Protein corona formation around extracellular vesicles

Doctoral Thesis

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1. Introduction

Not long after the first trials executed with therapeutic artificial nanoparticles (NPs) it has been revealed that once NPs get introduced into a biological *milieu*, a diverse protein attachment evolves on their surfaces. This protein attachment, the so-called "protein corona" then modifies the bioavailability as well as the bio-distribution of NPs as it may mask the molecules responsible for targeting these therapeutic nanovehicles. It was found that the NP protein corona may consist of several layers of proteins, where the inner layer, the "hard corona" shows stronger adhesion, while the outer "soft corona" is more dynamic as it is more easily sculptured by the forces (e.g. shear forces in the circulation) of the NP surroundings. While in the first instance, proteins with the highest concentration attach to NPs, over time, even less abundant proteins, with higher association rates may exchange these. Also, it seems, that the protein corona formation around NPs carries personal attributes, hence, its analysis might offer the opportunity to search for disease biomarkers as well.

Extracellular vesicles (EVs) are diverse, membrane-enclosed structures that all cells release. They are important participants of the intercellular communication system as they might carry a variety of molecules between cells. While there had been many studies focusing on the intravesicular cargo, only sporadic pieces of information have been available regarding the surface protein interactome of EVs. On the other hand, there has been an increasing growth of interest in the possible application of EVs as alternatives to artificial NPs to achieve even better therapeutic results. Therefore, we set to goal to examine the surface protein cargo of EVs in blood plasma in order to find out if we have to count on the formation of a protein corona around EVs similar to what is observed in the case of artificial NPs.

2. Objectives

Our specific aims were the followings:

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 into 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. Methods

Human blood sample collection

With the written informed consent of all involved subjects and ethical permission from the Hungarian Scientific and Research Ethics Committee, we collected peripheral blood samples from altogether 17 rheumatoid arthritis (RA) patients (58.5±16.5 years mean±SD age) and 20 healthy subjects (HS, 34.8±10.0 years mean±SD age). All involved RA patients were treated at the Department of Rheumatology and Clinical Immunology, Semmelweis University and had moderate median DAS28 (Disease Activity Score; 3.45 median score). In the HS group, we involved people only without any known history of inflammatory diseases.

We collected peripheral blood samples into acid-citrate-dextrose tubes in order to avoid anticoagulant-induced activation and related EV release of cells.

Preparation of EV-depleted blood plasma (EVDP) samples

We centrifuged blood samples twice at 2500 g for 15 min in order to obtain platelet-free plasma following the recommendation of the International Society on Thrombosis and Haemostasis. Next, we diluted samples two-folds with 0.2 μm filtered phosphate-buffered saline (PBS), filtered them consecutively with 5 μm and 0.8 μm filters followed by centrifugation at 20,000 g for 40 min and at 100,000 g for 16 hours after which supernatants (EVDP) were aliquot and stored at -80°C for maximum 6 months.

THP1 cell culture and separation of medium-sized nascent EVs (mEVs) from THP1 cell culture

We cultured THP1 monocytic cell line (ATCC) in RPMI 1640 medium with 10% fetal bovine serum (FBS), 1% Antibiotic Antimycotic Solution for Cell Culture and 1% glutamine at 2.5-5 x 10^5 /mL concentration.

We kept THP1 cells in serum-free conditioned media at 5-10 x 10^5/mL concentration for 18-20 hours prior to harvesting nascent mEVs. We separated mEVs with serial centrifugation (300 g, 2,500 g, and 12,500 g) and filtration (5 μm and 0.8 μm) steps. After the final round of centrifugation at 12,500 g, we washed the pellet once with 0.2 μm filtered 10 mM HEPES containing, pH 7.4 0.9% NaCl solution ("EV buffer") and then, we re-suspended the pellets again in EV buffer.

Labelling THP1 cells with a lipophilic fluorescent dye (Vybrant DiO)

In order to obtain fluorescent mEVs, we labelled THP1 cells with a fluorescent membrane dye (Vybrant Dio, from Thermo Fisher Scientific's

Multicolor Cell-Labelling kit) following the instructions of the manufacturer. We then performed mEV separation as described above.

Platelet isolation from platelet concentrates and separation of platelet mEVs

We purchased platelet concentrates from the Hungarian National Blood Transfusion Service and prepared platelet suspension with calcium- and magnesium-free Hanks' Balanced Salt Solution (HBSS) and prostaglandin E1 (PGE1) that we layered on 10-17% discontinuous iodixanol gradient (Optiprep, Sigma). We centrifuged samples at 300 g for 30 min and transferred the middle layer (rich in platelets) to new tubes. We spanned samples at 300 g for 5 min, subsequent to which we centrifuged the supernatant at 2200 g for 15 min and washed the pellet once. Finally, we re-suspended the pellet in calcium- and magnesium-containing HBSS at the concentration of 3×10^{11}/mL.

