially remove at least the largest EVs (>75nm) from plasma for transfusion (29). This process was shown to preserve the haemostatic effect of plasma coagulation factors (which are clinically important to treat patients with bleeding disorders), while concomitantly removing a detrimental EVs-associated thrombin generation that can be a cause for supra-normal thrombin generation and thrombotic episodes in transfused patients. Further evidence of the negative role of EVs in triggering cell-mediated thrombogenic events was identified, as subsets of PEVs were found to induce monocyte aggregation *in vitro* and the release of pro-coagulant monocyte-derived EVs. Therefore, excess of EVs in transfused plasma may cause thrombo-embolism either through direct thrombin generation or through pro-coagulant cell activation. Quite importantly, some populations of blood cell-derived EVs are suspected to

trigger at least one class of non-immune transfusion-related acute lung injury (TRALI) (21,30–32), and have recently been found to induce neutrophil extracellular trap (NETs) formation, a landmark of TRALI pathology (32,33). However, little is known on the type of blood EVs responsible for TRALI occurrence. Refined analytical technologies are much needed since the policies in place in several countries aiming at using only plasma from male donors, to avoid the risk of immunological TRALI due to allogenic leukocyte antigens or of neutrophil antigens (34) have not eliminated the occurrence of TRALIs (35).

So far, many studies to characterize and identify blood-borne EVs have used conventional flow-cytometry that lacks the sensibility needed to detect EVs of a size less than approximately 400 nm (8,36,37). To better understand the functional impact of blood EVs, smart sample processing methods and technologies to detect multiple subset in the 20 nm–1 μ m size range are needed (38). A global analytical approach would make it feasible to size, identify and characterize which type of EVs triggers thrombotic events, thereby providing a new “vision” of these complex biological materials. This could provide better understanding of the impact of blood and plasma collection process on EV formation, and serve the establishment of EVs depletion procedures.

In this study, we operate a recently validated nano-bio-analytical (NBA) platform, combining EV immunocapture biochip, surface plasmon resonance imaging (SPRi) and atomic force microscopy (AFM), to better characterize the different samples of EVs. In a first step, EVs released from resting (N-PEVs) and thrombin-activated platelets (T-PEVs) were investigated, then the NBA platform was used to characterize EVs contained in plasma subjected to a 75nm-nanofiltration process (NF-plasma) (39). We further establish a link between the presence of some PEVs and the generation of pro-thrombotic NETs *in vitro*, which *in vivo* are known to trigger lethal transfusion reactions like TRALI and thrombotic events.

Methods

Ethical approval

The supply of platelet concentrates (PC) from volunteer, non-remunerated blood donors (Taiwan Blood Service Foundation, Taipei Blood Center, Guandu, Taiwan) was approved by Taipei Medical University Hospital Institutional Review Board (TMUH-IRB N°201305054).

Reagents

Glutaraldehyde, sodium citrate and Bicinchoninic-acid (BCA) protein assay kit were purchased from Sigma-Aldrich. The SPR running buffer was 10 mM HEPES buffered, at pH 7.4, with 5 mM CaCl_2 and 150 mM NaCl, all purchased from Sigma-Aldrich and prepared using ultrapure water (18 M Ω /cm resistance; Purelab prima from ELGA labWater, Antony, France). Irrelevant control IgG were provided by Immutep (Châtenay Malabry, France). Anti-CD41a monoclonal rabbit antibody was purchased from Diaclone SAS (Besançon, France). Anti-CD61, Anti-CD62-P, Anti-CD9 and Anti-TF monoclonal antibodies were purchased from Abcam (Paris, France). Annexin-V was purchased from Biovision (France).

Instrumentation

Physical characterization of EVs in suspension was done by Nanoparticle Tracking Analysis (NTA), using Nanosight NS300 system (Malvern Instrument) and by Tunable Resistive Pulse Sensing (TRPS) using qNano (Izon, Christchurch, New Zealand). For multiplex, label-free detection and phenotyping of EVs, we used SPR experiments that were carried out using SPRiplex-II (Horiba Scientific) instrument at a temperature of 25°C. On-chip physical characterization (size and shape) of EVs was done by atomic force microscopy (AFM) using a Nanowizard III AFM (JPK Instruments, Germany) mounted on a Leica microscope (Z16 APOA).

