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Fast isolation of highly specific population of platelet-derived extracellular vesicles from blood plasma by affinity monolithic column, immobilized with anti-human CD61 antibody

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Abstract

A new, fast and selective immunoaffinity chromatographic method including a methacrylate-based convective interaction media (CIM[®]) disk monolithic column, immobilized with anti-human CD61 antibody, was developed for the isolation of CD61-containing platelet-derived extracellular vesicles (EVs) from plasma. The isolated EVs were detected and size characterized by asymmetrical flow field-flow fractionation (AsFIFFF) with multi-angle light-scattering (MALS) and dynamic light-scattering (DLS) detection, and further confirmed by nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM). The mean size of platelet-derived EVs isolates from the anti-CD61 CIM[®] disk monolithic column were 174 nm (SD 60 nm) based on the NTA results. These results indicated a successful isolation of platelet-derived EVs, which was confirmed by Western blotting the isolates against the EV-specific markers CD9 and TSG101 together with transmission electron microscopy. Additional elucidation of MALS and DLS data provided detailed information of the size distribution of the isolated fractions, confirming the successful isolation of also small platelet-derived EVs ranging from 30 to 130 nm based on the hydrodynamic radii. The isolation procedure took only 19 min and the time can be even further decreased by increasing the flow rate.

The same immunoaffinity chromatographic procedure, following AsFIFFF allowed also the isolation and characterization of platelet-derived EVs from plasma in under 60 minutes. Since it is possible to regenerate the anti-CD61 disk for multiple uses, the methodology developed in this study provides a viable substitution and addition to the conventional EV isolation procedures.

Keywords: Immunoaffinity chromatography; isolation; monolithic disk column; extracellular vesicles; platelet-derived vesicles; CD61

Graphical abstract

1 Introduction

Extracellular vesicles (EVs), including exosomes and microvesicles, are found in all types of body fluids, since most cells release EVs to carry molecular information (e.g. RNAs) between different cells [1,2]. EV subpopulations cover a wide ranges of sizes: 50-150 nm for exosomes, between 50-500 nm (up to 1 μ m) for microvesicles, and 50 nm-2 μ m for apoptotic bodies [3], and they have an important role in diagnosis of human pathologies and therapeutics [4–6]. Complex nature of the body

fluids poses challenges for EV isolation, and previously EVs have been predominantly isolated by ultracentrifugation or by combining many different methods. For complex body fluid samples, methods like density gradient centrifugation, filtration, size exclusion chromatography, and magnetic bead capture have been utilized [7]. Since most of the methods employed are relatively slow, costly and laborious, with relatively low reproducibility and isolation technique based bias, there is a great and urgent need for fast and efficient ways to isolate EVs from plasma. [4,7]

Affinity chromatography and ion exchange with highly permeable monoliths with large surface area have been successfully used for large-scale purification of plasmid DNA, viruses, cells and cell organelles [8]. A high affinity ligand attached to the monolith offers a useful system for fast and selective isolation of EVs. The monoliths with pore sizes of over 1 μm provide convective transport through the channels [9] with high flow rates [8], resulting in short separation times [10], and allow the isolation of large biomolecules without losing biological activity [11]. The monolithic columns, and especially poly (glycidyl methacrylate) ones, have usually unique separation properties for the large biomolecule separation [12–15], which makes them also suitable for the isolation of EVs. Antibody attached to monolithic silica microtips have been utilized with success for exosome profiling [16], and methacrylate-based anion-exchange monolithic columns, for separation and enrichment of EVs [17,18]. Surprisingly, the antibody immobilized methacrylate-based monolithic columns have not yet been utilized for the isolation of EVs. These polymeric columns are more biocompatible compared to silica and more capable of handling complex biofluids in short time compared to the anion-exchange columns. [4] Recently, we successfully employed methacrylate-based monolithic disk column immobilized with monoclonal antibody for selective isolation of low-density lipoproteins from plasma [19].