We then incubated platelets at 37°C, 5% CO2 for 180 min with continuous gentle agitation. We separated platelet mEVs with serial centrifugation (4,750 g, 11,000 g, and 20,000 g) and filtration (0.8 μm) steps. After the 20,000 g centrifugation round, we re-suspended the pellet and washed it once in EV buffer.

Incubation of nascent THP1 or platelet-derived mEVs in blood plasma samples

We separated nascent mEVs from the conditioned medium of THP1 cells as described above and incubated these mEVs in EVDP samples of either HS or RA patients for 30 min at room temperature with continuous low-speed rotation. As controls, we also used EVDP samples with the addition of EV buffer as well as nascent mEVs incubated in EV buffer. We then re-isolated

mEVs employing three different approaches (i) differential centrifugation (dC), (ii) density gradient ultracentrifugation (DGUC), (iii) size exclusion chromatography (SEC) as described below. Similarly to THP1 mEVs, we incubated platelet-derived mEVs (matched in protein-concentration to THP1 mEVs) in healthy EVDP samples (n=3) and re-separated mEVs by dC.

Re-separation of mEVs *via* three different methods

- **dC:** After incubation in EVDP samples, we re-separated THP1 mEVs by pelleting them at 12,500 g and washed them twice with EV buffer. We submitted n=12 HS and n=10 RA samples to mass spectrometry (MS), where we re-suspended the final pellets in high-performance liquid chromatography (HPLC) water. Platelet-derived mEVs were re-pelleted by 20,000 g.
- **DGUC:** We also re-separated nascent THP1 mEVs by DGUC. We layered HS EVDP samples (n=3) after incubating them with mEVs onto discontinuous iodixanol (Optiprep) gradients subsequent to which we centrifuged samples at 100,000 g for 18 hours. EVDP samples without THP1 mEVs were also used as controls. We collected fractions starting from the top of the gradient and washed each fraction in separate tubes centrifuging samples at 12,500 g for 80 min. Each fraction was analysed by flow-cytometry employing Annexin V-FITC (fluorescein isothiocyanate) staining and those fractions containing mEVs were subjected to MS and transmission electron microscopy (TEM).
- **SEC:** We re-separated aliquots of the same HS EVDP samples (n=3) as by DGUC utilising SEC with qEVoriginal (IZON) columns (separation size: 70 nm nominal, bed volume: 10 mL) following the instructions of the manufacturer. Out of the pooled 1-3, 4-6, 7-9, 10-12 fractions, only fractions 7-9 contained mEVs detected by Annexin V-FITC labelling. Therefore we

centrifuged the pooled 7-9 fractions at 12,500 g and subjected the re-suspended pellets to MS and TEM.

Isolation of blood plasma mEVs based on Annexin V affinity capture

In the case of three HSs, we separated mEVs directly from the 20,000 g pellets of the blood plasma with Annexin V MicroBeads (MACS, Miltenyi) according to the instructions of the manufacturer. We loaded samples onto Miltenyi µ Columns from where eluted samples with protein lysis buffer, added protease inhibitors to the samples after which we consecutively frozen-thawed, sonicated them and kept them frozen till Wes analysis.

Mass spectrometry (MS)

THP1 and platelet-derived mEVs incubated in EVDP samples as well as their controls were subjected to MS. Proteins after tryptic digestion were loaded into a Dionex Ultimate 3000 nanoRSLC coupled to a Bruker Maxis II mass spectrometer and peptides were separated on an ACQUITY UPLC M-Class Peptide BEH C18 column following trapping on an Acclaim PepMap100 C18 NanoTrap Column. We applied data-dependent analysis with a fix cycle time of 2.5 s. We processed raw data files with the Compass DataAnalysis software and for protein identification we utilised Mascot and X!Tandem search engines against the Swissprot *Homo sapiens* and for a few samples, against the *Bos taurus* databases with 10 ppm peptide mass tolerance, 0.15 fragment mass tolerance and 2 missed cleavages. As a fixed modification, carbamidomethylation on cysteine molecules was set, while deamidation and oxidation were set as variable modifications. We then analysed data with FunRich and Scaffold 4.5.3 software. We omitted skin proteins as potential contaminants from the final protein lists.

