PROTEIN CORONA FORMATION AROUND EXTRACELLULAR VESICLES

PhD thesis

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Table of contents

List of Abbreviations

ANOVA – analysis of variance

CD… – cluster of differentiation

DAMP – damage-associated molecular
pattern

dC – differential centrifugation

DC – dendritic cell

DGUC – density gradient
ultracentrifugation

ECM – extracellular matrix

ELISA – enzyme-linked immunosorbent
assay

EM – electronmicroscopy

EVDP – extracellular vesicle depleted
blood plasma

FITC – fluorescein-5-isothiocyanate

HLA-D – human leukocyte antigen-D

HSV – herpes simplex virus

IgG2 – immunoglobulin G2 and G4

IL-6 – interleukin-6

ISEV – International Society for
Extracellular Vesicles

lEV – large-sized EV

LDL – low-density lipoprotein

LNP – lipid nanoparticle

mEV – medium-sized EV

MISEV – Minimal Information for Studies
of Extracellular Vesicles

moDC – monocyte-derived dendritic cell

NTA – Nanoparticle Tracking Analysis

NP – nanoparticle

PAMP – pathogen-associated molecular
pattern

RA – rheumatoid arthritis

RNA – ribonucleic acid

RSV – respiratory syncytial virus

SEC – size exclusion chromatography

sEV – small EV

TEM – transmission electronmicroscopy

TNF-α – tumour necrosis factor alpha

TRPS – Tunable Resistive Pulse Sensing

1. Introduction

1.1. Protein corona formation on artificial nanoparticles (NPs)

The therapeutic potential attributed to artificial NPs have put them in the spotlight recently [1]. With the growing interest and foregoing studies, more and more information is gathered regarding these nanoscale particles that are one of the promising vehicles of targeted therapy [1]. Artificial NPs are usually employed to introduce drugs to the body and can be administered intravenously, or even *via* inhalation, ingestion or through the skin [2]. Early during the first trials when these particles were applied in biological fluids, it became clear that depending on the surface characteristics of the used NP and the *milieu* they have entered, proteins from the surroundings adhere onto the surface of the NPs [3]. This protein attachment can potentially mask surface molecules of the NPs as well as decrease NP mobility leading to worse therapeutic result as the NP drug content loses target [4-6]. The targeting efficiency due to the protein coverage was found to be reduced by 94% *in vitro* and 99% *in vivo* in one study [7]. Hence, much effort has been implemented to gain better knowledge on the so called "protein corona" of NPs.

While so far it proved impossible to avoid protein corona formation on the surface of NPs, with clever modifications of shell molecules, the directed adherence of proteins, with more predictable effects can be achieved [8]. For example, dextran pre-coating of NPs was found to reduce the percentage of certain proteins in the corona, and these proteins (e.g. complement components, apolipoproteins and coagulation factors) were found to have substantial influence on the NP-uptake by macrophages [9] (Figure 1.).
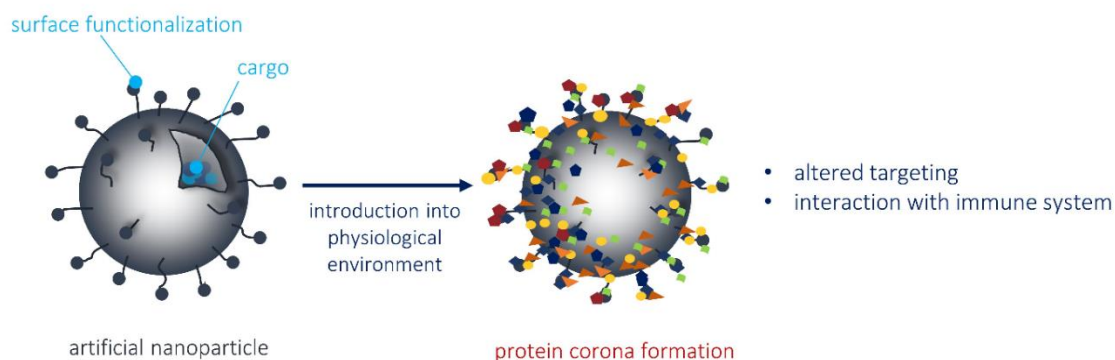
Figure 1. Protein corona formation around artificial nanoparticles.
Once nanoparticles are introduced into a physiological environment, e.g. blood plasma, a protein corona is formed around them, which substantially modifies the fate of NPs, by covering the surface molecules of NPs as well as by interacting with the immune system and the target cells. (based on [10])

While the surface properties of NPs determine the components of the protein corona, the size of the particles show only quantitative correlation with the proteins bound to the NPs [11-13]. It is hypothesized that the protein adsorption onto the NPs happens passively by non-specific interactions of the NP surface molecules and environmental proteins, as well as due to that the entropy of the adhering molecules increase when they attach to the NPs [14-16]. In the moment a NP is introduced to a system that contains proteins, e.g. the blood plasma, proteins start "sticking" onto them [17]. It was found, that particles become first surrounded by albumin after which albumin molecules switch place with less abundant plasma proteins that have higher association and lower dissociation rate constants [18]. Therefore, proteins with the highest plasma concentration are not necessarily the most profuse corona components [12, 19-21]. With time, several layers of proteins evolve, where inner layers, called the "hard corona" tend to be more stable, while the outer "soft corona" shows looser structure and more rapid exchange of components that primarily bind to other corona proteins and not the NP itself [22]. When predicting the function of the protein corona based on e.g. high throughput proteomics, it is important to consider that the protein-NP interaction may change the way in which a protein is exposed to its environment by rearranging critical binding or catalytic domains with a possible reduction or loss of function of protein activity [23]. Although recently questioned [11], it is also crucial to consider that *in vitro* protein corona formation seems remarkably different from what was described in *in vivo* experiments [23-25]. While *in vitro* the circumstances are usually well controlled, *in vivo* there are several factors, e.g. sheer-force in the circulation, alteration of pH, etc. that lead to the dynamic and mostly unpredictable changes of the protein corona around NPs [25]. Similarly, mostly unavoidable experimental steps, such as centrifugation, filtration, use of anticoagulants, etc. increase this discrepancy and have to be acknowledged when interpreting *in vitro* research data [23-25]. Besides, another interesting consideration could be that the protein corona formation might at some rate be personal, which, on the one hand may make it even more difficult to drive general conclusions without substantially high number of involved persons. On the other hand, it might offer the opportunity to employ the analysis of the protein corona for searching for disease biomarkers [26]. Also, recently, instead of a protein corona, the concept of a biomolecular corona is increasingly favoured

indicative of that not only proteins, but lipids, polysaccharides, protein metabolites, etc. attach to the NP surfaces [27].

1.2. Extracellular vesicles

1.2.1. Classification

Extracellular vesicles (EVs), our endogenous nanoparticles are diverse structures surrounded by lipid-bilayers that are released by all known types of cells either by budding from the cell membrane or by being released from the multivesicular bodies [28]. They can be found in all bodily fluids, and wide-ranging functions are attributed to them [28]. Since the first description of EVs in 1967 (referred to as "platelet dust" [29]), the EV research field has shown exponential expansion drawing the growing surge to unify the nomenclature and pave the basic requirements of their research [30]. Hence, the International Society for Extracellular Vesicles (ISEV) released the Minimal Information for Studies of Extracellular Vesicles (MISEV) criteria first in 2014, refreshed in 2018. It is being updated yearly ever since to serve as a supporting "hand rail" for scientists as well as a quality control for the field [31, 32]. In order to untangle the earlier sometimes incoherent nomenclature, MISEV2018 recommends to adhere to some basic rules in the classification of EVs. Based on the recommendation, EVs should either be classified based on their physical (e.g. size, density) or biochemical (e.g. specific molecules) characteristics and on a detailed description of the conditions of the cells that released them (e.g. during apoptosis, in hypoxia, etc.) [32]. Based on size, three categories are established: small EVs, with size <100 nm (some sources mention <200 nm), medium EVs (mEVs, 100-800 nm) and large EVs (lEVs, >800 nm), where the latter two groups are merged sometimes [32]. Despite the clear recommendations, several studies still use the term "exosomes" (EVs that are released from the endosomal compartment) for sEVs and "microvesicles" (ectosomes, EVs that originate from the plasma membrane) for mEVs-lEVs. In our studies, we worked with mEVs of approximately 200 nm mean diameter (based on our measurements with Nanoparticle Tracking Analysis (NTA) and Tunable Resistive Pulse Sensing (TRPS)).
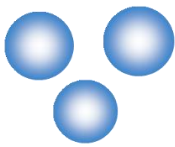
1.2.2. EV separation techniques and confounding factors of separation

In order to be able to study the properties of EVs, it is necessary to separate them from the biological *milieu* they reside in. The way of separation however may itself have effect on the attributes of vesicles. According to MISEV2018, differential centrifugation

(dC) has been the most commonly utilized method of EV separation worldwide by 2016 [32]. With different speeds of centrifugation, different size particles sediment. Thus, with specific settings it is feasible to separate vesicles from the fluid compartment. Meanwhile, due to the great physical forces, the biological function of EVs might be altered as well as several other, non-vesicular structures (e.g. immune complexes, protein aggregates, lipoproteins) might co-sediment together with the EVs [33-35]. Despite the aims to differentiate between vesicles and the other particles (e.g. protein aggregates, Table 1.), the field has not yet been fully capable to separate EVs from other EV-sized structures so far.

Table 1. Example for the similarities and the possibility for differentiation between EVs and protein aggregates.

EVs: extracellular vesicles; NTA: nanoparticle tracking analysis; TRPS: tunable resisitive pulse sensing; BCA: bicinchoninic acid assay; SPV: sulfo-phospho-vanillin assay (✓ signs indicate that the two groups might be distinguished based on the listed methods) (based on [36, 37])
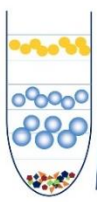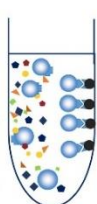
	EVs	protein aggregates
particle number (NTA, TRPS)	high particle number	high particle number
protein (BCA)	high concentration	high concentration
lipid (SPV)	high concentration	no lipid
differential detergent lysis		

✓ protein/lipid and or particle number ratio
✓ differential detergent lysis

Therefore many other techniques have been implemented to obtain EVs with better "purity" and more conserved structure as well as to offer quicker separation [32]. Most recently EV isolation from solid tissues (after enzymatic dissociation of the tissues) has also been achieved [38-41]. Out of the countless approaches of EV separation (and/or concentration), I shortly introduce four methods: dC, density gradient ultracentrifugation (DGUC), size exclusion chromatography (SEC) and affinity-based isolation in Table 2, highlighting the main advantages and disadvantages of each. Combining different methods, such as DGUC and SEC might result in a more pure population of separated EVs, however a big proportion of vesicles might be lost on the way not to mention the

time needed to obtain EVs [42]. Meanwhile, plasma protein contamination in blood plasma derived EVs has been a difficult and unavoidable issue for researchers of the field and getting rid of certain proteins, e.g. albumin appeared impossible even with the combination of these techniques [42, 43]. All in all, the choice of EV separation method should always consider the downstream analysis, ultimately the experimental question, and there is no universal "best way" [32]. The mEVs that we used in our studies were obtained by serial dC and gravity driven size filtration from conditioned cell culture medium. For mEV separation from blood plasma, either dC, DGUC, SEC, or an affinity-based approach was applied.

Table 2. Comparison of four different techniques of EV separation.
dC: differential centrifugation, DGUC: density gradient ultracentrifugation, EV: extracellular vesicle, SEC: size exclusion chromatography (based on [32, 44])

1.2.3. Function of EVs

Since the earliest report of EVs by Peter Wolf, who described these structures as "platelet dust" indicating that they represent the debris of platelets that exert coagulative properties [29], nowadays diverse functions are attributed to EVs both in physiology and pathophysiology [45]. Remarkably, besides that their surface can serve as a field for reactions (e.g. blood clotting), EVs are important participants of intercellular communication as they serve as well-packed information units that can transfer even as sensitive molecules as micro-RNA (ribonucleic acid) between distant sites of the body [46]. Moreover, cross-species (even cross-eukaryote-prokaryote) communication is carried out by EVs as vesicles of the gut flora as well as of several parasites were found to have immune-modulatory effects on mammals [47-51].