Future theranostic EV applications call for plasma protein and lipoprotein free populations of EVs. Although it is possible to immunoisolate contaminant free EV populations with current methods, in case of magnetic beads, either the beads or remaining antibodies are retained on the EVs, which

impedes the functional studies. [20] In addition, some of the affinity bead -based methods have problems to elute the EVs. Thus, there is still a great need for new methods, which are not multi-step nor time consuming [4,21]. The preferred isolation methods should elute the EVs and provide the contaminant free population of highly concentrated EVs in a fast and reproducible manner. Potential clinical trials on a large number of patient samples accelerate the need for large quantities of EVs and technological advancements in the isolation processes. At present, there are unfortunately no clearly defined manufacturing protocols. In addition, it is important to develop isolation methods that are scalable and cost-effective, with control of the final product. Previously mentioned challenges along with a detailed characterization of the isolates are the bottleneck that needs to be solved before the patients can access the EV-based therapies. [4]

Thus, the development of immunoaffinity chromatographic systems including immobilized EV-selective antibodies are important for the future development of EV isolation [4]. They can solve the bottlenecks in the isolation, since selective interactions allow fast and easy single step isolations with enrichment of hundred to thousand-fold [22]. Platelet EV surface markers such as CD61 have proved to play an important role in detecting pathologies [23], and in providing non-invasive detection and therapeutic methods [23,24], and CD61 is also beneficial for the specific capture of platelet-derived EVs [25]. Newly emerging fractionation technologies, such as asymmetrical flow field-flow fractionation (AsFIFFF), have also been successfully utilized for the isolation of EVs including exosomes, e.g. from cell cultures and cell lines, as well as for the size characterization of exosomes when coupled with multiangle light scattering (MALS) and dynamic light-scattering (DLS). [5,26–28]

In this study, a novel and fast EV immunoaffinity chromatographic isolation method was developed based on polymeric monolithic disk immobilized with mouse anti-human CD61 antibody. The antibody allowed selective isolation and elution of highly specific and contaminant free platelet-derived EVs from plasma and platelet-concentrate derived platelet-free plasma (PC-PFP) samples.

The isolation of CD61 positive EV fraction of platelet EVs from clinical human plasma samples has potential in biomarker discovery. Levels of platelet-derived EVs are increased in several diseases, e.g. hematological disorders, atherothrombotic, autoimmune and infectious diseases, and cancer [23], but so are often the EV levels in general. Thus, the isolation of a platelet-specific EV population can be used for the discovery of biomarkers to improve the sensitivity. The effect of flow rates on the isolation yields was studied and protein concentration, particle concentration, and morphology of the isolates were evaluated. In addition, the isolates were Western blotted against EV-markers: surface protein CD9 and a cytosolic protein TSG101 [5,20,23,29,30] were utilized to confirm the presence of platelet-derived EVs in the isolates. Further, AsFIFFF was exploited in combination with MALS and DLS detectors to determine the sizes of the isolates.

2 Materials and methods

2.1 Chemicals and materials

Anhydrous Na_2CO_3 , ethanolamine, glycine, HCl, NaHCO_3 , phosphate buffer saline (PBS), and Tris were purchased from Sigma-Aldrich (USA). 25% ammonia was obtained from Riedel-de Haën (Germany). 1 M NaOH and 1M HCl were from FF-Chemicals Ab Oy (Finland). CIM[®] CDI -0.34 mL disk and the disk housing were from BIA Separations (Slovenia). Purified mouse anti-human CD61 (clone VI-PL2) antibody was purchased from BD Biosciences (USA), TSG101 (clone 51/TSG101) was from BD Transduction Laboratories (USA), and CD9 (clone C-4) was from Santa Cruz Biotechnology (USA), while HRP-conjugated sheep anti-mouse IgG antibody was from GE Healthcare (USA). Healthy human plasma and outdated platelet concentrates containing plasma and platelets were purchased Finnish Red Cross Blood Service, Helsinki, Finland. Clarity ECL substrate and the DC Protein assay were from Bio-Rad (USA). Polyvinylidene difluoride (PVDF) membranes, 12% SDS-PAGE, and PageRuler prestain protein ladder were purchased from ThermoFisher Scientific (USA).