Capillary Western (Wes) analysis of blood plasma mEVs

We analysed the lysates of Annexin V affinity captured blood plasma mEVs with the 12-230 kDa Separation Module (ProteinSimple) of a ProteinSimple-Biotechne 004-600 Wes System according to the instructions of the manufacturer. We applied primary antibodies specific for either CD63, fibrinogen α-chain, complement C3, complement C4b, ApoA1 or ApoE as well as horseradish peroxidase (HRP)-conjugated secondary antibodies, followed by the addition of chemiluminescent substrates, Peroxidase and Luminol-S. We then analysed gel pictures using Compass for SW (ProteinSimple) software.

Tunable resistive pulse sensing (TRPS)

We analysed THP1 mEVs either nascent or after incubation in HS EVDP (n=3) as well as the pellets of the same EVDP samples without the addition of mEVs produced by dC. We employed an IZON qNano instrument using a NP400 nanopore membrane calibrated with 200 nm beads according to the instructions of the manufacturer. All samples were also analysed after applying detergent (0.1% Triton X) lysis to them.

Transmission electron microscopy (TEM)

- **Immune EM**

For immune EM, we used dC re-isolated THP1 mEVs (either nascent or coated), or pellets of EVDP samples without the addition of EVs. We applied overnight staining on samples dried onto nickel grids with primary antibodies (either alone or in combination) specific for the α-chain of fibrinogen, complement component C3, haptoglobin, ApoA1 and CD63. Next, secondary antibody labelling (conjugated either with 5 or 10 nm gold particles (Sigma and Abcam)) was applied after which we fixed samples with 2%

glutaraldehyde, and stained them with phosphotungstic acid. Finally, we examined the samples with a JEOL 1011 transmission electron microscope (Japan).

- **Ultrathin sections without immunogold labelling**

For EM of ultrathin sections, we used the simplified model of the protein corona: we incubated THP1 mEVs in either 1 mg/mL fibrinogen (Sigma) or in EV buffer. We re-isolated mEVs by 12,500 g dC and fixed the pellets with 2% glutaraldehyde and 2% paraformaldehyde and postfixed them in 1% osmium tetroxide. Subsequently, we dehydrated samples in graded ethanol, applied 1% uranyl-acetate in 50% ethanol as block staining and embedded samples in Taam 812 (Taab). After an overnight polymerization at 60°C, we analysed the ultrathin sections with a Hitachi 7100 electron microscope (Japan) equipped by Veleta, a 2000 × 2000 MegaPixel side-mounted TEM CCD camera (Olympus).

Confocal microscopy

We isolated the mEVs from the serum-free cultured medium of DiO stained THP1 cells and carried out the HS EVDP coating as well as the re-separation of mEVs as described above. We then immune-stained samples with primary antibodies specific for the α-chain of fibrinogen and complement component C3. Then, we applied secondary antibody labelling with Alexa 594 and Alexa 647 (Thermo Fisher Scientific). We then dried samples into gelatine-coated glass slides, mounted them with Aqua-Poly/Mount medium (Polysciences, USA) and analysed the samples employing a Nikon Eclipse Ti-E inverted microscope (Nikon Instruments Europe B.V, The Netherlands) with a CFI Plan Apochromat VC 60XH oil immersion objective (numerical aperture 1.4) and an A1R laser confocal system using 488, 561 and 647 nm lasers (CVI

Melles Griot) with serial scanning. A NIS-Elements AR software was employed to obtain image stacks.

Determination of protein and lipid concentrations in serially centrifuged EVDP samples

We carried out the serial dC of three HS EVDP samples at 12,500 g for 40 min each round (six rounds altogether). After each round, we carefully moved the supernatant to a new tube, while we washed the pellet with EV buffer. We then re-suspended the washed pellets in HPLC distilled water and measured both the protein and the lipid concentrations (with micro bicinchoninic acid (BCA) assay and sulfo-phospho-vanilin (SPV) assay) of the samples.

Exposure of dendritic cells to nascent and coated EVs as well as protein aggregates

- **Human monocyte-derived dendritic cell (moDC) cultures**

We obtained HS leukocyte-enriched buffy coats from the Regional Blood Centre of the Hungarian National Blood Transfusion Service (Debrecen, Hungary), separated peripheral blood mononuclear cells by standard density gradient centrifugation, subsequent to which we purified monocytes by anti-CD14 immuno-magnetic microbeads, positive selection cell separation (Miltenyi Biotec). We seeded isolated monocytes at 1x10^6 cell/mL in the presence of IL-4 and GM-CSF for five days.