Preparation and generation of Platelet derived EVs

Two types of PEVs were prepared as described before (40,41) and present in Figure 1/I and in **supplementary data–S1**. “N-PEVs” designate the PEVs naturally present in the plasma supernatant of PC, and “T-PEVs” those generated by thrombin activation of isolated platelets. Isolated PEVs were resuspended in 1 mL of Tyrode’s buffer and immediately frozen at -80°C until use for further experiments.

Nanofiltration of plasma

Plasma nanofiltration was performed as previously described (29). In brief, fresh plasma supernatant was immediately subjected to leucoreduction prior to nanofiltration on a Planova 75-nm nanofilter (Asahi Kasei Medical). Samples of plasma before and after 75-nm nanofiltration were taken, aliquoted and frozen at -80°C until analyses.

Characterization by NTA and TRPS

The size distribution and concentration of EVs were determined by video-recording by NTA. Samples were thawed at 35°C and diluted in a commercial, particle-free saline solution and immediately analyzed as before (29). Quantification of EVs by TRPS used qNano, elastomeric membranes with nanoscale pores of 100, 200, 400, or 800 nm, and standard particle sets (CPC100, CPC400, CPC200 or CPC800, Izon) for calibration.

Selection of markers

Exosome express CD9 abundantly on their surface (42). CD9 antigen is also present on platelets surface and monoclonal antibodies against CD9 can activate platelets (43). Integrin alpha-IIb (CD41) and beta-III (CD61) form the fibrinogen receptor (integrin alpha-IIb/beta-III) expressed on platelet membrane and is essential for coagulation. CD41 and CD61 are considered as markers of PEVs (10,44). P-selectin (CD62P) is normally stored in alpha-granules in non-activated platelets and is expressed on platelet membranes upon activation

(45). Annexin V (Annex-V) interacts with phosphatidylserine (PS), a component of EV membrane (46). Tissue factor (TF) is expressed by monocytes and is known as an initiator of coagulation (47). An irrelevant IgG was used as negative control in immunocapture experiments.

Neutrophils extra-cellular trap (NET) formation

Blood was collected from the forearm vein of healthy donors using a scalp vein set (21G x 3/4) mounted on a 50 mL syringe. The ACD anticoagulant (C3821, SIGMA) was used at a 1/6 ACD/blood ratio. After centrifugation (230xg, 15 min, room temperature RT) the platelet rich plasma supernatant was discarded and the sedimented cells put in Ficoll and centrifuged (2000 rpm, 15 min, RT, without break). The upper layer above the buffy coat was discarded, and the red blood cells lysed using a lysis buffer. The neutrophils were suspended in RPMI medium containing 10% autologous heat inactivated serum and heparin (20 µg/ml). To stimulate NETs formation, neutrophils (8×10^5) were seeded on poly-L-lysine pre-coated coverslip and let to rest at 37°C for 2 hours. EVs samples, obtained by spiking PEVs in buffer or in 75-nm nanofiltered plasma, were diluted in Tyrode's buffer based on initial EVs content. EVs samples were then subjected or not to 75-nm nanofiltration prior to incubation with neutrophils, for 1 hour at 37°C, at neutrophil to EVs ratios of 1:10, 1:30, and 1:100. Bright field images were captured by inverted microscopy (Ti-U, Nikon). For confocal microscopy observations, the coverslips were pre-coated with 10 µg/ml poly-L-lysine at 4°C overnight. The neutrophils (8×10^5) were seeded on the coverslip and let to rest. After the treatment with the EVs samples, the neutrophils were fixed (4% paraformaldehyde, 400 nM sucrose, 2mM EDTA in PBS) for 1 hour at RT. Samples were washed using PBS, and cells permeabilized by 0.5% Triton-X100 for 15 min at RT. After blocking with 3% BSA in PBS, the anti-MPO

(myeloperoxidase) antibody (Santa Cruz) and anti-histone H1 antibody (Santa Cruz) were incubated with the samples at 4°C overnight. Samples were washed by PBS, then stained with goat anti-mouse IgG (H+L) conjugated Alexa 488 (109-035-003, Jackson ImmunoResearch) and goat anti-rabbit IgG conjugated TRITC (111-025-045, Jackson ImmunoResearch) for 1 hour at RT. DNA was stained by Hoechst33342 for 20 min at RT. The samples were mounted in mounting solution for confocal microscopy (TCS SP5, Leica).