1.2.3.1. EVs in rheumatoid arthritis (RA)

RA is a systemic autoimmune disease that's most striking manifestation is the debilitating chronic joint destruction [52]. Besides, RA also involves the cardiovascular system leading to significantly increased cardiovascular morbidity and mortality of patients [52]. Worldwide, RA appears with a 0.1-2% prevalence, hence being among the most prevalent autoimmune diseases and thus being not only burdensome to the affected individuals but globally, to the healthcare system as well [53]. So far, even though the multifactorial background and pathogenesis of RA is getting more and more understood, there are still many blank spaces to be filled and thus the possibility of introducing novel, more targeted therapies also seems promising [54].

The contribution of EVs to RA is extensively studied and many parts of the pathophysiology where EVs play role have already been described (extensively reviewed in [55-57]. According to the cited reviews, with a few added examples (reference added to each) EVs may:

- serve as pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs);
- carry autoantigens;
- transfer inflammatory lipid metabolites e.g. prostaglandins, leukotrienes;
- transfer microRNA;
- carry cytokines;

- interfere with the effect of cytokines [58];
- carry enzymes that contribute to cartilage destruction – e.g. matrix metalloproteinases;
- contribute to immune complex formation;
- contribute to the alteration of surface oxidation status of immune cells related to cell activation as well as to getting rid of over-oxidised molecules e.g. peroxiredoxin 1 [59];
- contribute to anti-inflammatory pathway activation;
- serve as therapeutic targets, therapeutic vehicles and biomarkers.

1.2.4. Surface cargo of EVs

While most studies focus on the intravesicular cargo of EVs, only sporadic pieces of information are available regarding the "package" vesicles carry on their surfaces. Nonetheless, as we have reviewed recently in [60], familiarising with the EV surface molecules might be of great importance to understand how EVs:

- create connections with their surroundings (e.g. anchoring to extracellular matrix (ECM) or cell membranes);
- initiate their uptake by cells and modify immune recognition;
- migrate in the interstitial space, or on a wider perspective, in the body;
- exert effector functions (e.g. mediate apoptosis by expressing Fas ligand; provide cell-free antigen presentation);
- can be recognised in our experimental settings, or as clinical biomarkers of disease.

Figure 2 presents EV surface interactions with divergent blood plasma molecules, while Figure 3 displays some examples where EVs interact with the ECM or with other cells *via* EV surface proteins.
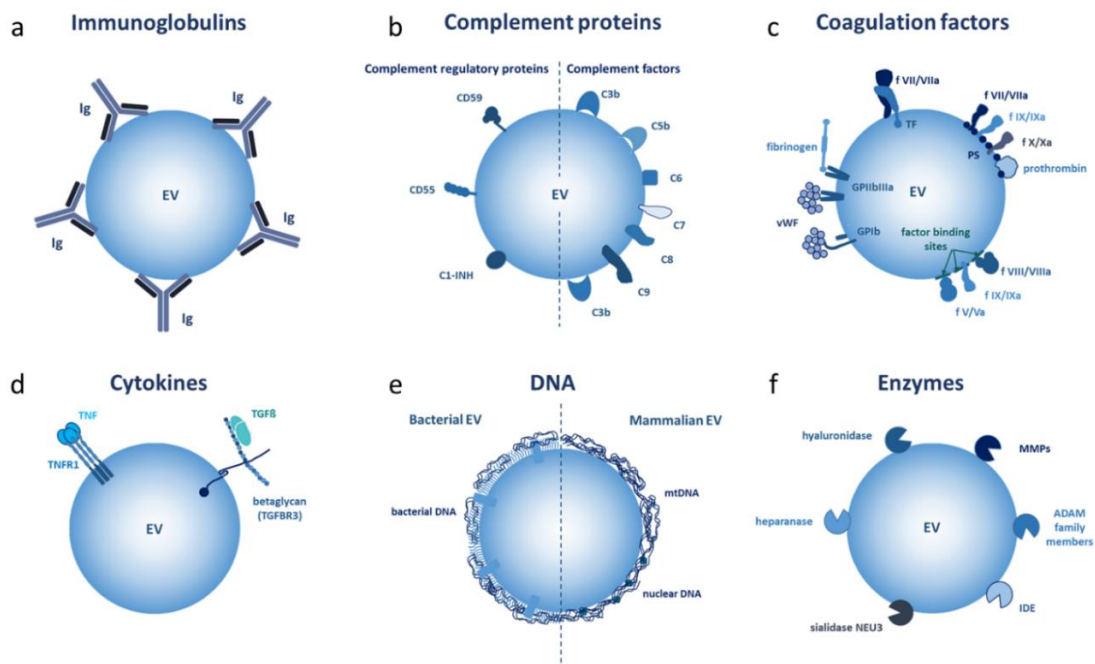
Figure 2. Examples for EV surface-associated molecules.

(a): Antibody binding to EVs has been demonstrated, e.g., in numerous autoimmune diseases. (b): Both complement factors and complement regulatory proteins have been shown to associate with EV surfaces. (c): On EVs from blood plasma, different coagulation factors are also identified. (d): EV-associated cytokines include TNF bound to TNF receptor as well as TGFβ bound to TGFβR3 (betaglycan) on EV surfaces. (e): Both bacterial and mammalian EVs have been demonstrated to carry surface-associated DNA and DNA-binding proteins. In the case of mammalian EVs, both mitochondrial and nuclear DNA were found on EV surfaces. (f): A surprisingly large variety of EV surface enzymes were identified that can bind and cleave protein or glycan substrates of the EV microenvironment. [60]
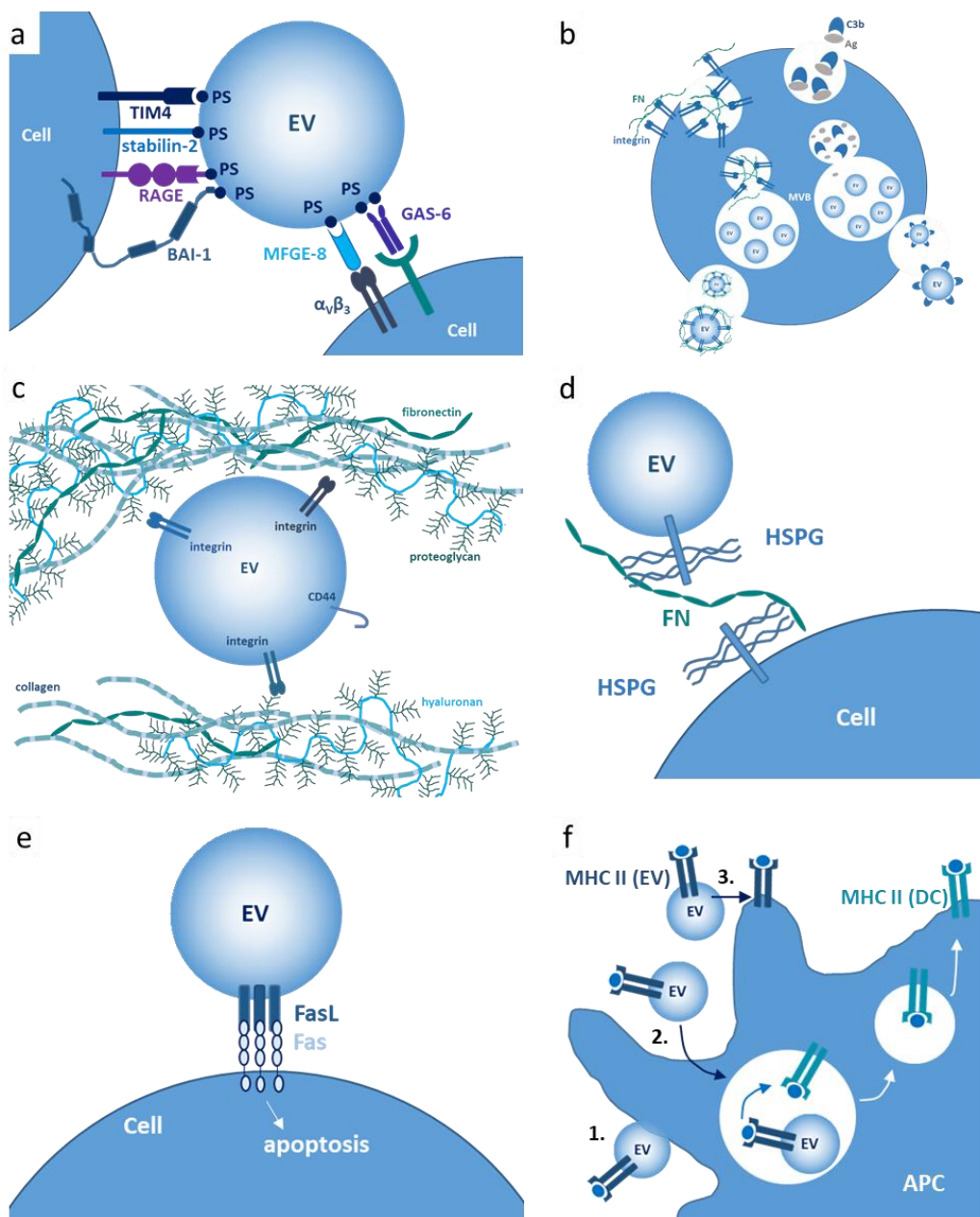
Figure 3. Examples for EV surface interactions with the plasma membrane of cells and components of the extracellular matrix (ECM).

(a): One of the best characterized interactions between the plasma membrane and the surface of EVs is mediated by proteins that recognize externalized phosphatidyl serine (PS) on EVs. Direct interactions with PS include those with TIM4, stabilin-2, RAGE, or BAI-1. Indirect interactions include those between the PS-binding MFGE-8 and $\alpha v \beta 3$ integrin as well as the PS-binder GAS-6 and the MER tyrosine kinase on the cell. (b): Endocytosis of fibronectin (FN) or C3b complement protein is followed by an association of these molecules with intraluminal vesicles within MVBs followed by secretion of exosomes with surface-associated FN or C3b. (c): Interaction of EVs with ECM is mediated by integrins or CD44. (d): FN forms a bridge between HSPGs present on both EV surface and plasma membrane, and mediates EV uptake by cells (e): some EVs carry Fas ligand (FasL) that can initiate apoptosis upon interaction with Fas on cells. (f): Three different ways of antigen presentation by EVs are shown. 1.) Direct way: EVs carry major histocompatibility (MHC) II molecules; 2.) Indirect way: the antigen is transferred from the EV MHC molecule onto the MHC II of the antigen presenting cell (APC); 3.) Cross-dressing: MHC II molecules of EVs are transferred onto the membrane of APCs. (modified from [60])

12

2. Objectives

There have been many reports of undesirable "protein contamination" of EV proteomic studies and it seems nearly impossible to avoid the presence of attaching proteins despite the utilization of the most careful handling of samples. In the case of artificial NPs, the adherence of proteins from the surrounding *milieu* is a well described process and formation of this so called protein corona has long been studied in order to reach an even better therapeutic targeting with NPs. In contrast, there is little known about the surface protein cargo of EVs.

Therefore, in this study, our specific aims were to:

1.) Find out if a protein corona similar to what was observed in the case of artificial NPs forms around EVs in blood plasma.

If the hypothesis of EV protein corona formation proves correct, to:

2.) Describe the molecular composition of the EV protein corona.
3.) Analyse interactions between EVs and surface proteins as well as between the attaching proteins themselves.
4.) Compare the EV protein corona to that of artificial NPs.
5.) Gain better insight on the way protein corona evolves on EVs by employing different methods of microscopy.
6.) Study the function of the surface protein cargo of EVs.