2.2 Instrumentation

2.2.1 Instrumentation used for isolation and characterization of EVs

Distilled water was deionized using a Millipore water purification system (Millipore, France). Sp100i syringe pump (WPI, USA) was used for immobilization of the anti-human CD61 antibody on CIM® CDI disk and EV isolation experiments. For the measurement of protein concentration, the DC Protein assay was used following the manufacturer's protocol. The colorimetric measurements were performed using Multiscan EX microplate reader (Labsystems Diagnostics, Finland) at wavelength 750 nm. In Western blotting, the chemiluminescent signal was detected using LAS-3000 Imaging system (Fujifilm, Japan). Stained samples were viewed with Tecnai 12 (FEI Company) transmission electron microscope at 80 kV.

Isolated EV samples were analyzed by Nanosight model LM14 (Nanosight, UK) equipped with blue (404 nm, 70 mW) laser and SCMOS camera. The samples were diluted in phosphate-buffered saline (PBS) and three videos of 60 seconds each were recorded using camera level 14. The data was analyzed using NTA software 3.0 with the detection threshold set at 5 and screen gain set at 10 to maximize the particles tracking with minimal background.

For the elution profiles of EV isolations, UV detector (SPD-20A Prominence, Shimadzu, Japan) with PostNova AF2000 program was used. The samples were injected using the syringe pump with a flow rate of 0.5 mL/min. The outlet of the anti-CD61 disk was connected to the UV detector and the measurements were performed at the wavelength of 280 nm.

2.2.2 Asymmetrical flow field-flow fractionation system

The asymmetrical flow field-flow fractionation (AsFFFF system from Postnova Analytics AF2000 system (Landsberg, Germany) equipped with a solvent organizer (PIN PN7140) and a solvent degasser (PN7520), was utilized. The carrier flow was generated using two isocratic high-performance liquid chromatography (HPLC) pumps (PN1130). In addition, a syringe pump (Kloehn

v6) was used to generate cross-flow and a purging port (PN1610) was used for rinsing. Injection was done manually utilizing a 500 μ L injection loop. AsFIFFF separation channel had a 350 μ m spacer (Postnova Af2000 MF spacer) and a 10 kDa mass cut-off regenerated cellulose membrane (Postnova AF2000 MT series Membrane). The channel was kite shaped (total length 27.5 cm) with a large triangle at the inlet (height/base = 3.5 cm / 2.0 cm) and a small triangle at the outlet (height/base = 1.0 cm / 0.5 cm) and a trapezoid in between. Detection was done with ultraviolet detector (UV) at 214 nm and multiangle laser light scattering (MALS) detector (BI-MwA Molecular Weight Analyzer) at 35°, 50°, 75°, 90°, 105°, 130°, and 145° at 635 nm followed by a dynamic light scattering (DLS) detector (Zetasizer Nano, Malvern Instruments, Malvern Works, UK). The AsFIFFF-MALS was controlled with PostNova AF2000 software and the DLS with Malvern Zetasizer software.