SPR biochip preparation

Home-made gold-biochips, provided by the “Mimento” technology center (Besançon, France), were used to immobilize different ligands into an array format (16 spots: 4x4) as described previously (39,48). Each ligand was deposited ($\approx 2500 \text{ pg/mm}^2/\text{spot}$) in duplicate or triplicate on the biochip. The capture level of EVs by each ligand was determined as the mean value from at least 2 spots per immunocapture experiment.

Injection of the different analytes

SPR experiments were carried out in HEPES buffer (running buffer). The analytes were diluted in the running buffer and injected, at different concentrations, over the biochip surface presenting specific ligands. The capture levels of analytes were determined from the SPRi signal converted into mass per surface unit (1% of reflectivity variation = 125 pg/mm^2).

AFM imaging

Following EV capture on the gold biochip, glutaraldehyde (0.5%) was injected for 10 min to fix the captured materials for further AFM experiments operating in air. An injection of water was applied to remove buffer salts from the biochip surface; the biochip was then dried under gentle airflow. AFM imaging was obtained in contact-mode and in air using V-shaped silicon nitride cantilevers (Digital instruments, NP-S10) with a spring constant of 0.32 N/m. Nanoscale investigations of the biochip by AFM were engaged by rigorously imaging

different areas (from 100 μm^2 down to 2 μm^2 zones) of each spot to ensure representative sampling. EV size distribution was measured mainly using AFM images of 5 x 5 and 10 x 10 μm^2 . The typical scan rate was 0.5 Hz for 512 x 512-pixel images. Using Gwyddion software, a height threshold of 3 nm was applied to AFM images, in respect to the ligand-modified biochip surface roughness, to identify/mark the captured EVs prior to their size measurement (see Supplementary data – S2). At least 1000 individual EVs were measured on each spot. The results were statistically analysed using the Wilcoxon rank sum test, and a *P* value less than 0.001 was considered to be statistically significant.

Results

1. N- & T-PEVs: functional characterization

Neutrophil aggregation by PEVs

Apheresis platelet concentrates (PC) were collected for N-PEVs and T-PEVs isolation/generation as presented in Figure 1/I.

Figure 1

We first tested whether N-PEVs and T-PEVs induced neutrophil aggregation. When neutrophils were incubated with N-PEVs at different ratio, there was no obvious aggregation (Figure 1/II, A,B,C). In addition, no NETs formation was observed by immunofluorescent staining (Figure 1/II, D,E). When neutrophils were incubated at 1:10, 1:30, or 1:100 T-PEVs ratios, a dose-dependent aggregation was observed (Figure 1/II,F,G,H respectively). Moreover, there was strong NETs formation confirmed by immunofluorescent staining (Figure 1/II,I,J). As T-PEVs are prepared from isolated platelets, they are expected to contain more platelet-derived EVs than N-PEVs that are extracted from the plasma supernatant and therefore may contain EVs from other cellular origins.

2. Qualification of N-PEVs and T-PEVs : Physical characterization and investigation by the NanoBioAnalytical platform

Physical characterization by NTA and TRPS

The size of N- and T-PEVs determined by two different techniques recommended by EV scientific community, one based on light scattering (NTA) and the other on electrical current monitoring through a nanopore (TRPS) (Table 1). NTA allows the determination of the size of individual particles in a polydisperse mixture in the size range of 50 nm-1 μ m. NTA showed a mean diameter of 216 and 238 nm for N- and T-PEVs respectively.

TRPS size measurement depends on the pore size that is used. With a pore size of 800 nm (NP800), allowing detection of particles in the size range of 400-1600 nm, the mean diameter measured for N- and T-PEVs was of 670 and 633 nm respectively. When a pore size of 400 nm (NP400) was used, allowing detection of particles in the size range of 200-800 nm, the mean diameter of PEVs was of 384 and 378 for N- and T-PEVs respectively. Moreover, the size measurement using smaller pore size of 100 nm (NP100), with analysis range of 50-300 nm, was impossible due to the repetitive clogging of the pore by large EVs. NTA and TRPS, in their window of relevancy, do not reveal major differences in mean size between the two EVs sample populations.

EVs concentration was also determined by TRPS and NTA. The concentrations of EVs in N- and T-PEVs with NP400 were 5.10×10^9 and 8.65×10^8 EVs/ml respectively; EVs concentration determined by NTA for both samples were ≈ 400 times greater than the values determined by TRPS (NP400); this is due to the lower detection limit of NTA (≈ 50 nm), compared to TRPS (≈ 200 nm with NP400), and its wider range of detection allowing detection of smaller EVs (< 200 nm) which seems to be more abundant than large EVs (> 200 nm). Interestingly, both NTA and TRPS showed that N-PEVs are ≈ 6 fold more concentrated than T-PEVs.