3. Results

3.1. A variety of proteins newly associate with nascent THP1-derived and platelet-derived mEVs in blood plasma

We separated the nascent mEVs of THP1 monocytic cell line cells and of washed (purified on Optiprep gradient) platelets (both kept in serum-free conditioned medium prior to EV separation) by serial centrifugation and filtration steps. We considered that these mEVs carry no or little proteins on their surfaces. Nascent mEVs were then incubated in EV-depleted blood plasma (EVDP) samples of healthy subjects, or of RA patients. After the incubation period of 30 minutes, mEVs were re-isolated by differential centrifugation (dC) and were washed twice. As controls, more pure re-isolation methods, such as i) density gradient ultracentrifugation (DGUC), or ii) size exclusion chromatography (SEC) were utilized. Also, EVDP samples without the addition of mEVs and nascent mEVs incubated in buffer instead of EVDP were used. The re-isolated mEVs (and EVDP controls) were then subjected to mass-spectrometry. For better understanding, a schematic illustration of the workflow is provided below (Figure 4).
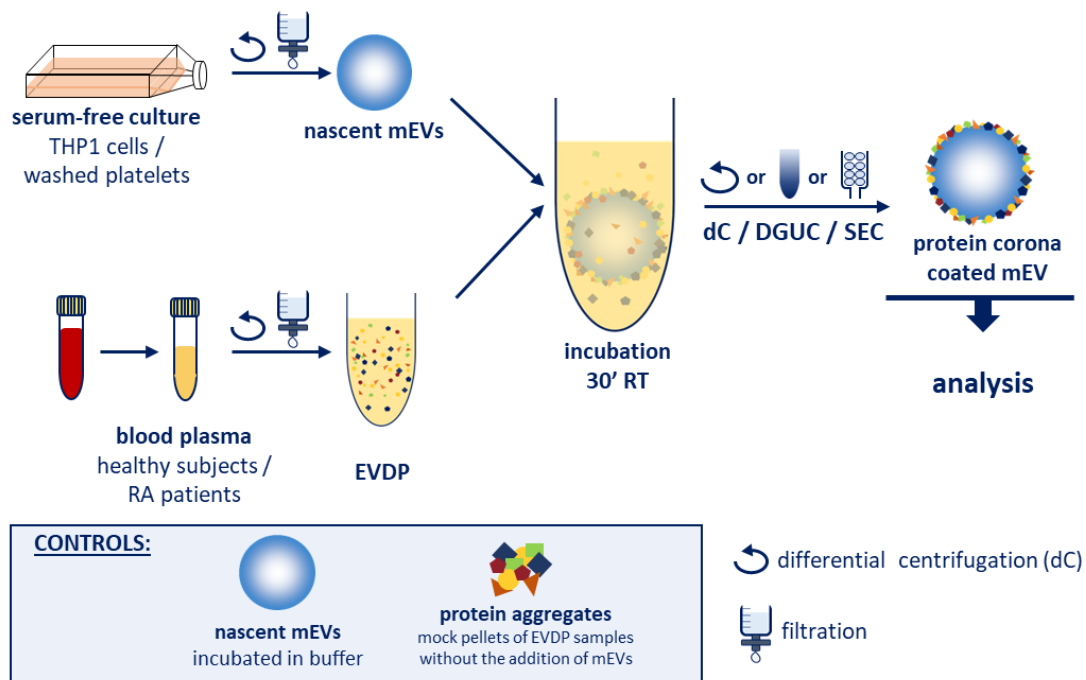
Figure 4. Schematic illustration of the work-flow.

dC: differential centrifugation; DGUC: density gradient ultracentrifugation; EVDP: extracellular vesicle-depleted blood plasma; mEV: medium-sized extracellular vesicle; RA: rheumatoid arthritis; RT: room temperature; SEC: size exclusion chromatography (modified from [61])

To receive the list of proteins that newly associated to the vesicles, we subtracted the proteins that were detected in the nascent mEV samples from the protein lists of EVDP-incubated, plasma protein coated mEVs. We identified the difference of the lists (coated mEV proteins minus the nascent mEV proteins) as protein corona proteins. In the case of coated mEVs re-isolated by dC, altogether, 144 corona proteins were identified, out of which 20 were present in ≥90%, and 61 were present in ≥30% of all the 22 samples. Figure 5 a shows these latter 61 proteins out of which 42 were found in equal ratios among healthy subject-derived and among RA patient-derived EVDP-incubated mEVs, whilst 16 appeared at least 1.5-fold more frequently in the RA EVDP coated mEV lists. Moreover there were 66 proteins in the intersection of the protein coated and the nascent mEV datasets (Figure 5 b). Interestingly, albumin, along with some other typical plasma proteins that are widespread in EV-proteomic works [32] was present in this list. We considered those 9 proteins from this list that have earlier been found to have secreted forms [62] as "extended protein corona" proteins. Of note, we did not include them in our downstream analyses.

Compared to dC, both DGUC and SEC re-isolation yielded lower number of proteins (Figure 5 a). We hypothesize that this partially can be due to the higher purity of these methods as well as due to a greater dilution of the samples resulting in a bigger portion of lost vesicles. Employing DGUC, the presence of associating molecules was also indicated by a higher flotation density of coated mEVs as compared to nascent ones. With flow cytometry applying Annexin V-FITC labelling, nascent mEVs appeared in higher percentage in the 1.10 mg/mL fraction, while coated mEVs were more abundant in the 1.15 mg/mL fraction (multiple t-test $p < 0.001$.and $p < 0.01$, respectively) (Figure 5 c).

The protein corona of washed platelet-derived mEVs incubated in healthy EVDP samples was also analysed in comparison to THP1-derived vesicles both re-isolated by dC. Proteins of nascent platelet-mEVs were subtracted to obtain the list of protein corona proteins same as to how we performed in the case of THP1-mEV studies. A 44% overlap was found between the proteins identified in the THP1-mEV and platelet-mEV protein coronas (considering the proteins that were present in ≥30% of all samples in each group) (Figure 6).

a

RA
| 4.5-5.5x | 3.5-4.5x | 2.5-3.5x | 1.5-2.5x | RA≈HS | 1.5-2.5x | 2.5-3.5x | 3.5-4.5x | 4.5-5.5x |

HS

↺ dC   DGUC   SEC

b

ALBU* (100%) VTDB* (100%)   PPIA* (64%)
AT1A1* (100%) GELS* (86%)   AMPN* (59%)
FETUA* (100%) HS90B* (82%)   LEG1* (45%)

c

16
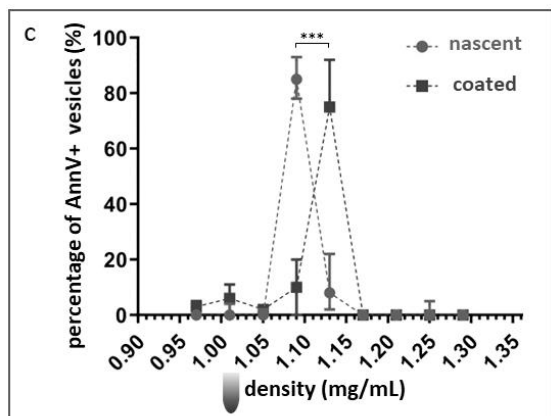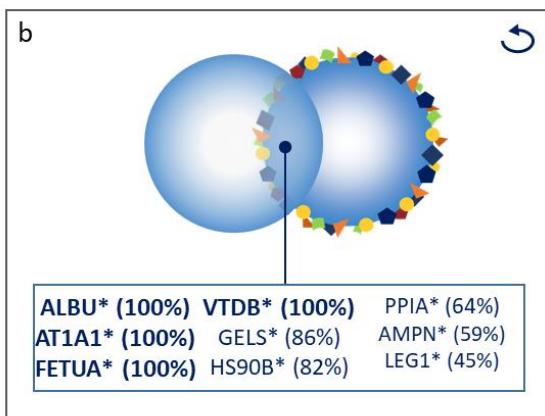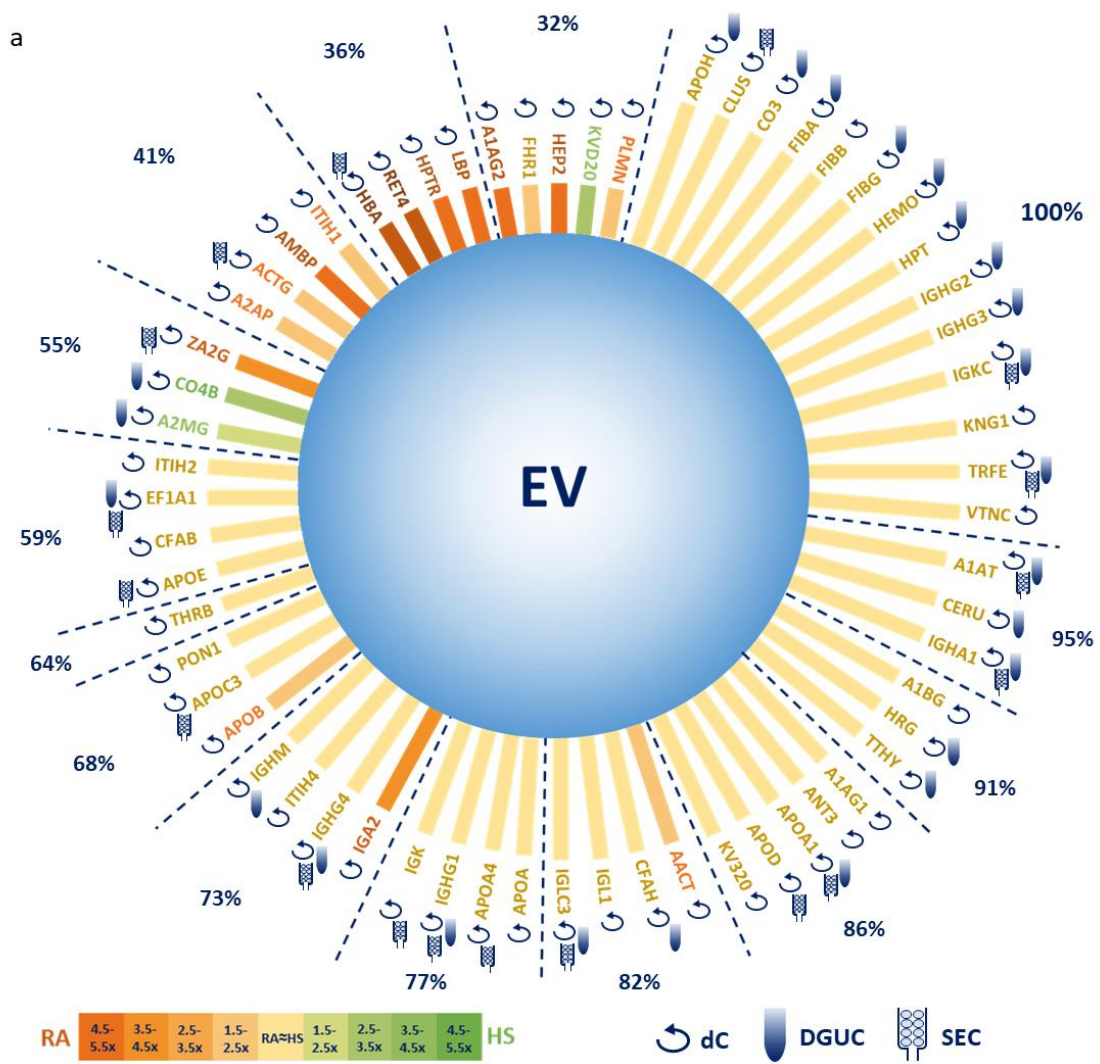
Figure 5. A protein corona is formed around nascent THP1 EVs upon incubation in blood plasma.