2.3 Buffers and solutions

Phosphate buffer saline (PBS), pH 7.4 was prepared by dissolving one PBS tablet into 200 mL Milli-Q water. 1.06 g of anhydrous Na_2CO_3 was dissolved in 100 mL Milli-Q water to prepare 0.1 M Na_2CO_3 stock solution. 0.8401 g of NaHCO_3 was dissolved in 100 mL Milli-Q water to prepare 0.1 M NaHCO_3 stock solution. 10 mL of 0.1 M NaHCO_3 stock solution was mixed with 90 mL 0.1 M Na_2CO_3 stock solution and pH was adjusted to pH 11.3 with the addition of 200 μ L 5 M NaOH. 2 M ethanolamine, pH 9, was prepared by adding 0.603 mL of ethanolamine in small amount of Milli-Q water and then 900 μ L of fuming 37% HCl was added by pipetting it directly inside the ethanolamine solution. The final solution was prepared by topping up the total volume to 5 mL with Milli-Q water. 0.15 mM NH_4OH , pH 11.3, was prepared by adding 2.26 mL of 25% ammonia and filling it to a final volume of 100 mL with Milli-Q water. 0.1 M glycine-HCl (pH 3.0) was prepared by dissolving 7.5 g of glycine to 800 mL of Milli-Q water and adjusting the pH to 3.0 with 1 M HCl. Milli-Q water was added until volume was 1 L. 1 M Tris-HCl (pH 8.5) was prepared by adding 12.11 g of Tris base to 80 mL of Milli-Q water. The pH was adjusted to 8.5 with 1 M HCl, and Milli-Q water was added to a final volume of 100 mL.

For the isolation experiments, 1 mL aliquots of plasma or platelet-concentrate derived platelet-free plasma (PC-PFP) samples were centrifuged for 5 minutes at 13,000 g to remove cellular debris and large protein aggregates. 250 μ L of the plasma, diluted in 4750 μ L of PBS buffer, pH 7.4, was used in the studies unless otherwise stated.

2.4. Preparation of affinity monolithic column and isolation procedure

2.4.1 Immobilization of mouse anti-human CD61 antibody on CIM[®] CDI - 0.34 mL disk

Immobilization was done according to the manufacturer's protocol, with a slight modification. In brief, CIM[®] CDI -0.34 mL disk, with the pore size of 1.3 μ m, was placed in the disk housing and washed with 6.8 mL of Milli-Q water followed by 15 mL of PBS buffer. 1 mL of 0.5 mg/mL purified mouse anti-human CD61 antibody was injected through the housing back and forth using a syringe pump and two syringes with a flow rate of 0.5 mL/min for 1.5 hours at room temperature. The cycle of injecting back and forth was repeated five times daily for three consecutive days. The disk was left over a weekend to ensure maximum antibody immobilization, the disk was then washed with 3 mL of PBS, and the remaining CDI groups were deactivated by passing 1 mL of 2 M ethanolamine (pH 9) through the system twice and left overnight with stoppers. The system was finally washed with 20 mL PBS buffer to remove the excess ethanolamine.

2.4.2 EV isolation procedure

Before the disk was used to collect the isolates, it was first conditioned by injecting and eluting three plasma samples (250 μ L of plasma in PBS, final volume 5 mL) through it. Target sample for the method use in future is plasma. In this study, also another platelet EV source, an outdated clinical platelet concentrate was used to test the feasibility of the method. 5 mL of diluted plasma samples were injected into the setup using a syringe pump at flow rates of 0.25-1.0 mL/min. The flow through was collected after the void volume. Then the system was washed with 3 mL of PBS. Elution of EVs was done with 1.5 mL of carbonate-bicarbonate solution (pH 11.3). After the void volume, 0.5 mL of

the isolate was collected and neutralized immediately with the addition of 50 μ L 1M HCl. Next, 2 mL of PBS was injected to remove the carbonate-bicarbonate solution. Then, 1.5 mL NH_4OH , (0.15M pH 11.3) was injected to wash any residual compounds bound to the disk. Finally, 3 mL PBS was injected to regenerate the system before the next sample injection.

2.5 Characterization of isolated EVs

2.5.1 Lowry Method (DC protein assay)

Protein concentrations were measured using Lowry assay (DC protein assay) at 690 nm, with bovine serum albumin as a standard [31].

2.5.2 Western blotting

Proteins from the isolated EVs were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. PageRuler prestain protein ladder of 10 to 250 kDa was used as the ladder marker. The membranes were then probed with antibodies against TSG101 at 1:500 dilution and CD9 at 1:500 dilution. Proteins of interest were detected with 1:3000 diluted HRP-conjugated sheep anti-mouse IgG antibody and visualized with the Clarity ECL substrate according to the provided protocol.