Biochemical characterization

The protein concentration of N- and T-PEVs samples was measured using BCA protein assay kit. Protein concentration in N-PEVs sample was higher than in T-PEVs with mean values of 6.62 and 2.42 mg/mL respectively. This result is in agreement with the NTA and TRPS analysis that showed a higher EVs concentration in N-PEVs sample compared to T-PEVs.

Investigation by the NanoBioAnalytical Platform

To characterize exhaustively micro- and nanovesicles in solution at the biochemical and physical levels, we have developed a NanoBioAnalytical (NBA) platform consisting of an on-chip immunocapture and of combination of Surface Plasmon Resonance Imaging (SPRi) with Atomic Force Microscopy (AFM) for a real-time, label-free, direct detection, phenotyping and size characterization of particles. This platform was successfully applied to study EVs isolated from platelet concentrates (39). Figure 2-A shows an image of a gold biochip presenting 16 spots (4x4) of different ligands with at least 2 spots per ligands. Figure 2-B shows a SPRi contrast image of the gold biochip when inserted inside the SPRi instrument. The grafted amount of each ligand was calculated based on the shift of plasmon curves generated by each spot compared to the plasmon curve of the surface (outside the spots) (Figure 2-C). We noted that plasmon curves from the different spots were shifted slightly to the left compared to the surface, which confirmed the grafting of ligands. We obtained a good interspot homogeneity of the grafting for ligand-antibodies comprised between 13.6 and 18.5 fmoles/mm² (Figure 2-D). Due to its low molecular weight (35 kDa vs 150 kDa), we grafted Annex-V with a higher density reaching 92.2 fmoles/mm².

Figure 2

i) Capture of N- and T-PEVs

N- and T-PEVs were injected on the biochip surface presenting Annex-V, α -CD41, α -CD62P, α -CD9 and an irrelevant antibody as negative control (IgG ctrl). Each sample was diluted in the running buffer to 1/100 and injected onto the biochip surface for 10 minutes. Three successive injections were applied. Figure 3-A, C shows the sensorgrams recorded during the injections of the samples. The immunocapture tests were applied in duplicate for each sample, and the capture level was reproducible on each ligand (Figure 3-E). Results showed a variation in the capture level with regards to different spots but also between the

two samples. This can be explained by the presence of different subpopulations of EVs in both samples and at different levels. Also, the weak non-specific signals on the control IgG spots and on the ligands-free surface reflected a high specific capture of vesicles onto the surface. On the other hand, we can note that the SPRi signal recorded for the different spots remained stable at the end of the injection which confirmed the stability of the capture of EVs with their ligands on the surface. The capture level of T-PEVs was higher than N-PEVs on the two receptors specific of platelet, i.e. α -CD41 (5.8 ± 0.7 against $1.8 \pm 0.3\%$), α -CD62P (5.8 ± 0.7 against $0.97 \pm 0.2\%$) and comparable on α -CD9 (2.2 ± 1.2 against $1.1 \pm 0.3\%$), and Annex-V ($\approx 4\%$) (Figure 3-C). This may reflect either a high level of EVs expressing these markers (CD41, CD62P) and/or an abundance of the expression level of these markers at the surface of vesicles in the T-PEVs sample which promoted their capture on the surface by their ligands.

Figure 3

ii) AFM analysis of captured N- and T-PEVs

Following the injection of N- and T-PEVs, separately, each on the surface of a biochip presenting different specific ligands, a nanoscale investigation of the different spots was engaged afterwards with AFM.

The analysis of different AFM images of each spot showed that the majority of EVs were appearing as individual and disc-shaped objects (Figure 4-A, B; [supplementary data-S4](#)), taking into account the deformability of EVs and the recalculation of effective diameter ([Supplementary data-S3](#)).

The effective diameter of the subpopulations of EVs, captured on the different ligands, in both N- and T-PEVs samples, was in the range 25-1000 nm, with a majority ($\approx 90\%$) of vesicles ≤ 100 nm (Figure 4-D). Moreover, some large objects, such as aggregates or clusters

of small vesicles and large individual objects of different shapes were detected especially in the case of T-PEVs ([supplementary data–S4, S6](#)). Statistical analysis showed that CD41⁺ and PS⁺ subpopulations in N-PEVs were significantly ($p < 0.001$) larger in size than their counterparts in the T-PEVs sample. CD62P⁺ and CD9⁺ PEVs were significantly ($p < 0.001$) smaller in N-PEVs samples compared to those in T-PEVs samples. Moreover, PEVs-CD9⁺ were the smallest in size distribution compared to other PEVs subpopulations in both N- and T-PEVs, with median sizes of 30.7 and 36.6 nm respectively.