(a): THP1 monocytes were cultured under serum free conditions, and nascent mEVs were isolated. These mEVs were next incubated in EV-depleted blood plasma (EVDP) samples from healthy subjects (HS) and patients with rheumatoid arthritis (RA). Plasma-incubated mEVs were subsequently separated by differential centrifugation (dC) (HS, n = 12, RA n = 10), density gradient ultracentrifugation (DGUC) (HS, n = 3) or size exclusion chromatography (SEC) (HS, n = 3). The protein content of the re-isolated mEVs was analysed by MS. As controls, nascent mEVs were used. We identified 61 corona proteins, which were present in more than 30% of the plasma-coated THP1 mEV samples but not in nascent mEVs. A protein was considered to be preferentially found in RA plasma-coated THP1 mEV samples, if it was found at least 1.5-fold more frequently in the RA coronas than in the healthy ones. The proteins that could be identified by DGUC and/or SEC besides the standard dC are indicated by symbols next to the abbreviation of the name of each human protein. Protein name abbreviations are derived from the UniProt IDs of each protein, omitting the species of origin (_HUMAN). (b): The primary protein corona list (identified in dC samples) did not include some proteins due to their presence also in nascent mEVs. These proteins are considered as members of an extended protein corona list and are indicated with asterisk. Their frequency among all samples is indicated in the figure. (c): Flow cytometry analysis of Annexin V (AnnV) positive events in density gradient ultracentrifugation fractions of THP1 mEVs (either nascent (n = 3) or corona coated (n = 3)). The density of THP1-derived mEVs is shifted to a higher density upon incubation in EVDP samples prior to DGUC as compared to nascent mEVs. $p < 0.001$ and $p < 0.01$; multiple t-test [61]
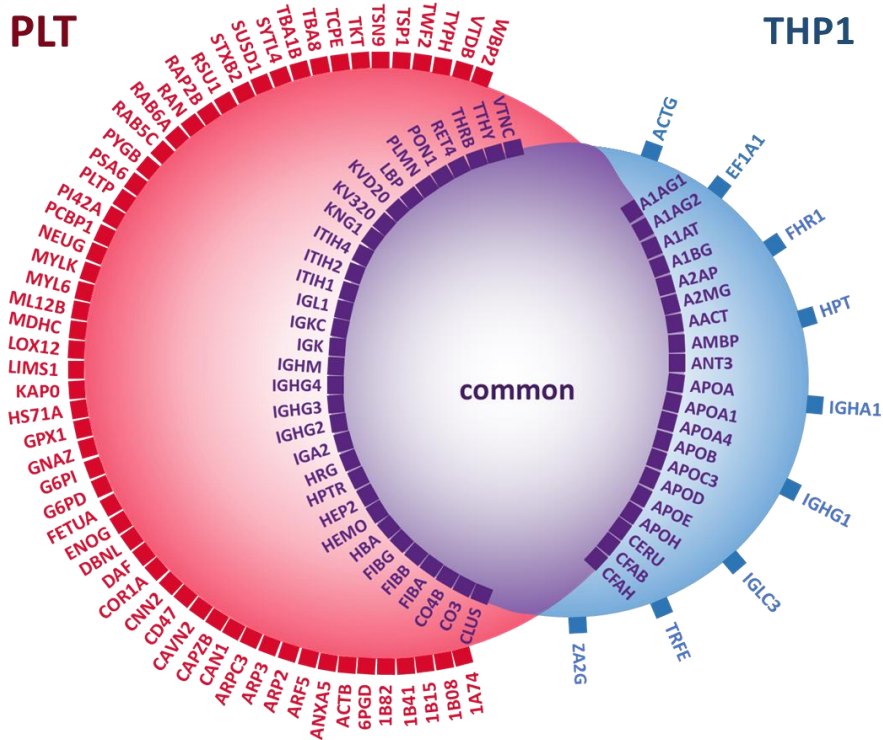
Figure 6. Comparison of the THP1-mEV and the platelet (PLT)-mEV protein corona.

Nascent medium sized EVs (mEVs) released by THP1 cells and by twice washed platelets were incubated in EV-depleted blood plasma samples (n=3), were re-isolated by dC and were studied by MS/MS. The proteins of nascent mEVs were subtracted from the protein list of plasma protein-coated ones. We compared corona proteins found in ≥30% of THP1 and platelet mEVs, and we found a 44% overlap. [61]

3.2. Protein-protein interactions among protein corona proteins and/or EV surface proteins

In order to better understand how the protein corona is formed around EVs, we investigated the interactions of the corona proteins with i) the predicted membrane proteins of nascent THP1-mEVs as well as with ii) other members of the protein corona. We selected those 180 nascent THP1-mEV proteins that had predicted membrane localization based on an online database [62]. For the analysis of protein-protein interactions, we uploaded these proteins as well as the corona proteins (present in ≥30% of dC re-isolated samples) into the online database and web resource, STRING [63]. We included only high confidence physical and functional interactions (confidence score ≥0.700). The predicted map of associations is shown in Figure 7. mEV membrane proteins and corona proteins as well as the type of interaction (between corona proteins or between corona and membrane proteins) are displayed with different colours. It appeared that 13 out of the 61 included corona proteins showed no direct association to any of the mEV membrane proteins, and just interacted with the other members of the protein corona.
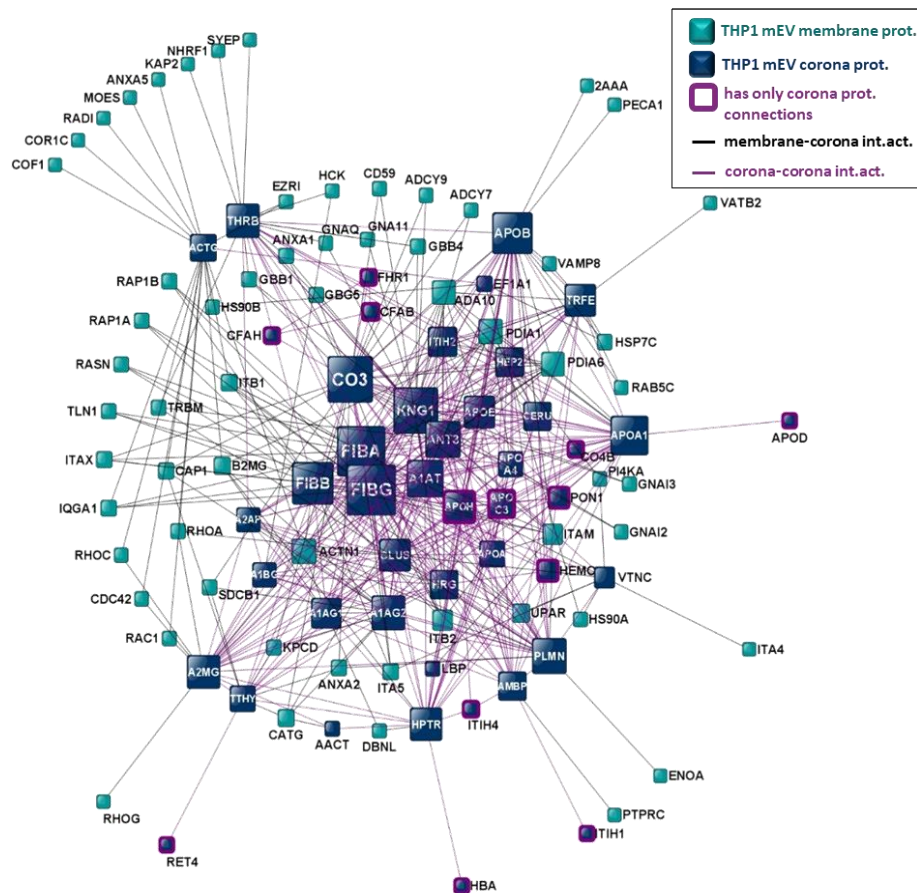
Figure 7. Interactions among corona proteins of mEVs and of corona proteins with the mEV surface.

Representation of protein-protein interactions identified with a high confidence (interaction score ≥ 0.700) with the STRING database and web resource ([63], https://string-db.org). Of note, this database exclude histone proteins and immunoglobulins. Interactions between the nascent THP1 mEV membrane proteins (with predicted membrane localisation according to the UniProt database [62]) and corona proteins are shown. Furthermore, interactions among the corona proteins are also indicated in purple colour. Out of the 107 network nodes, 61 represent membrane proteins while 46 represent corona proteins. Out of 515 edges, 203 show corona protein interactions with membrane proteins and 312 reflect interactions among corona proteins. We found 13 corona proteins (displayed with a purple node frame) to interact with other protein corona proteins only. Non-interacting proteins are not shown. All interaction sources (including physical and functional associations based on text mining, experiments, databases, co-expression, neighbourhood, gene fusion and co-occurrence) were considered in the analysis. Graph centrality measures were set based on the number of connected edges to each node. (modified from [61])

In addition, we used the approach of high-salt concentration washing of fibrinogen, a corona protein present in all dC-re-isolated samples, showing association with not just other corona proteins but THP1-mEV membrane proteins as well. Figure 8 a shows that FITC-labelled recombinant fibrinogen attached to THP1 mEVs (indicated by the appearance of membrane detergent (Triton X [34])-sensitive signal in the mEV gate during flow cytometry) in physiological salt concentration (0.154 M NaCl) buffer, and was successfully removed by increasing the salt concentration to 0.75 M and further to 1.5 M NaCl (t-test $p < 0.05$). We observed the same phenomenon using platelet-mEVs as well (t-test $p < 0.01$) as shown in Figure 8 b.
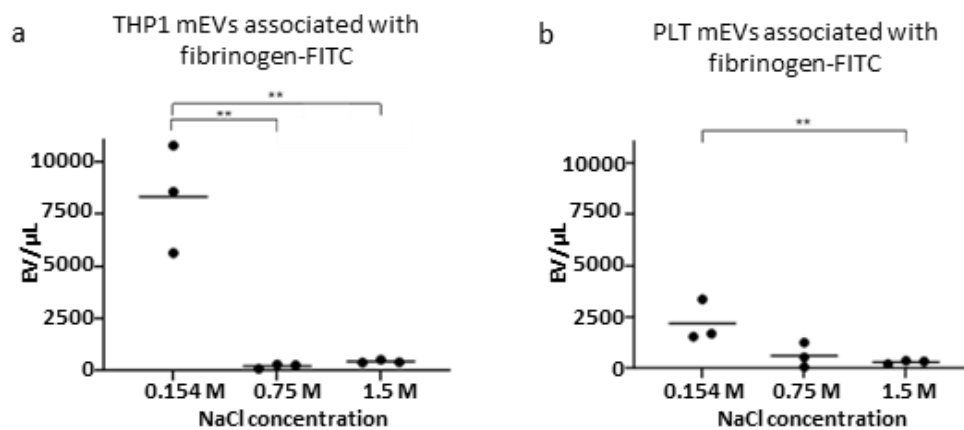
Figure 8. Fibrinogen dissociates from mEVs upon high salt concentration washing.