2.5.3 Transmission electron microscopy (TEM)

EM samples were prepared by negative staining as described earlier [32]. Briefly, EVs were loaded on 200 mesh grids, fixed with 2% PFA, stained with 2% neutral uranyl acetate and embedded in methyl cellulose uranyl acetate mixture.

2.5.4 Asymmetrical flow field-flow fractionation (AsFIFFF) connected to UV, multi-angle light scattering (MALS), and dynamic light scattering (DLS) detectors

AsFIFFF was employed at room temperature after the isolation step with anti-CD61 disk. The EVs collected from anti-CD61 disk were then injected to the AsFIFFF system. Total injection volume was

500 μ L with injection flow of 0.1 mL/min over 5 minutes during the focus mode at the cross-flow rate of 3 mL/min. Detector flow rate was set to 1.0 mL/min and PBS was used as eluent. After the focusing step and 1 min transition time, started a 5 min linear decrease in cross-flow to 1.0 mL/min, followed by a linear decrease over 15 min to 0 mL/min. Only the detector flow was applied for 10 min after the cross-flow reached 0 mL/min. This was followed by a rinse step with only tip flow of 0.1 mL/min for 0.6 min, making a total run time of 36.6 min. Optimal conditions for the AsFIFFF experiments are collected in Table S1.

The root mean square radius of gyration (R_g) was determined by the MALS and the flow DLS was used to determine the hydrodynamic radius (R_h) of EVs. The MALS data was treated with the Zimm first order model [33] found in PostNova AF2000 software. The flow DLS data was treated with Malvern Zetasizer software utilizing refractive indices of 1.39 for EVs [34] and 1.332 for PBS.

3 Results and discussion

The applicability of immunoaffinity chromatography using methacrylate-based monolithic disk column for the isolation of platelet-derived extracellular vesicles (EVs) from plasma was studied. The disk was immobilized with mouse anti-human CD61 antibody, the most prominent surface marker on platelets and their EVs. CD61 (integrin β_3) in platelets is a subunit of two heterodimeric integrin complexes namely with CD41 (also known as integrin α_{IIb}) and with CD51 (integrin α_V). There are 80-100,000 copies of CD41/CD61 complex on a platelet, which makes 3% of the total protein and 17% of membrane proteins [35]. This is a highly abundant platelet receptor making it ideal to detect the majority membranous vesicles originating from platelets. CD41/CD61 has been shown to be present in different EV subtypes, e.g. on exosomes and plasma membrane originating EVs [36,37]. The success of the isolation was evaluated by measuring their protein and particle concentrations, as well as the expression of EV-specific proteins CD9 and TSG101 by Western blotting. AsFIFFF was employed for accurate characterization of size distributions for EV isolates. Finally, TEM was used to elucidate the morphology of the isolates.

3.1 Development and optimization of the immunoaffinity chromatographic isolation method

Polymeric carboxyimidazole (CDI) monolithic disk was used as chromatographic column in affinity chromatography (Fig. 1). Mouse anti-human CD61 antibody was covalently immobilized to CDI disk to target platelet-derived EVs in plasma. The selected CDI disk material has a broad working pH range and good chemical stability, and the disk has homogeneous channels of 1.3 μm , which are suitable for EV isolation. The methacrylate-based monolithic disk column has also advantages compared to silica monoliths, such as a wide pH range and high mechanical stability, and advantages in biocompatibility when working with complex biofluids, as plasma used in this study. The column is also easier to use compared to magnetic beads immunoisolation, since usually either the beads or remaining antibodies are retained on the EVs.

Fig. 1. Schematic description of the overall EV isolation procedure from plasma using the anti-CD61 CIM[®] disk. Void volume for the system was 0.5 mL.