- **Exposure of moDCs to THP1 mEVs with/without coating or to pellets of EVDPs**

We exposed moDCs for 48 h to (i) EV buffer, (ii) nascent, (iii) HS EVDP coated, (iv) RA EVDP coated THP1 mEVS as well as pellets of (v) HS EVDP and (vi) RA EVDP samples (all re-separated with dC) subsequent to which

we analysed cell surface activation markers with flow cytometry and the conditioned media with enzyme-linked immune-sorbent assay (ELISA).

Flow cytometry of (i) mEVs, (ii) THP1 cells and (iii) moDCs

(i) We carried out the flow cytometric detection of mEVs with a FACS Calibur Flow Cytometer applying Annexin V-fluorescein isothiocyanate (FITC) staining. The EV gate was set with 160-200-240-500 nm Megamix Beads (Biocytex) and was optimized with 1μm Silica Beads Fluo-Green (Kisker). We also tested dye-only and unstained EV controls. We determined the vesicle concentration employing count check beads (Sysmex) and the vesicular nature of the detected events was checked by detergent (0.1% Triton-X) lysis. We analysed fibrinogen binding and removal by high salt concentration washing by incubating the THP1 and platelet-derived mEVs in the presence of 1 μg/mL FITC-labelled fibrinogen (Abcam) subsequent to which we applied 0.75 M and 1.5 M NaCl washing of samples.

(ii) We also tested the binding of FITC-labelled fibrinogen to THP1 cells (cultured in serum-free conditioned medium prior to testing).

(iii) We tested the CD83, HLA-DR and CD86 expression of moDCs by a NovoCyte flow cytometer (ACEA Biosciences).

Measurement of cytokine concentrations by ELISA

We measured the concentration of interleukin-6 (IL-6) and tumour necrosis factor α (TNF- α) in the conditioned medium of moDCs by OptEIA kits (BD Biosciences) according to the instructions of the manufacturer.

Bioinformatic tools and databases

We obtained data for Venn diagrams based on the analysis by either FunRich Functional Enrichment Analysis Tool or by InteractiVenn (an online

platform). We analysed data of flow cytometric measurements by FlowJo vX.0.7. (Tree Star). We predicted protein-protein interactions utilizing the online platform and database, STRING. We set the minimum required interaction score to high confidence (0.700) and we considered all interaction sources (text mining, experiments, databases, co-expression, neighbourhood, gene fusion and co-occurrence) and considered both physical and functional protein associations. We then illustrated the network with the yEd Graph Editor software in which we set graph centrality measures based on the number of connected edges to nodes. We predicted the membrane localization of THP1 mEV proteins based on the UniProt database. For the comparative analysis of the protein corona of THP1 and platelet-derived mEVs, we analysed the proteomic data of viral and lipid nanoparticle protein corona provided in the supplemental material of the publication of Ezzat *et al*. considering only the human proteins identified by the authors.

Statistics

We evaluated numerical data in Microsoft Excel and carried out statistics with GraphPad Prism 8 (GraphPad Software Inc.). Prior to analysis, we determined the normality of data with D'Agostiono&Pearson test or in case the number of data elements was too low to perform this test, we used Shapiro-Wilk test. We employed Student's t-test, Mann-Whitney U test, Kruskal-Wallis test with Dunn's post-test and one-way ANOVA test with Tukey's post-test according to the distribution of the data with *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ significance levels.

4. Results

We obtained the list of proteins of dC re-separated nascent, as well as of HS, or RA EVDP coated THP1 and platelet-derived mEVs and of pellets of HS or RA EVDP samples without the addition of mEVs by MS. By subtracting the proteins that we also identified in nascent mEVs from the protein list of coated mEVs, we received the list of proteins that have newly associated with mEVs. We consider that these proteins might be the corona proteins We identified 144 corona proteins, out of which there were 20 that were detectable in ≥90% of the samples (10 RA and 12 HS blood plasma) and 61 were present in ≥30% of all the 22 samples. 16 proteins showed up a1.5 more preferentially in the corona of RA EVDP coated mEVs. Repeating these measurements with n=3 HS EVDP coated platelet-derived mEVs, we found a 44% overlap between the THP1 and the platelet mEV protein corona. In the case of n=3 HSs, we also repeated the MS analysis of THP1 mEV protein corona after utilizing either DGUC or SEC to re-separate the vesicles. With these two latter methods of EV separation, we identified a lower number of proteins, which might be attributed to better purity of these methods as well as to a greater dilution of the samples. With DGUC, we also found indirect evidence for the presence of an EV-attachment from the blood plasma: we found that EVDP coated mEVs had a significantly higher flotation density.