(a) and (b) show the association of fibrinogen-FITC with nascent THP1 and platelet mEVs, respectively. EV binding of the fluorescently labelled fibrinogen decreased significantly upon exposure of the samples to high concentration salt solutions. (**P < 0.01; t-test). (modified from [61])

3.3. Nanoparticles, viruses and mEVs in the same size range share components of their protein coronas

Ezzat *et al*. studied the protein corona formation around viruses (herpes simplex virus (HSV) and respiratory syncytial virus (RSV)) as well as around positively, or negatively charged lipid nanoparticles (LNPs) [64]. These particles fell into the size-range of the mEVs of our work (approximately 200 nm). Using the same approach as in our studies (subtracting proteins that were present in the non-coated viruses as well from the corona protein lists) and excluding non-human proteins from the analysis, we compared the protein coronas of THP1-mEVs and of platelet-mEVs to the corona of viruses and lipid nanoparticles. As shown in Figure 9 a, 9 proteins were shared by all datasets: apolipoproteins A1, B, C3 and E, complement factors C3 and 4b, the α-chain of fibrinogen and immunoglobulin heavy constants γ2 and γ4 (IgG2 and IgG4 respectively). We also detected the 9 shared proteins together with the pan-EV marker, CD63 in blood plasma mEVs isolated directly from human plasma samples by Annexin V-covered magnetic bead based affinity capture and downstream capillary Western blotting (Wes system) (Figure 9 b, showing six out of the nine proteins). Of note, albumin, the most abundant plasma protein does not appear in this list as it was excluded from our mEV protein corona lists due to its presence in the nascent mEV samples. The fact, that we identified shared components among the protein coronas of different particles points to that there are universal factors in protein corona formation independent of the properties of what the protein corona forms around.
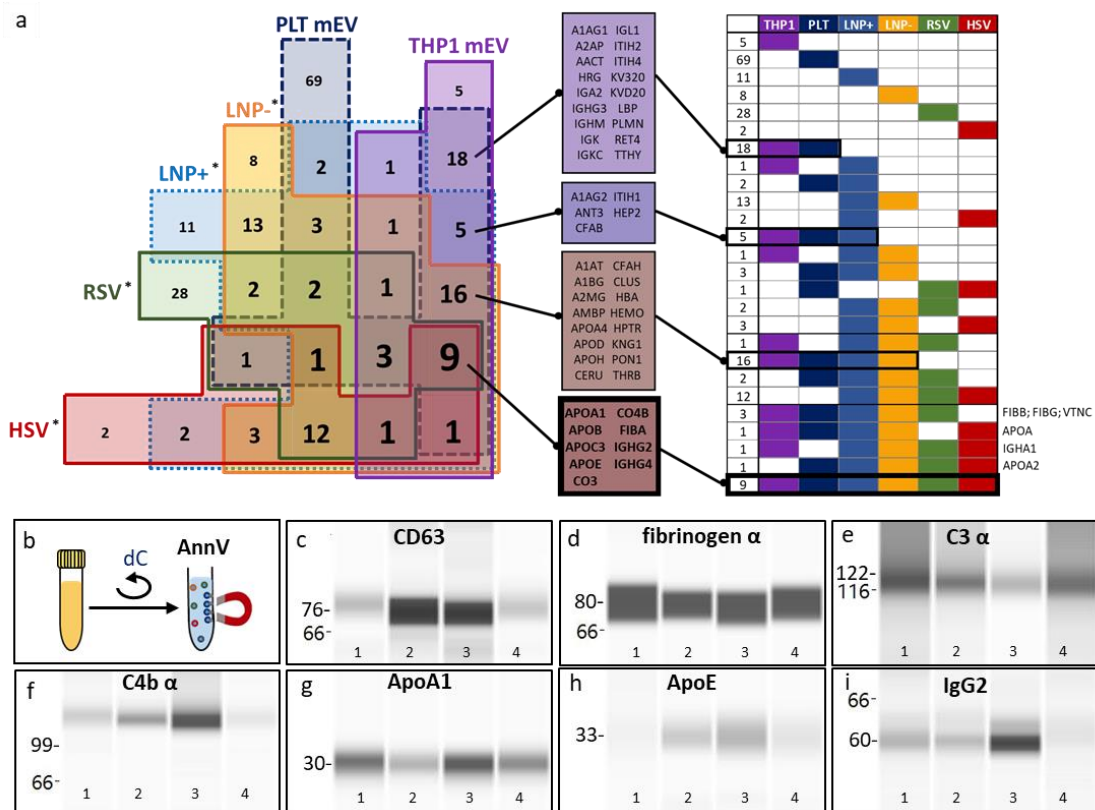
Figure 9. Comparison of viral, nanoparticle- and mEV-associated protein coronas formed in human plasma samples.

(a): The proteomic data of Ezzat *et al*. [64], marked with asterisks) on herpes simplex virus (HSV) and respiratory syncytial virus (RSV) as well as on positively (NP+) and negatively charged (NP-) artificial nanoparticles were re-analysed using a similar approach that we applied to identify EV corona proteins. The obtained protein lists were compared with proteins detected in ≥ 30% of the coated THP1 and platelet mEV samples (re-isolated by differential centrifugation (dC)). On the bottom right, next to the chart, we indicated those proteins that were missing from only one dataset. (b): Schematic illustration of the Annexin V-based affinity capture of blood plasma EV isolation from healthy samples (n = 4; individual samples are marked as 1–4 on each gel line) for Capillary Western (Wes) analysis. (c): CD63, (d): α chain of fibrinogen, (e): α chain of complement C3, (f): α chain of complement C4b, (g): ApoA1, (h): ApoE, (i): immunoglobulin G2. Molecular weights are indicated (kDa). (modified from[61])

3.4. Imaging the protein corona with different methods of microscopy

In order to receive direct evidence for the presence of a protein corona around plasma protein coated mEVs, we employed three different methods of microscopy.

3.4.1. Ultrathin cross-section transmission electron microscopy (TEM)

We were interested if the protein corona was visible in ultrathin cross-section TEM images. However, when following our regular protocol and incubated THP1-mEVs in EVDP with dC re-isolation, the excess amount of proteins veiled the images and so the round-shape EV membranes could hardly be detected. Hence, we came up with the approach of working with a simplified "corona", and incubated the mEVs with only one corona protein, fibrinogen. With this method, while the membrane structure of mEVs was still well visible, it appeared, that fibrinogen-incubated vesicles more likely acquired a "fluffy" surrounding in contrast to the sharp-edged nascent ones. Comparing the numbers of "fluffy"-looking and bare-membrane mEVs in six independent fields, we found, that fibrinogen-incubated vesicles appeared significantly more frequently coated (t-test, $p <$ 0.0001) (Figure 10).
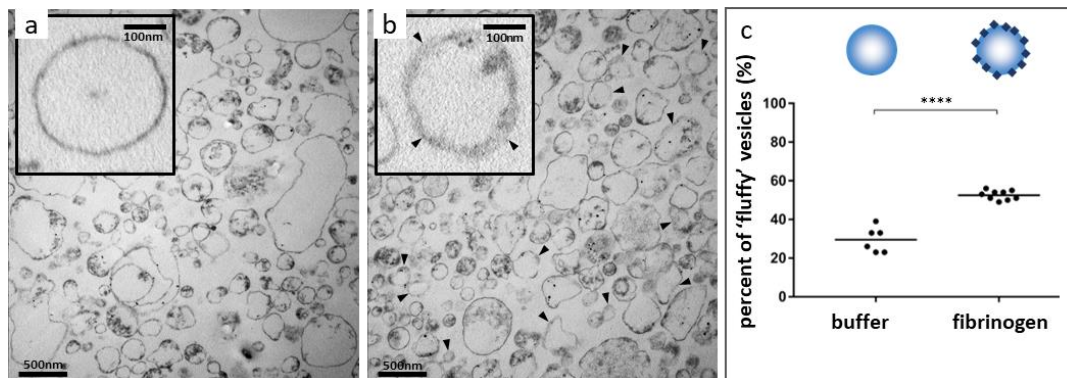
Figure 10. Imaging of fibrinogen-coated THP1 mEVs.

Electron micrographs of mEVs either incubated in buffer (nascent mEVs, a), or in 1 mg/mL fibrinogen (coated mEVs, b). Arrowheads point to some mEVs with 'fluffy' (thickened) membrane. (c): Image analysis of nascent mEVs (six independent fields, n = 596 vesicles) and fibrinogen-coated EVs (eight independent fields, n = 838 vesicles) P < 0.0001, t-test. [61]

3.4.2. Immune electron microscopy (EM)

Besides visualizing a single-component corona in ultrathin cross-sections, with a different approach, we also detected other members of the protein corona on EVDP-coated mEV preparations. Applying immunogold labelling, the co-localization of several corona proteins (fibrinogen α-chain, apolipoprotein A1, haptoglobin, complement C3) as well as the pan-EV-marker CD63 was demonstrated (Figure 11).
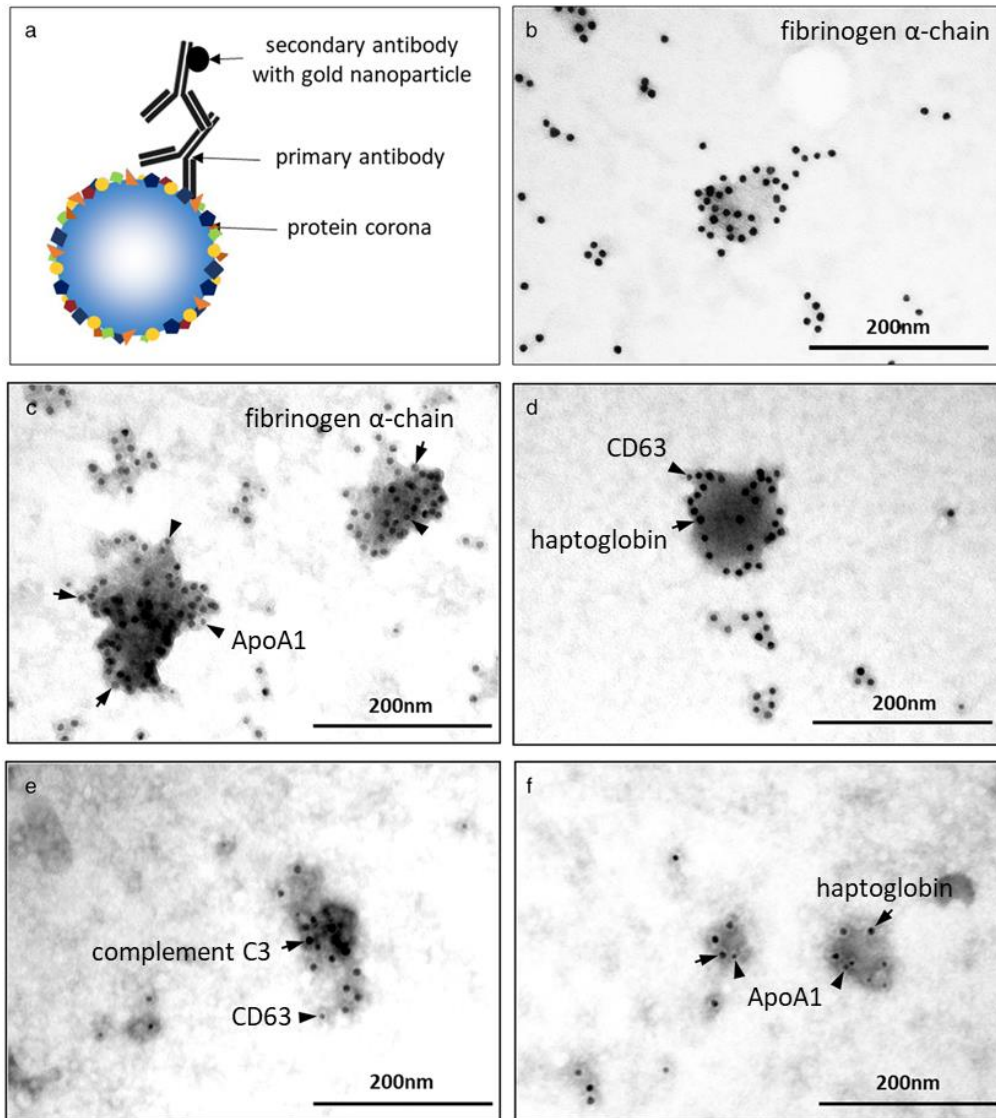
Figure 11. Detection of co-localization of corona proteins and mEV membrane proteins by immune electron microscopy.