After the adsorption of EVs onto disk, the effect of different pHs (pH 3.0, 8.5, and 11.3) was evaluated for the desorption of the analytes. The latter pH was selected based on our recent studies on lipoprotein isolation [19]. Surprisingly, the elution of EVs was possible only under very high alkaline

conditions of pH 11.3, which indicates that the antigen-antibody interaction was of high affinity. After the release of the EV isolates at pH 11.3, the collection vial was immediately adjusted with 1 M HCl to neutralize the sample pH. For the isolation procedure, the carbonate-bicarbonate solution (pH 11.3) was selected for the eluent, since it reduced the amount of the unspecifically bound proteins in the isolate. Fortunately, these were easily removed from the disk with 0.15 mM NH_4OH , the same solution was used for the regeneration of the anti-CD61 CIM[®] column that was reusable for hundreds of EV isolations after regeneration.

Different flow rates for step 2 in Fig.1 and their effect on isolation yields were assessed by isolating EVs from 5 mL of diluted plasma samples (250 μL of plasma in PBS, final volume 5 mL). The isolates were Western blotted and assessed with EV markers (CD9 and TSG101). CD9 and TSG101 are considered to be proteins that are enriched specifically in exosomes [38], but in platelets CD9 is a plasmamembrane tetraspanin which associates with many EV inducing signaling receptors. Both microvesicle- and exosome-enriched fractions derived by differential ultracentrifugation at 10-20,000 and 100,000 g, respectively, show the presence of CD9 and TSG101, a soluble protein [37,39]. Hence, in platelet context these markers are not specific for any EV subtype. Fig. 2A shows the protein content of the samples along with the Western blot results. The doubling of flow rate resulted in a decrease in protein concentration of 17 % on average. Western blot intensities of EV markers also became less distinct, indicating the decrease in the EV concentration with the increase of the flow rate. Thus, a flow rate of 0.25 mL/min was selected for the further studies for the highest possible isolation yields. TEM analysis of plasma isolated EVs (Fig. 2B) confirmed the presence of membrane encapsulated EVs. In addition, the platelet-depleted plasma isolated EVs showed typical morphology in the EM (Fig. S1).

Fig. 2. A) Western blotting and protein concentrations of isolated EVs from plasma with the anti-CD61 disk at flow rates 0.25-1.0 mL/min. B) TEM morphology of platelet-derived EVs, isolated with anti-CD61 disk. The isolates show vesicles with different diameters and a typical “cup-shaped” EV morphology.

3.2 Yields and purity of EV isolates

To assess the efficiency of anti-CD61 disk in the isolation of platelet-derived EVs, plasma and platelet-concentrates were used. The particle concentrations and mean sizes (Figs. S2-S3) were determined with a nanoparticle tracking analysis (NTA). The averaged results have been collected to Table 1. There was an approximately ten-fold increase in the particle concentration of the isolates compared to the flow through. The plasma isolates contained over three times higher protein concentration compared to the platelet concentrate-derived isolates, and both had similar mean sizes. In addition, particle:protein ratio of the isolates increased hundred-fold indicating a highly concentrated EV isolates.

Table 1. EV isolation yields from plasma and platelet-depleted concentrate derived platelet-free plasma (PC-PFP). Particle yields and mean sizes were determined with the nanoparticle tracking analysis.

	Plasma		Platelet-concentrate derived platelet-free plasma (PC-PFP)	
Average	Flow through (n=3)	Isolate (n=3)	Flow through (n=2)	Isolate (n=2)
Protein yield ($\mu\text{g mL}^{-1}$)	2637 $\pm$ 79	338 $\pm$ 72	1147 $\pm$ 47	97.7
Mean size (nm)	157 $\pm$ 50	174 $\pm$ 60	123 $\pm$ 61	175 $\pm$ 64
Particles (mL^{-1})	9.70 $\pm$ 3.26 $\cdot 10^{10}$	1.17 $\pm$ 0.41 $\cdot 10^{12}$	1.51 $\pm$ 0.13 $\cdot 10^8$	1.29 $\pm$ 0.17 $\cdot 10^9$
Particle:Protein Ratio	3.70 $\pm$ 1.32 $\cdot 10^7$	3.47 $\pm$ 1.02 $\cdot 10^9$	1.32 $\pm$ 0.16 $\cdot 10^5$	1.32 $\pm$ 0.17 $\cdot 10^7$