Figure 4

Furthermore, a positive correlation was found between the capture level of EVs on the different spots, given as SPRi signals (reflectivity %), and the number of vesicles captured onto the surface of these spots (EVs/ μm^2), which was obtained through AFM analysis ([Figure 4-E](#)).

3. Qualification of EVs in plasma

The presence of numerous EVs subpopulations in plasma was already reported in various studies (49–52). For plasma EVs qualification, we have chosen a set of ligands or antibodies that targets all blood-borne EVs (Annex-V), EVs derived from platelet (α -CD41, α -CD61, α -CD62P), EVs wearing tissue factor (α -TF) and exosomes (α -CD9).

We engaged TRPS measurements and NBA analysis on crude and NF-plasma, as a mean to appreciate the efficiency of EV depletion through a 75-nm plasma nanofiltration process and to characterize EVs subpopulations in terms of concentration, size and cell origin.

TRPS measurements were realized using two different nanopore membranes, a NP200 (*limit of detection*= 80 nm) and a NP100 (*limit of detection*= 50 nm) for crude and NF-plasma

respectively, since the use of NP100 was impossible in crude plasma, due to membrane clogging.

TRPS results showed that crude plasma contained EVs with a size spreading from 80 to 500 nm, with a mean diameter of 110 nm (Figure 5-A), while 75-nm NF-plasma presents smaller EVs, with a diameter spreading from 50 to 300 nm, with a mean diameter of 60 nm (Figure 5-D).

The EVs concentration, evaluated using these two different NP200 and NP100 nanopores, reached 4.4×10^{11} and 2.0×10^{12} EVs/ml in crude and NF-plasma respectively, but with an underestimation of small EV content in crude plasma due to the use of NP200 membrane.

i) Capture of EVs from plasma

For immunocapture experiments, each plasma sample was injected, at a dilution of 1/50 in HEPES buffer, during 10 minutes on the surface of a gold biochip presenting Annex-V, α -CD41, α -CD61, α -CD62P, α -TF, α -CD9 and an IgG control. Figure 5-B and E shows the SPR sensorgrams recorded during the injection of the crude and NF-plasma samples, and the SPRi contrast images (Figure 5-C and F, respectively) taken after stability of the SPRi signal at the end of the injection. The immunocapture experiments were done in triplicate for each sample. Results firstly showed that EVs subpopulations remained in the NF-plasma since significant biodetection is observed on the different spots (Figure 5-E, F). Secondly, results in respect of ligands show that the relative EVs capture levels in the two plasma samples were comparable (Figure 5-B, E). The capture levels were higher on α -CD9 and Annex-V spots, followed by that on α -CD61, α -CD41, α -TF and α -CD62P (Figure 5-G). Also, systematically, the capture levels on the different spots were lower for NF-plasma than for crude plasma.

Figure 5

ii) AFM analysis of captured EVs from plasma

A nano-characterization of the multiplexed biochips that have been exposed to either crude plasma or NF-plasma showed that the subpopulations of vesicles, captured on the different spots of the biochips, appeared essentially as individual disc-shaped objects with an effective diameter between 25–1000 nm in plasma, with a majority ($\approx 90\%$) showing a size ≤ 100 nm, similar to PEVs. As an example of differential analysis of a specific CD61⁺ EVs subset, the figure 6-A & B shows a significant difference in EVs surface density and size, before and after nanofiltration, with median sizes of 44.9 versus 24.2 respectively (additional images are shown in supplementary data-S5).

In 75-nm NF-plasma, and whatever the spot, most of the vesicles ($\approx 99\%$) present a diameter ≤ 75 nm, and never exceeded roughly 100 nm, while EVs size in crude plasma could reach 500 or even 1000 nm (Figure 6-C).

Figure 6

4. A biological prospect: neutrophil aggregation by plasma EVs

To unveil whether T-PEVs induced neutrophil aggregation and whether NETs formation can be decreased by 75-nm nanofiltration, T-PEVs were spiked into tyrode's buffer or into NF-plasma and then subjected or not to nanofiltration before incubation with neutrophils.