(a): Schematic illustration of the immunogold labelling. (b): THP-1 mEVs were re-isolated by differential centrifugation after incubation in EV-depleted blood plasma sample of a healthy person and were immuno-stained for the alpha chain of fibrinogen (10 nm gold particles), (c): for the alpha chain of fibrinogen and ApoA1 (10 and 5 nm gold particles, respectively), (d): for haptoglobin and CD63 (10 and 5 nm gold particles, respectively), (e): for complement C3 and CD63 (10 and 5 nm gold particles, respectively) and (f): for haptoglobin and ApoA1 (10 and 5 nm gold particles, respectively). Arrows indicate 10 nm, while arrowheads point to 5 nm gold particles. [modified from 61]

3.4.3. Confocal microscopy

For confocal microscopy we stained THP1 cells with a membrane dye, Vybrant DiO. Hence all secreted THP1-mEVs also showed green fluorescence and became visible with confocal microscopy. After incubating these fluorescent nascent mEVs in EVDP samples, we applied anti-fibrinogen α-chain and anti-complement C3 antibodies with consequent labelling by Alexa647 and Alexa594 anti-antibodies. With this method, as Figure 12 shows, we observed different forms of protein corona formation around vesicles as well as protein aggregates without DiO signal indicating mEVs. It seems that besides a diffuse appearance, proteins also bind to vesicles in big aggregate-like structures.
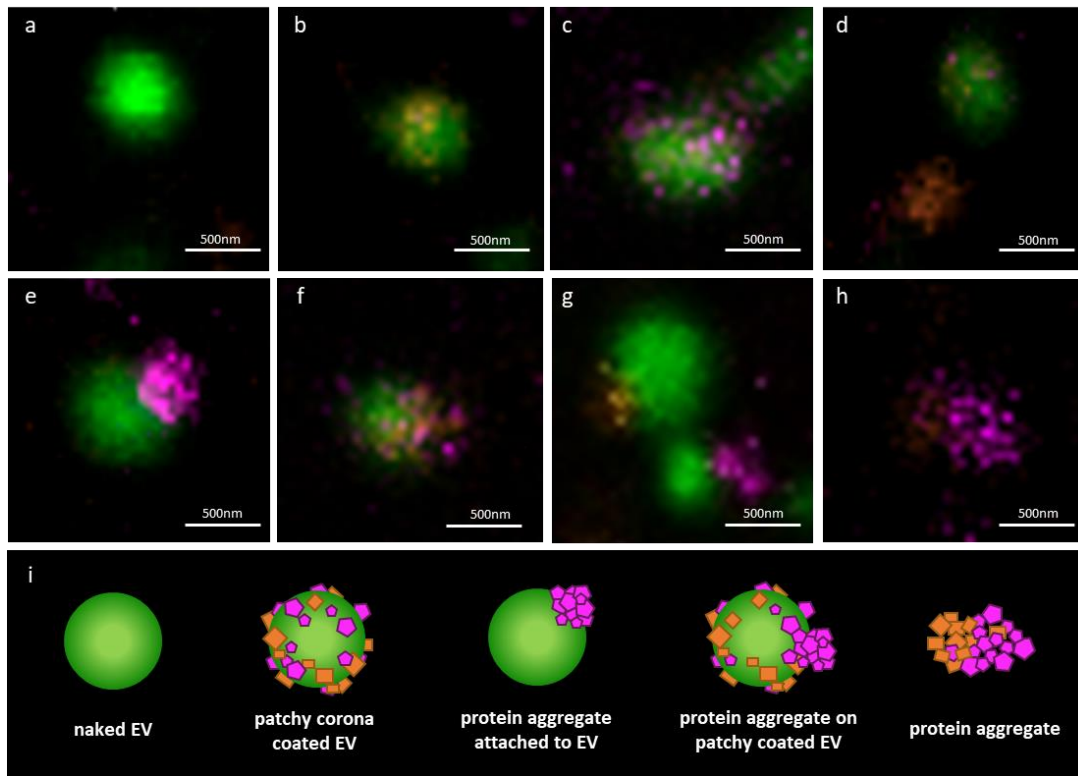
Figure 12. Confocal microscopy of EV-depleted blood plasma (EVDP)-coated mEVs.

DiO-stained THP1 cell-derived nascent mEVs were incubated with healthy EVDP, washed twice and were immuno-stained with anti-fibrinogen α chain and anti-complement C3 antibodies, followed by Alexa647 and Alexa594 donkey anti-mouse and anti-rabbit antibodies, respectively. (a): mEV; (b): mEV with a patchy complement C3 corona; (c): mEVs with a patchy fibrinogen corona; (d): mEV with patchy fibrinogen and complement C3 deposition as well as a C3 aggregate; (e): mEV with associated fibrinogen aggregate; (f): mEV with patchy fibrinogen and complement C3 deposition as well as with a fibrinogen aggregate; (G): mEVs with associated fibrinogen and C3 aggregates; (h): aggregate of C3 and fibrinogen; (i): schematic illustration of the types of interactions of mEVs with proteins. [61]

3.5. Protein aggregates show similar characteristics to the mEV protein corona

As mentioned above, EVDP samples without the addition of nascent mEVs were also included in all our studies as controls. The "mock pellets" of the same three EVDP samples with all three re-isolation methods, dC, DGUC and SEC were subjected to mass spectrometry. Even in the pellets obtained by the purer ways of separation we detected a high number of proteins with 17 of them shared by all sets (Figure 13 a.). The 55 proteins that we identified in the samples re-isolated by dC are shown in Figure 13 b. We next compared the list of proteins of the EVDP "mock pellets" to that of the protein corona. Despite DGUC and SEC re-isolation resulted in a smaller overlap than dC, in all three datasets there was a striking overlap between proteins of aggregates and the protein corona (Figure 13 c). Employing TRPS to better characterize the "mock pellets", we found that events in these samples appeared in the same size-range as THP1-mEVs, although in one order of magnitude lower concentration (Figure 13 d). On the contrary, while mEVs vanished upon membrane detergent (Triton-X, 0.1%) lysis, these samples showed resistance to it, which supports the hypothesis that the events in these "mock pellet" samples refer to protein aggregates. Also, with immune EM, immunogold particles appeared randomly in images of EVDP samples, no EV-like structures were visible, neither CD63-colocalization was observed with plasma proteins (Figure 13 e). Similarly, with confocal microscopy, we detected clumps of proteins without the presence of DiO signal indicative of mEV membranes (Figure 13 f) Eager to find out whether it was plausible to eliminate protein aggregates by serial centrifugation (analysing the pellet after each round and re-centrifuging the supernatant), we subjected EVDP samples to six consecutive rounds of 12,500 g centrifugation. We measured the protein and lipid concentration of the pellets of each round after a washing step. In contrast to nascent THP1-mEVs, that had both lipid and protein content measurable with the applied methods, in "mock pellets" the lipid concentration was below the threshold, while proteins remain detectable even after the sixth round of centrifugation (Figure 13 g). This is in line with our hypothesis that EVDP samples include protein aggregates and also indicates that serial centrifugation itself is not sufficient to eradicate these confounding events.

a

SEC

DGUC

23

1

12

17

6

13

19

dC

b

C4BPA
CFAH
IGKC
ITIH4
VTNC
IGG1
CO3
A2MG
THRB
HEMO
IGHG3
APOA4
FIBA
APOD
THY
TRFE
*
IGHM
APOC3
IGHG4
CLUS
GELS
FIBB
HPT
ALB1
IGHA1
APOH
FIBG
CO4B
IGHG2
ITIH1
IGLC3
APOA
A1AT
PON1
A1BG
CERU
APOE
CO4A
APOA1
ANT3
A1AG1
FETUA
*
APOL1
IGHG1
IGLC2
CFAB
VTDB
APOA2
KNG1

c

protein aggregate   coated mEVs

6   49   41   dC

21   22   11   DGUC

17   30   42   SEC

d

concentration (part./mL)

1,20E+08
1,00E+08
8,00E+07
6,00E+07
4,00E+07
2,00E+07
0,00E+00

0   200   400   600   800   1000 1200
particle diameter (nm)

before Triton-X
after Triton-X

mEVs

8,00E+08
7,00E+08
6,00E+08
5,00E+08
4,00E+08
3,00E+08
2,00E+08
1,00E+08
0,00E+00
0   200   400   600   800 1000 1200

e

200nm

f

500nm

g

concentration (mg/mL)

0.5
0.4
0.3

0.20
0.15
0.10
0.05
0.00

1x   2x   3x   4x   5x   6x
rounds of centrifugation (pellets of EVDP)

protein
lipid

mEVs

0.5
0.4
0.35
0.3
0.25
0.20
0.15
0.10
0.05
0.00

EVDP   SN   SN   SN   SN   SN

**Figure 13. Characterization of plasma protein aggregates.**

(a): The Venn-diagram indicates the number of proteins identified by mass spectrometry in the washed pellets of healthy EV-depleted blood plasma (EVDP) samples (n = 3) corresponding to protein aggregates. Identical EVDP samples were processed by three different methods (differential centrifugation (dC), DGUC and SEC). (b): Word cloud illustration of the coloured section of panel A. The different colours mark the proteins detected either only with dC, or also with SEC or DGUC or with all of these methods. The font size correlates with the percentage of detection of a given protein among the samples. Asterisks indicate those proteins that were also identified in the nascent THP1 mEV samples and therefore were not included in the primary list of corona proteins. (c): Overlaps of the corona proteins with protein aggregates separated from the same three healthy EVDP samples with three different methods. (d): Representative TRPS histogram of a twice-washed EVDP pellet separated by dC (measured before and after 0.1% Triton-X lysis). For comparison, the insert shows the effect of the detergent lysis on a nascent THP1 mEVs. (e): Immunogold-stained electron micrograph of a twice-washed 12,500 g healthy EVDP pellet. The sample was stained for haptoglobin and CD63 (10 and 5 nm gold particles, respectively). Arrows indicate 10 nm gold particles (CD63 was not identified with 5 nm gold particles in the sample). (f): Confocal microscopic image of a mixed fibrinogen and complement C3 aggregate in a EVDP-coated mEV preparation (immuno-stained with anti-fibrinogen α chain and anti-complement C3 antibodies and Alexa 647 and Alexa 594 donkey anti-mouse and anti-rabbit antibodies respectively) (g): Healthy EVDP samples (n = 3) were subjected to serial centrifugation at 12,500 g for 40 min. Protein and lipid concentrations of the washed pellets were determined after each run. While the lipid concentration was under the detection limit throughout the analysis, proteins were detectable in the pellet even after the 6th round of centrifugation. For comparison, the insert shows protein and lipid concentrations of nascent mEV samples (n = 3) [61]

3.6. Functional analysis of protein corona coated mEVs

Finally, we studied whether protein corona coated mEVs had altered biological effect compared to nascent mEVs.