3.3 Size fractionation and characterization by AsFIFFF-UV-MALS-flow DLS

AsFIFFF connected to multidetector system (UV-MALS-DLS) was utilized to determine the sizes of the isolates. The dimensional analysis of EVs such as performed with AsFIFFF enables also subpopulation isolation, which will hugely improve our understanding of the physiopathological roles of different EVs. As the EV formation is spatiotemporally dynamic and carefully regulated, it will be imperative to isolate EVs based on “umbrella” properties, e.g. CD61 positive EVs, which will catch the majority of EVs from a certain cellular source and then perform further analysis to identify the changes occurring in the subpopulations.

After the plasma-derived EVs were isolated with the anti-CD61 disk at the flow rate of 0.25 mL/min, the collected eluate of 0.5 mL was manually injected into the AsFIFFF system without additional neutralization. The run was started after the loop was filled. MALS was used to determine the root mean square radius of gyration (R_g) and flow DLS to determine the hydrodynamic radius (R_h). The results of UV-MALS are shown in Fig. 3. The LS showed two peak maxima at 16.1 min and 25.7 min elution times, while the UV had two clear maxima at 9.7 min and 15.5 min (Fig. 3). The UV and

MALS profiles resembled those of previous AsFIFFF study of exosomes [27], which confirmed a successful isolation of EVs with the anti-CD61 disk. The range of diameters of the isolated EVs were 20-110 nm based on the radius of gyration calculated from the MALS data. These results were in the size range of the exosomes, with smaller particles having size range of exomeres (size <50 nm), a new type of nanoparticles found just recently [28]. This population could be identified in the range 10-20 min (Fig.3). The UV signal at 15.5 min indicated that the exomere-sized particles ranging 10-50 nm also had the highest concentration in the isolate. To exclude the presence of possible contaminant lipoproteins was studied by running plasma sample through the deactivated CIM® CDI disk and by analyzing the isolated fraction (Fig. S4) with fast protein liquid chromatography (FPLC). The sample contained twice as much plasma (500 µL), and was more concentrated (diluted to 2.5 mL with PBS) compared to plasma samples used for the isolation experiments, thus creating an experiment where exaggerated amount of non-specific adsorption was induced. The lipoproteins were not seen from the chromatogram at 280 nm, nor any non-specifically adsorbed proteins, indicating that EV isolates from anti-CD61 disk were of high purity. Fig. S5 shows 1 mg/mL of low-density lipoproteins (LDLs) isolated by ultracentrifugation injected to the same FPLC system, which confirmed that LDLs were not adsorbed on the deactivated monolithic column utilized in this study.

Fig. 3. Normalized AsFIFFF-UV (blue dashed line) and AsFIFFF-MALS (grey line) fractograms of isolated EVs in PBS, with the root mean square radius of gyration, R_g (red circles). Fractionation conditions utilized were: 350 μm spacer, cross-flow rate of 3 mL/min, detector flow rate 1.0 mL/min, focusing step 5 min, linear decrease in cross-flow to 1.0 mL/min in 5 min, followed by a linear decrease over 15 min to 0 mL/min. Detector flow was applied for 10 min after the cross-flow was off.

The flow DLS fractogram (Fig. 4) resembled that of MALS, but depicted the hydrodynamic radius (R_h) as Z-average. The range of diameters of the EV isolates, based on the hydrodynamic radius was 30-130 nm. Here as well as in the Fig.3, we can see that elution times 11-20 min represent the exomere-sized particles [28], followed by exosome-sized particles from 20 min onward. Thus, the MALS and the flow DLS results supported the successful isolation of platelet-derived EVs of varying size classes.

Fig. 4. Raw flow DLS detector signal (blue line) with Z-average or the hydrodynamic radius, R_h (red circles).