T-PEVs, spiked to buffer or plasma, induced a dose-dependent aggregation of neutrophils and NETs formation (NETosis). The strong formation of clusters of neutrophils was occurring when incubating them at a 1:100 ratio with T-PEVs spiked to buffer (Figure 7-A right) or plasma (Figure 8-A right). Moreover, the NETosis was found by immunofluorescent staining when neutrophils were incubated at 1:100 ratio with T-PEVs in buffer (Figure 7-B) and plasma (Figure 8-B,C). On the other hand, when the T-PEVs were spiked in buffer or in

plasma and were then subjected to 75-nm filtration, there was no cell aggregation in both conditions (Figure 7-C and 8-D). There was no NETosis when neutrophils were incubated with T-PEVs spiked-buffer subjected to nanofiltration (Figure 7-D) whereas only mild NETosis was observed in the presence of T-PEVs spiked plasma following nanofiltration (Figure 8-E, F), while strong NETosis occurred with these samples prior to nanofiltration (Fig. 7-B and 8-B, C).

Figure 7

Figure 8

Discussion

Red blood cell concentrates, platelet concentrates, and plasma for transfusion are listed by the World Health Organisation as essential medicinal products. This listing emphasizes the critical life-saving role of these blood products in all countries at global level, and illustrates the need to ensure optimal production and safety of these products (53–55). Although the quality and safety of blood products has dramatically increased over the last decades, their clinical use remains associated with sometimes lethal side-effects. For instance, plasma transfusion can lead to the occurrence of thromboembolic events, TRALI, and inflammatory events (56,57). Attempts have been made to identify the possible cause of these adverse events but have not all been successful (26). In particular, how blood-borne EVs could play a role in the occurrence of thromboembolic events after plasma transfusion could not be assessed due to a lack of sensitive methods capable to discriminate among EV subpopulations. Recently we have been able to provide indirect evidence of the detrimental role of at least some plasma-borne EVs (>75 nm) by showing that plasma nanofiltered on 75-nm membrane maintained its hemostatic activity but evidenced lower pro-thrombotic activity *in vitro* (29). In further studies we also found that some of the PEVs, in particular the T-PEVs,

may be capable to induce thrombogenic events through an activation of THP-1 monocytes (40). Here we show that these T-PEVs may also be responsible for the generation of NETs *in vitro*. However, a main issue in transfusion medicine today is that, due to their nanosize, no analytical system has been made available to validate or control neither the impact of production procedures of plasma for transfusion on EVs generation, nor the content of end-products in EVs (22). There is a rationale need to better realize the link between subpopulations of EVs in blood products and the trigger of transfusion reactions.

Complementary to conventional technics and methods, the NBA platform allowed real time, label-free and semi-quantitative detection of different EVs subpopulations, in PEVs samples but also in plasma, via their specific capture onto a multiplexed biochip surface, presenting spots of different specific ligands, using the SPR technique. Moreover, the AFM analysis of the captured vesicles presented the advantage to provide dimensional and morphological information of the relevant captured particles and information about their morphology and size distributions over their wide size-range (30-1000 nm); also, it allowed to discriminate between EVs aggregates and individual vesicles. The NBA platform showed high capture specificity of EVs in buffer and in plasma, which was confirmed by the low level of SPR signals recorded on control IgG spots in the different experiments. In addition, the AFM analysis showed very low number of adsorbed vesicles on control IgG spots in correlation with SPR results. In all this study, the size distribution of EVs in the different samples was essentially located in the range of 30-300 nm, which is in agreement with previous studies that used different techniques (AFM, NTA and electron microscopy) to measure the size of EVs (8,58–60). The efficiency of the NBA platform to discriminate subpopulations of EVs is illustrated through the EVs-CD9⁺ species: their smallest size distributions reflect the biogenesis. Indeed, CD9 is mainly expressed by exosomes which are characterized by their small size compared to other EVs subpopulations (1,61,62).