3.6.1. Gene enrichment analysis

First, we loaded the list of the 61 corona proteins detected in ≥30% dC re-isolated samples into FunRich gene enrichment analyser [65, 66] to see which biological processes these proteins are involved. Figure 14 shows the predicted biological processes, where protein metabolism, immune response and transport appeared with the highest percentages out of all (34.8%, 26.1% and 26.1% respectively).
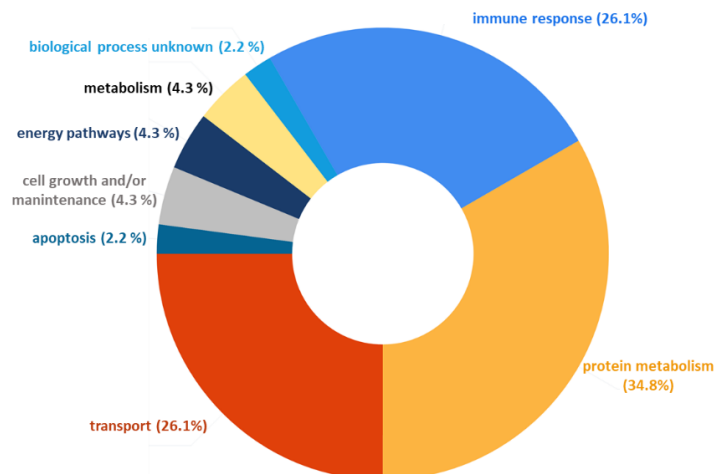
Figure 14. Gene enrichment analysis of THP1 mEV protein corona proteins.
Genes encoding for proteins found in ≥30% of the coated EV samples (re-isolated from EVDP samples with differential centrifugation, n=22) were analysed by the FunRich gene enrichment analyser [65, 66]. The following biological processes were represented with the highest percentages among the analysed genes: protein metabolism (34.8%), immune response (26.1%) and transport (26.1%). [61]

3.6.2. The effect of the protein corona on monocyte-derived dendritic cells (moDCs)

With immune response receiving the second highest percentage among the predicted processes, we next employed moDCs to investigate the biological effect of the protein corona. We treated moDCs with either i) empty buffer control ii) nascent THP1-mEVs, iii) protein corona coated THP1-mEVs, or iv) "mock pellets" of EVDPs (all re-isolated by dC). For this study, EVDP was obtained from both healthy subjects and RA patients and the effect of the different protein coronas was compared (Figure 15 a). We subjected the treated moDCs to flow cytometry and their conditioned media was analysed by enzyme-linked immunosorbent assay (ELISA). In comparison with nascent mEVs, both healthy and RA EVDP-coated mEVs induced a significantly higher tumour necrosis factor α (TNF-α) production (Kruskal-Wallis analysis with Dunn's post-test $p < 0.05$ and $p < 0.001$ respectively) (Figure 15 b). The RA protein coat also activated more significant production of both TNF-α and interleukin-6 (IL-6) when compared to protein aggregates of the same plasma samples (Kruskal-Wallis analysis with Dunn's post-test $p < 0.01$ and $p < 0.05$ respectively) (Figure 15 c). Looking at the surface activation markers of moDCs, we observed that CD83 frequency and HLA-DR mean fluorescence intensity was significantly elevated in all nascent mEV, healthy and RA coated mEV samples when compared to cells treated with empty buffer or with matching protein aggregates as well as CD86 frequency was significantly higher among moDCs treated with coated (either healthy or RA) mEVs than empty buffer treated or protein aggregate treated cells. (One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test, $p < 0.05$, $p < 0.01$ or $p < 0.001$ – as marked in the respective figures) (Figure 15 d-f).
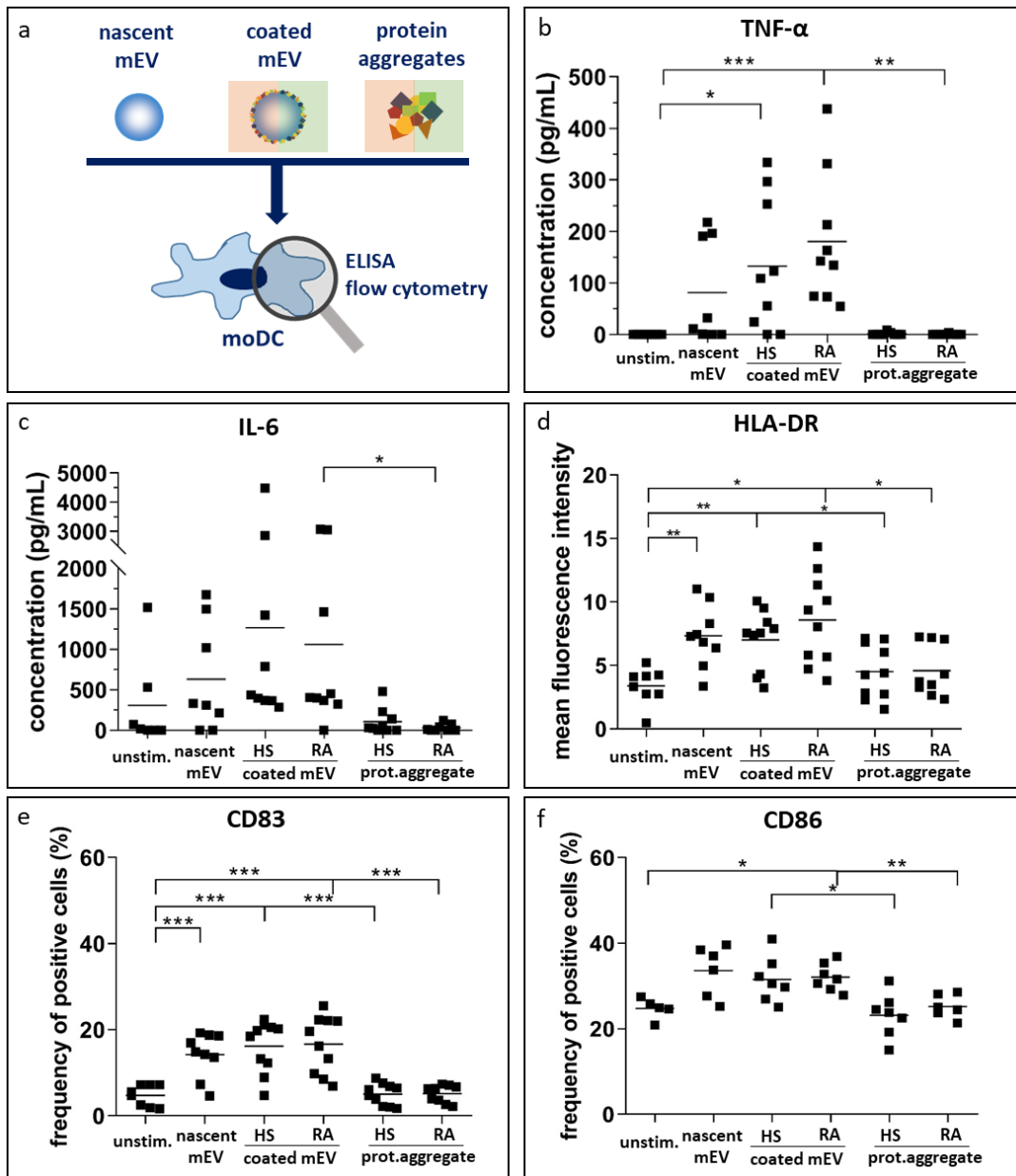
Figure 15. The effects of corona-coated mEVs on human monocyte-derived dendritic cells.
Human monocyte-derived dendritic cells (moDCs) were differentiated *ex vivo* in the presence of IL-4 and GM-CSF for 5 days. MoDCs were then exposed to nascent- or plasma protein-coated vesicles or to pellets of EVDP samples (protein aggregates) separated with differential centrifugation. The production of TNF-α and IL-6 was determined by ELISA. The frequency of CD83 and CD86 positive cells as well as the mean fluorescence intensity of HLA-DR positive cells was assessed by flow cytometry. HS: healthy subjects; RA: patients with rheumatoid arthritis; unstim.: unstimulated, cells treated with EV buffer; prot. aggregate: protein aggregate. Kruskal-Wallis analysis with Dunn's post-test (TNF-α and IL-6), One-way ANOVA with Tukey's multiple comparisons test (CD83, CD86 and HLA-DR), *: P < 0.05, **: P < 0.01, ***: P < 0.001 [61]

4. Discussion

EVs, our endogenous nanoparticles play wide-ranging roles in all known organisms contributing to health and disease. They are the carriers of distinct molecules even in between the furthest cells in the body thus act as important players of intercellular communication [28, 67]. Therefore EVs have long been in the field of interest of studies aiming to develop better therapeutic targeting. While there are already much data available about the intravesicular cargo, only sporadic pieces of information are available regarding the molecules that are carried on the surface of EVs [60]. Also, the studies of EVs are hindered by that it so far proved impossible to isolate pure vesicles without proteins and other molecules "contaminating" the samples. Despite the most careful ways of separation, blood-plasma derived EV samples were still found to contain traces of the blood plasma, e.g. albumin, which is one of the best known culprits that EV-researchers have been unable to "get rid-off" during EV-separation [68]. On the other hand, in the case of artificial NPs, the cutting-edge nano-vehicles of targeted therapy (mostly in experimental stage), it is a well-described phenomenon that once they are introduced into a biological *milieu*, a protein corona, moreover, a bio-molecular corona forms on their surfaces, which corona alters the pharmacokinetics and pharmacodynamics of these particles [8]. Also, recently, the protein corona of viruses has been described and viruses and EVs are known to share many attributes [69]. While preliminary data already suggested that similarly to artificial NPs and viruses, a protein corona might also be formed around EVs, this study was based on indirect evidence comparing pellets of ultracentrifuged blood plasma to previously published EV proteomes as well as to NP protein corona studies [70]. Therefore our aim was to provide direct information whether a protein corona is also formed around EVs in the blood plasma similar to artificial NPs and viruses.

However, in the case the EV protein corona, distinguishing what proteins belong to the protein corona from the composing proteins of the heterogeneous EVs themselves proves more challenging than in the case of artificial NPs and viruses, where the composition of these structures are better defined. Our approach was to separate nascent mEVs of THP1 cells or from Optipep-purified platelets cultured in serum-free conditioned medium, incubate these mEVs in blood plasma that was prior depleted in EVs and consequently re-separate the vesicles with different methods. In some

30

experiments, we also applied a simplified setting, where instead of the whole EVDP, just one selected protein was incubated with the nascent mEVs, also, we isolated mEVs with Annexin V-affinity isolation directly from blood plasma. Our results obtained from the analysis of the re-separated, "coated" mEVs by MS, different techniques of EM, confocal microscopy and capillary Western-blotting provide evidence that proteins newly associate to the surface of mEVs in blood plasma. The association of a protein corona is also suggested by that "coated" mEVs were found to have an elevated floatation density when compared to nascent ones in our DGUC studies. While in our work we focused on the protein corona formation of mEVs, another group received similar results in the case of sEVs, where sEVs with an increased flotation density ("dense sEVs") were found to be associated with distinct proteins of the blood plasma (e.g. albumin, fibronectin, complement factors) [71].

In our work, we provided direct evidence for the first time for the presence and also information about the components of the mEV protein corona. Analysing the predicted interactions between the different corona proteins we found that several of them were predicted to only interact with other corona components but not with the surface proteins of mEVs. This indicates that similar to artificial nanoparticles, the protein corona of mEVs might also be multi-layered where an inner hard corona and a looser, "less adhesive" soft corona can be distinguished [17]. The finding, that in the simplified corona model we could dissociate fibrinogen from the surface of mEVs with increasing salt concentrations indicate the presence of electrostatic bindings among the mEV-corona or corona-corona interactions. It is also presumable that besides aspecific interactions, receptor-ligand binding might also play role in the corona formation. In a study that was released after our work, it is proposed, that EVs might carry innate protein corona being present at the time of their release as well as an acquired corona that develops and changes dynamically in the environment of the discharged vesicles [72]. Earlier to this, it already has been suggested that several proteins, e.g. complement C3 and fibronectin are already present on the surface of sEVs in the multivesicular bodies and therefore can be detected on nascent sEVs [73-75]. In the case of mEVs, as we have proposed [61] and as Yerneni *et al*. also suggested, the molecules attached to the plasma membrane are transmitted onto the mEVs that are shed from the cells, hence they serve as the innate corona of the vesicles. In our studies albumin was present in our nascent mEV samples and therefore

had to be subtracted from the list of corona proteins. This came as a surprise as albumin is the most widely present "contaminant" in all EV researches [32, 76]. However, considering it as an innate corona protein in our experiments might serve as an explanation for this finding as i) we found the gene of albumin to be expressed by THP1 cells themselves and ii) running an analysis of our MS data on bovine databases we found that in the case of twenty-six proteins, the human or bovine origin could not be ascertained, furthermore, two proteins were detected as bovine indicative of the reminiscence of foetal bovine serum despite washing the cells and culturing them without serum prior to EV-separation. This is also in line with our previous findings [77] and an earlier study, where bovine protein contamination from the foetal calf serum seemed unavoidable despite applying a careful, affinity-based isolation method [78]. The work of Yerneni *et al*. used the approach of applying radio-labelled iodine for the follow-up of the structure and the real-time fluctuation of the surface molecules of EVs and found that not only proteins, but lipids and nucleic acids are also part of the EV corona similar to what was described in the case of artificial NPs [72, 79-88]. This is also in line with our earlier findings where we described that low-density lipoprotein (LDL) particles associate with the surface of nascent EVs *in vitro* [33] as well that DNA (mostly that of mitochondrial origin) adheres onto the sEVs of cells undergoing oxidative stress induced mitochondrial dysfunction [89].