3.3.1 Size and shape of the isolated EVs

Based on major peak intensities obtained from the flow DLS, the smallest particles (size <50nm) were identified at the elution time range of 18.6-19.0 min, small EVs or EV-S (60-80 nm) at 23.2 - 23.5 min, and large EVs or EV-L (90-120 nm) at 27.2-28.1 min. The mean hydrodynamic diameter was obtained from the flow DLS data (Fig. 5A) and the diameter of gyration from the MALS data (Fig. 5B).

Fig. 5. Measured mean hydrodynamic diameter (A) and root mean square diameter of gyration (B) of small particles, EV-S and EV-L of the anti-CD61 isolates from plasma. Hydrodynamic diameter

was obtained with Zetasizer software and root mean square diameter of gyration was obtained from the MALS data with the Zimm first order model.

A shape factor/an asymmetry factor (ρ) calculated (Table 2) was used to estimate the structural characteristics of the different EVs. Value of 0.75 represents the ideal homogeneous spheres and values over 2 resemble more elongated ellipsoids [27,40–42]. The *bona fide* EVs are membrane-encapsulated, while the exomeres lack the membrane structure and are non-encapsulated [28]. The L-EV had the lowest factor of 0.85, indicating that larger vesicles were packed with cargo, and resembled the ideal homogeneous spheres in shape. Similar shape factor has also been previously described for large exosomes in the literature [27]. In addition, the S-EVs and the smaller particles had similar shape factors (0.95-0.96), and thus resembled each other in shape, although the exomeres are considered to be non-encapsulated [28].

Table 2. Shape factor (ρ) of platelet-derived EVs based on the results of AsFIFFF. The MALS data was utilized for (D_g) and the flow DLS data for (D_h).

	Small particles	EV-S	EV-L
D_g (nm)	43.4	64.9	100.5
D_h (nm)	45.0	68.5	118.9
$\rho = R_g/R_h$	0.96	0.95	0.85

This exciting finding confirms with a non-electron microscopy-based method that platelets do secrete exosome-sized particles despite not having multivesicular bodies, but rather MV- α -granules, as a source of S-EVs [36]. Furthermore, this is the first time that three separate classes of EVs within the exosomal size range have been reported in platelets [43]. In future, large batch isolations of these <130 nm platelet EV subpopulations by AsFIFFF will be needed to establish their molecular content

and identity e.g. regarding exomeres to determine their relevance in platelet physiology [23]. This finding adds to the well-established heterogeneity of platelet EVome [23,44]. In this study, the AsFIFFF was used as a fractionation and characterization technique to discern EV subpopulations of different sizes and their shape. However, it will be possible to apply AsFIFFF in a preparative mode to isolate different CD61-positive subpopulations from platelets and further characterize them in downstream analyses e.g. for their molecular cargo.

4 Conclusions

The immunoaffinity chromatographic procedure including the polymeric anti-CD61 CIM[®] affinity monolithic disk column was developed and successfully utilized for selective and fast isolation of platelet-derived EVs from plasma. The isolation procedure, carried out in under 10 minutes at flow rate of 1.0 mL/min with 5 mL of diluted plasma sample, resulted in highly concentrated platelet-derived EVs in a final volume of 500 μ L. However, 0.5 mL/min was found to be optimal, due to the compromise between the yield and the speed of the isolation. The procedure can be regenerated for hundreds of isolations by using the same disk. For increasing yields, the monolithic disk can be loaded with higher antibody amount. The plasma sample volumes and flow rates can also be further modulated resulting in even larger yields. Further studies are needed to assess the epitope functionality after the elution and the effect of elution conditions on the EVs. There might be a need for concentrating step in some applications, since the isolates are diluted. AsFIFFF combined with MALS and flow DLS gave important and valuable knowledge of the different EV populations and their size and shape distributions. TEM confirmed the presence of EVs with the typical morphology, and Western blotting the presence of EV markers. Finally, this study gives for the first time support for the existence of platelet-derived EVs in the exosomal size range < 100 nm.

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