Moreover, our results showed that the higher capture level observed for T-PEVs especially on α -CD41 and α -CD62P spots is not induced by a higher EVs concentration in N-PEVs sample, but is probably more likely due to a higher protein expression level (especially CD41 and CD62P) on the surface of T-PEVs which was validated by a semi-quantitative proteomic analysis of N- and T-PEVs through mass spectrometry (unpublished results). However, EVs characterization in crude and nanofiltered plasma gave low capture levels on α -TF and α -CD62P spots, a finding that is in agreement with the low presence of TF+ and CD62P+ EVs in normal plasma samples (50–52). Nevertheless, CD62P is known to be a platelet activation marker, and its abundance in the T-PEVs sample confirms the activation of platelets, used to generate T-PEVs, by thrombin. In addition, in the literature, CD62P protein (also known as P-selectin) present on activated platelet membranes appears to be also implied in cell adhesion and cell aggregation, and in particular to bind to P-selectin glycoprotein ligand 1 (PSGL-1) that is expressed by neutrophils, monocytes and THP-1 cells (55). Our data are also consistent with previous finding that CD62P expressed by PEVs is more avidly involved in activation and aggregation of neutrophils *in vitro* than activated platelets themselves (56). These *in vitro* observation of the importance of the removal of T-PEVs to decrease TRALI incidence should be confirmed in *in vitro* models (63) but are outside the scope of the current analytical study.

Therefore in this study we have assessed whether our instrumental combination based NBA platform could be used to discriminate EV subpopulations (a) in plasma prior to and after 75-nm nanofiltration and in (b) isolated EVs capable to induce differential actions on monocytes and neutrophils. Our results provide a proof-of-concept of the capacity of the developed NBA platform to perform discrete and fine phenotypical, dimensional and morphological analysis of platelet-derived EVs present in plasma or platelet concentrate samples. In addition, our data could relate the presence of specific PEVs to the possible

occurrence of thrombotic or inflammatory transfusion side effects due to NETosis.

Among perspectives of our work, and since our analytical solution lies on combining different techniques with their own sensitivity and reproducibility performances, we plan to engage a strict/rigorous dimensional study of EVs subpopulations pertaining to a fine analytical and metrological characterization of these biological nanoobjects. Such a physical investigation, based on deep instruments calibrations and focusing on accuracy, sensitivity, and traceability of EVs measurements, is crucial to achieve standardization of EVs subsets measurements (64).

At last, our biochips developments take place in the aim to limit pre-analytical operations by developing new sensitive tools for EVs characterization even in crude sample. These works allowed us to claim the usefulness of our NBA platform for EVs characterization in the latest MISEV guidelines (65).

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Figure legends

Figure 1: (I) Preparation of N-PEVs (A) and T-PEVs (B), and (II) effect on neutrophils. Neutrophils were incubated with N-PEVs or T-PEVs at a ratio of 1:10 (A for N-PEVs – F for T-PEVs), 1:30 (B for N-PEVs – G for T-PEVs), and 1:100 (C for N-PEVs – H for T-PEVs). The images were obtained under bright field with an inverted microscopy. The scale bar is 100 μm . NETosis was determined by immunofluorescent staining using antibodies targeted to DNA (blue), histone H1 (green), and MPO (red). The superimposed images were obtained under confocal microscopy. Neutrophils were incubated at 1:100 ratio with N-PEVs (D) and larger magnification of the area from the white square (E), or with T-PEVs (I) and larger magnification of the area from the white square (J). The scale bar is 50 μm (D,I) and 10 μm (E,J).

Figure 2 : (A) Photo of a multiplexed gold-biochip presenting 16 spots of different ligands deposited using a manual arrayer. Dimensions of the biochip are 12.5 x 28 mm. (B) SPRi contrast image of the biochip with the different spots. Scale bar of 1 mm. (C) Plasmon curves measured on the different spots of ligands and on the ligands-free surface (surface). (D) Table showing the mean grafted amount (fmol/mm^2) of each ligand onto the biochip surface through $n=10$ immunocapture experiments applied for platelet-EVs and EVs from plasma. $\alpha\text{-CD61}$ and $\alpha\text{-TF}$ was only used for immunocapture of EVs from plasma.

Figure 3 : SPRi capture levels recorded during the injection of N-PEVs (A) and T-PEVs (C) on the surface of multiplexed biochips. Three successive injections were applied for each sample at a dilution of 1/100 in HEPES buffer. Each injection was performed during 10 minutes with a flow rate of 20 $\mu\text{l}/\text{min}$. (B) & (D) show, respectively, two SPRi phase-contrast images of different spots on the surface of biochips after the injection of N- and T-PEVs (scale bar is 500 μm). (E) Histogram showing the mean capture level of EVs on the different spots from two independent immunocapture experiments for each sample.