We were interested whether the protein corona of THP1 and platelet-derived mEVs share components with that of the artificial NPs and of viruses. Therefore we chose the study of Ezzat *et al*., in which the proteins gathered around artificial lipid NPs with positive and negative surface charges as well as to the surface proteins of viruses (HSV and RSV) of the same size as these NPs were analysed [64]. Importantly, the size range of these particles were similar to that of the mEVs we used in our work, approximately 200 nm. To our surprise, we found nine shared proteins (apolipoproteins A1, B, C3, E, complement factors 3 and 4B, fibrinogen α-chain, immunoglobulin heavy constant γ2 and γ4 chains) that were present in all datasets and these proteins could also be identified in several other studies analysing the protein coronas of artificial NPs [90-95]. Once again, albumin could not be included in this list due to that it also present in our nascent mEV samples. It is important to highlight that all included studies applied similar, but in some means different methodology, hence the finding of shared proteins is even more

interesting as it suggests that irrespective of the particles protein coronas form around, there might be universal elements of protein corona formation. Also, despite the immensely different approach of the preliminary study of the EV protein corona by Palviainen *et al.*, several of the shared proteins mentioned above were present in the protein lists of the EV protein corona proposed by this group [70] as well. Moreover, we also identified these proteins together with the pan-EV marker, CD63 by capillary Western-blotting in mEVs isolated directly from blood plasma by a more pure method, Annexin V-based affinity isolation.

In our Wes studies, we observed that there seemed to be an individualised pattern in the protein coronas depending on the donor of the blood plasma. This is in line with the findings in the case of artificial NPs, where it already has been described, that depending on several factors, there can be significant interpersonal differences between the protein coronas of different subjects, and these differences could also be indicative of several diseases [96]. Following this idea, the application of NPs for diagnostics employing liquid biopsy has also been suggested [90, 97-100]. Hence, we also compared the THP1 mEV protein coronas of healthy subjects to that of patients suffering from RA. While the most abundant proteins did not show significant disease-correlation, among the proteins present in < 50% of all samples, there were some that were more likely to show up in the coronas of RA patients. Of note, only careful assumptions should be taken as RA patients involved in our work had significantly higher median age than the healthy people and the relatively low number of involved subjects also suggest being precautious when extrapolating these data.

Immune response was among the pathways with the highest percentage predicted to be affected by the corona proteins of our THP1 mEVs. Also, the shared corona proteins are well-known contributors of immune mechanisms, e.g. opsonisation. Therefore, in order to study the functional relevance of the protein corona, we decided to analyse its effect on dendritic cells, one of the immune cells with highest sensitivity to alterations of their surroundings. We treated moDCs with THP1 mEVs coated with protein coronas from the EVDP samples of either healthy subjects or of RA patients. Remarkably, while we could not notice any significant differences between the effect of the two types of protein coronas on the moDCs, both healthy and RA coronas proved to increase the TNF-α secretion as well as the maturation (based on the more prominent expression of CD83,

CD86 and HLA-DR by cells) of the treated moDCs as compared to pellets of EVDP samples without the addition of mEVs. While more experiments might be necessary to further map the effect of the protein corona, these results may suggest that the protein coating of EVs may lead to the tonic activation of pro-inflammatory pathways, e.g. sustain the responsiveness of T-cells in inflammation, where the release of EVs is known to be increased [55].

Applying controls of EVDP samples without the addition of mEVs in our study proved unexpectedly important as we observed the presence of protein aggregates in these samples despite (or maybe partially due to) that the blood plasma samples were subjected to several steps of filtration and centrifugation prior to that THP1 or platelet-derived mEVs were incubated in them (at last, an overnight 100,000 g centrifugation was included where the supernatant was carefully pipetted to a new tube). Even after the sixth round of consecutive centrifugation at the speed of 12,500 g (a speed usually applied for mEV isolation), where the supernatants were transferred to new tubes for the next step spinning there was a considerable amount of proteins detected by Bicinchoninic Acid (BCA) method in the pellet indicative of the presence of remaining protein aggregates. Of note, the pro-aggregative effect of centrifugation should also be taken into account as even low speed centrifugation was found to induce protein aggregation before [101]. Similarly to centrifugation, freezing-thawing of plasma samples is also a well-known factor of protein aggregation [101]. Hence, we also should consider the effect that we kept our EVDP samples frozen at -80°C for up to 6 months before usage. On the other hand, as most research with EVs apply freezing of biological samples prior to experiments and even more likely use centrifugation at different speeds, our results may serve as important reminders that protein aggregation may hinder the analysis of EVs in the case of several generally utilized methods, like particle number detection, or protein concentration measurement techniques. Of note, the occurrence of protein aggregation could not be avoided despite applying DGUC or SEC re-isolation methods. While the overlap between the corona proteins and aggregate proteins was smaller, the possible confounding effect of a greater sample dilution than in dC separation should also be taken into consideration in the analysis.

For the visualization of the protein corona, we employed two different methods of EM as well as confocal microscopy. With the latter, we applied fluorescently labelled

anti-protein antibodies specific against some components of the protein corona, while mEVs appeared green due to the staining of their mother cells with the fluorescent membrane dye, DiO. On the surface of green vesicles, we observed two main types of protein attachments: i) a flower-meadow like patchy binding of the labelled proteins and ii) big, clumpy aggregates of proteins. This latter witnessed appearance could serve as an explanation for the great overlap between the proteins of protein aggregates and of the protein corona of mEVs. In the case of immune EM, the co-localization of the pan-EV marker tetraspanin CD63, together with the signal of corona proteins indicated that the proteins did not only form protein aggregates but bound to the EVs as well.

Despite the structural and compositional similarity of protein aggregates to the EV protein corona, it is important to highlight that in contrast to coated mEVs, in our functional study with moDCs, proteins aggregates failed to induce the activation of these cells. It is clear that for understanding the role of the EV protein corona, there is still a long way to go despite there have been many pieces of information gathered in the last few years. We already know that EVs with surface protein cargo have larger diameter as detected by non-optical methods of particle size-measurement [102] and earlier findings also indicated that the membrane proteins of sEVs significantly limit the mobility of these vesicles in the ECM [103]. Also, there have been implications, that specific surface proteins might modify the effect of EVs. In the murine model of an autoimmune neurodegenerative disorder, multiple sclerosis, EV-associated fibrinogen enhanced the differentiation of encephalitogenic killer T-cells [104]. Most recently, investigating the protein corona formation of EVs has emerged into the centre of interest of many EV-researchers leading to the arousal of more advanced studies. In one research, it was found that corona coated placental cell EVs enhanced angiogenesis and improved skin regeneration in an *in vivo* model, while the removal of the corona led to the inhibition of these processes [105]. The work of Yerneni *et al*. utilising radio-iodinated molecules also aimed to improve the methodology of studying the corona of EVs *in vivo* [72], which is a rather important next step in order to broaden the field into the direction of the therapeutic or diagnostic application of EVs. A good example of this tendency is the work of Wu *et al*., who designed exosome-mimetic nano-vehicles to shuttle targeted therapy against a malignant brain neoplasm, glioblastoma and manipulated the protein corona in order to enhance the blood brain barrier penetration of these particles [106]. While

designing artificial NPs in a way that enhances therapeutic targeting has longer been encouraged [107], engineering EVs in a similar way may open the doors of even more possibilities.

5. Conclusions

The major conclusions of our study are:

1.) A variety of proteins newly associate with nascent THP1-derived and platelet-derived mEVs in blood plasma.

2.) The associating proteins interact not only with the vesicular surface but with each other and the interactions are partially established by electrostatic binding.

3.) Nanoparticles, viruses and mEVs in the same size range share components of their protein coronas suggesting the presence of a universal protein corona.

4.) Besides a diffuse protein coating of EVs, protein aggregate-like structures also attach to the EV surfaces.

5.) The presence of protein aggregates even in DGUC or SEC separated samples encourages precaution when analysing EVs, as protein aggregates show similar characteristics to the EV protein corona.

6.) While the main components of the protein corona show universality, less abundant proteins appear in a personalised manner in the protein corona and might show disease-specificity.

7.) The protein corona coated mEVs (irrespective of that the corona was derived from the blood plasma of healthy subjects or RA patients) induced significant activation of moDCs when compared to the effect of protein aggregates.

6. Summary

EVs are important carriers of molecules and therefore contribute to the complex intercellular communication of the body [67]. Compared to the number of studies that focus on the intravesicular cargo of EVs, there is far less information available on the molecules that are carried on the surface of vesicles. On the contrary, the surface cargo of therapeutic NPs, the artificial relatives of EVs has been extensively studied revealing that the molecular surface attachment might alter both the bio-availability and bio-distribution of these particles [22]. This protein corona formation was also observed on the surface of some viruses [64]. Hence, the question whether EVs also develop protein coronas in a biological *milieu* arouse.

In summary, here we present direct evidence for the first time for the formation of a protein corona around EVs. With our approach, we identified several proteins that newly associated with nascent mEVs in blood plasma. Besides a more personalised corona that also showed correlation with health and disease, our data point to the presence of a universal surface protein cargo on blood plasma EVs. This also overlapped with the protein coronas described by others previously around artificial NPs and viruses. We also analysed the predicted interactions of the participants of the protein corona and found that while most proteins established connections with the membrane surface proteins of the nascent mEVs, several proteins only showed interaction with other members of the corona. This might be indicative of a multi-layered corona formation. With confocal microscopy we revealed that proteins do not only attach diffusely onto EVs, but some adhere as big clumps of aggregates. Whether EVs act as nuclei for aggregation or pre-formed protein aggregates tend to bind onto them needs further clarification as well as whether this process has actual relevance *in vivo* or it just appears as the artefact of our *in vitro* sample handling. Our data may also provide explanation for the widely observed "protein contamination" of EV samples implying that these "contaminants" might actually be imminent participants of the protein coronas of EVs. Finally, the better understanding of the protein (and biomolecular) corona is inevitable for the therapeutic application of EVs as there is already evidence suggesting that the modification of the protein corona alters the function of the EVs themselves and EVs after all may prove to be better vehicles of targeted therapy than artificial NPs [105, 108, 109].

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8. Bibliography of the candidate's publications

8.1. List of publications used for the thesis

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8.2. List of publications not used for the thesis

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NYILATKOZAT EREDETISÉGRŐL ÉS SZERZŐI JOGRÓL
a PhD disszertáció elkészítésére vonatkozó szabályok betartásáról

Alulírott dr. Tóth Eszter Ágnes jelen nyilatkozat aláírásával kijelentem, hogy a „PROTEIN CORONA FORMATION AROUND EXTRACELLULAR VESICLES" című PhD értekezésem önálló munkám, a dolgozat készítése során betartottam a szerzői jogról szóló 1999. évi LXXVI tv. vonatkozó rendelkezéseit, a már megjelent vagy közlés alatt álló közlemény(ek)ből felhasznált ábra/szöveg nem sérti a kiadó vagy más jogi vagy természetes személy jogait.

Jelen nyilatkozat aláírásával tudomásul veszem, hogy amennyiben igazolható, hogy a dolgozatban nem saját eredményeimet használtam fel vagy a dolgozattal kapcsolatban szerzői jog megsértése merül fel, a Semmelweis Egyetem megtagadja PhD dolgozatom befogadását, velem szemben fegyelmi eljárást indít, illetve visszavonja a már odaítélt PhD fokozatot.

A dolgozat befogadásának megtagadása és a fegyelmi eljárás indítása nem érinti a szerzői jogsértés miatti egyéb (polgári jogi, szabálysértési jogi, büntetőjogi) jogkövetkezményeket.

Tudomásul veszem, hogy a PhD értekezés nyilvánosan elérhető formában feltöltésre kerül az Országos Doktori Tanács honlapjára.

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aláírás