It came surprising that albumin was not among the list of corona proteins due to that it was also present in nascent mEV samples and therefore was subtracted from the corona protein lists. We consider, that its presence among the proteins of nascent mEVs might be attributed to that (i) we found that THP1 cells also express albumin, (ii) albumin might remain from the FBS used for cell culturing either inside the cells or attached to their surfaces and

might be misidentified as a human protein, hence it may be the part of both the innate and the acquired protein corona of EVs.

In order to gain better insight into how the protein corona forms around EVs, we analysed the protein-protein interactions among (i) EV surface proteins and corona proteins, or (ii) protein corona proteins themselves. We found that 13 out of the 61 corona proteins showed association only to other members of the corona and not the mEV surface proteins indicative of the evolution of a multi-layered corona. With a simplified corona model, where we incubated THP1 mEVs in the presence of fluorescent fibrinogen, we found that upon applying high concentration salt washing to the samples, fluorescent signal indicative of mEV-associated fibrinogen molecules in the mEV gate decreases. This suggests that electrostatic interactions might also play role in the formation of the protein corona.

We compared our list of corona proteins to that of the study of Ezzat *et al*. in which the protein coronas of herpes simplex and respiratory syncytial viruses as well as of lipid nanoparticles with either positive or negative surface charges were analysed. We found nine shared corona proteins that were present in all lists: 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). Of note, we also detected these proteins by capillary Western blotting together with the pan EV-marker CD63 in EVs isolated directly from human blood plasma employing an Annexin V-based affinity isolation method.

In order to visualize, and thus obtain direct evidence for the presence of the protein corona around EVs, we utilised two methods of electron microscopy as well as confocal microscopy. For TEM imaging of ultra-thin sections, we applied a simplified corona model and incubated THP1 mEVs with

fibrinogen. As compared to nascent mEVs, we observed that fibrinogen-incubated mEVs appeared significantly more likely with a "fluffy" surrounding (t-test, $p < 0.0001$). With immune EM, we detected several members (fibrinogen α-chain, apolipoprotein A1, haptoglobin, complement component C3) of the protein corona together with CD63 in EVDP-coated mEV preparations. For confocal microscopy, we marked for fibrinogen α-chain, and complement component C3 with fluorescently labelled antibodies in the corona of DiO stained mEVs. We found, that besides a diffuse "patchy" appearance of EV surface proteins, big aggregate-like structures also attach to EVs.

An unexpected aspect of our work was the high number of proteins detected by MS in the "mock pellets" of EVDP controls (without the addition of mEVs) regardless of the method of re-separation (dC, DGUC and SEC). Importantly, these proteins also showed a high overlap with corona proteins. With TRPS, we found events in these "mock pellets" in the same size range (although in lower concentration) as mEVs, however, they showed resistance to membrane detergent (0.1% Triton-X) lysis also pointing to that these particles are most likely protein aggregates. We could neither identify EV-like structure in these samples with immune EM, nor with confocal microscopy. In order to test whether it was plausible to get rid of these events by serial centrifugation, we subjected EVDP samples to six consecutive rounds of dC at 12,500 g. While with SPV assay we could not detect lipids in the "mock pellets", there was a considerable amount of proteins measured by BCA even after the sixth round of centrifugation. This result point to that serial centrifugation itself is not enough to eradicate these confounding events and also might imply that centrifugation itself may lead to the formation of further protein aggregates.

To gain information on the function of the EV protein corona, we first applied FunRich gene enrichment analyser to find out in which biological processes are corona proteins predicted to be involved. With the highest percentages, protein metabolism, immune response and transport appeared in the analysis (34.8%, 26.1% and 26.1% respectively). Next, we settled to investigate the biological effect of the protein corona by treating moDCs with either (i) EV buffer, (ii) nascent THP1 mEVs, (iii) HS EVDP coated or (iv) RA EVDP coated THP1 mEVs, (v) HS EVDP-derived and (vi) RA EVDP-derived protein aggregates. We analysed the activation of cells by subjecting them to flow cytometry to detect their surface activation markers (CD86, CD83 and HLA-DR) as well as by measuring the concentration of IL-6 and TNFα in their conditioned medium by ELISA. We found, that there was no significant difference in between the effect of HS or RA protein corona in this model, and only in the case of TNFα was a significant difference between the effect of nascent and HS or RA coated mEVs (Kruskal-Wallis analysis with Dunn's post-test $p < 0.05$ and $p < 0.001$ respectively). Meanwhile, in contrast to EVs, protein aggregates did not induce moDC cell activation.

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 the origin being plasma from healthy subjects or RA patients) induced significant activation of moDCs when compared to the effect of protein aggregates.

Bibliography of the candidate's publications

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