Figure 4 : AFM analysis: (A) & (B) shows AFM images of N & T-PEVs, respectively, captured on the biochip surface via $\alpha\text{-CD41}$. “D” for effective diameter. (C) Shows an AFM image of an IgG ctrl spot after the injection of T-PEVs. (D) Boxplots showing the size distribution, represented as log of the effective diameters, of the different subpopulations of EVs ($\text{CD41}^+/\text{CD62P}^+/\text{PS}^+/\text{CD9}^+$), in N- & T-PEVs, captured onto the surface via different specific ligands ($n \geq 1000$ vesicles for each subpopulation). The median is marked with bold line. The p values were calculated by a Wilcoxon test, *** $p < 0.001$. (E) Histogram showing the correlation between the capture level of EVs on each spot (Reflectivity %) and the surface density of captured vesicles (number of EVs/ μm^2), measured by AFM.

Figure 5 : TRPS characterization and on chip capture of EVs from crude plasma (A to C) and NF-Plasma (D to F) on the surface of multiplexed biochips with SPRi phase-contrast images

of the different spots after the injections (scale bar is 500 μm). TRPS results obtained on NP200 (A) or NP100 (D) nanopore membranes respectively for crude and NF-plasma. For SPRi experiments, each sample was injected at a dilution of 1/50 in HEPES buffer during 10 minutes with a flow rate of 20 $\mu\text{l}/\text{min}$: sensorgrams (B,E) and contrast images (C,F). (G) Histogram showing the mean capture level of EVs on the different spots through three immunocapture experiments for each sample.

Figure 6 : AFM characterization of EVs captured on the biochip surfaces, via $\alpha\text{-CD61}$, through the injection of either crude plasma (A) or NF-Plasma (B). “D” for effective diameter. (C) Boxplots showing the size distribution, represented as log of the effective diameters, of the different EVs subpopulations, in Plasma and NF-Plasma, captured onto the surface via their specific ligands ($n \geq 1000$ vesicles for each subpopulation). The median is marked with bold line.

Figure 7 : T-PEVs spiked in tyrode’s buffer at neutrophils/EVs ratios of 1:10 (A, left), 1:30 (A, middle) or 1:100 (A, right and B). The scale bar is (A) 100 μm and (B) 50 μm . T-PEVs were spiked to tyrode’s buffer then subjected to a 75-nm nanofiltration, and incubated with neutrophils at ratios of 1:10 (C, left), 1:30 (C, middle) or 1:100 (C, right and D). The scale bar is (C) 100 μm and (D) 50 μm . Pictures (A) and (C) are bright field images captured by inverted microscopy, and (B) and (D) by fluorescence confocal microscopy. NETosis was determined by immunofluorescent staining using antibodies targeted to DNA (blue), histone H1 (green), and MPO (red) shown from left to right in (B) and (D); the 1st image from the right in (B) and (D) is a merge of the three other pictures.

Figure 8 : T-PEVs spiked in plasma and incubated with neutrophils at ratios of 1:10 (A, B, C, left), 1:30 (A, B, C, middle), or 1:100 (A, B, C, right). The scale bar is (A) 100 μm , (B) 50 μm , and (C) 10 μm . T-PEVs were spiked in plasma then subjected to 75-nm nanofiltration, and incubated with neutrophils at ratios of 1:10 (D, E, F, left), 1:30 (D, E, F, middle) or 1:100 (D, E, F, right). The scale bar is (D) 100 μm , (E) 50 μm and (F) 10 μm . Pictures (A) and (D) are bright field images captured by inverted microscopy, and (B), (C) and E) by fluorescence confocal microscopy. NETosis was determined by immunofluorescent staining using antibodies targeted to DNA (blue), histone H1 (green), and MPO (red) and only the merged pictures are shown (left, middle, and right in B, C, and E).

Tables

Table 1 : PEVs characterization by TRPS and NTA

Table 1

		N-PEVs	T-PEVs
TRPS		Average	Average
Membrane: 400nm	Mean (nm)	384 ± 13.4	378 ± 29.3
	Conc. (particles/mL)	5.10 x 10 ⁹	8.65 x 10 ⁸
TRPS		Average	Average
Membrane: 800nm	Mean (nm)	670 ± 47.5	633 ± 26.0
	Conc. (particles/mL)	6.95 x 10 ⁷	1.66 x 10 ⁷
NTA		Average	Average
Mean (nm)		216 ± 17.7	238 ± 29.0
Conc. (particles/mL)		2.02 x 10 ¹²	3.56 x 10 ¹¹

Figure 1

II

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Buffer spiked experiments

Figure 8

Plasma spiked experiments