Final report (NKFIH OTKA K120237)

Studies on extracellular vesicle heterogeneity

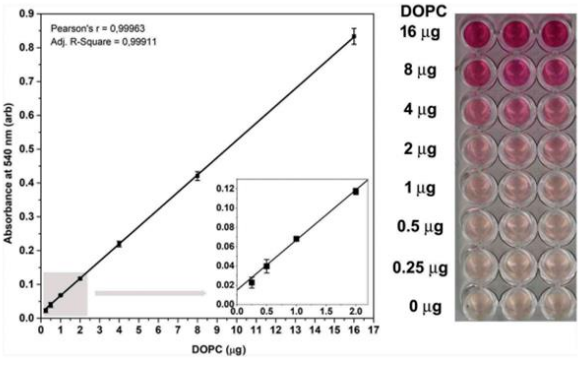
The goal of this project was to unfold extracellular vesicle (EV) heterogeneity in order to have a better understanding of the different EV types. EVs are phospholipid bilayer extracellular particles which have attracted substantial attention in the past two decades, and are considered to represent next generation biomarkers and therapeutic platforms.

In spite of the significant research efforts worldwide, there are still very important knowledge gaps that prevent immediate translation of the EV-related findings to clinical laboratory diagnostics and therapy. In the currently closing project, we aimed at performing studies on EVs and EV subtypes in order to have a better understanding of the heterogeneity of small (50-150 nm), medium sized (200-800 nm) and large (>1000 nm) EVs.

Development of a colorimetric 96 well lipid assay suitable to characterize all (small, medium and large) EV populations

During our work in the field of EVs, we realized a significant unmet need in EV characterization. While the current analytical platforms determine the particle number or the protein content of a given EV preparations, these platforms fail to distinguish EVs from protein aggregates/complexes which share biophysical parameters with EVs.

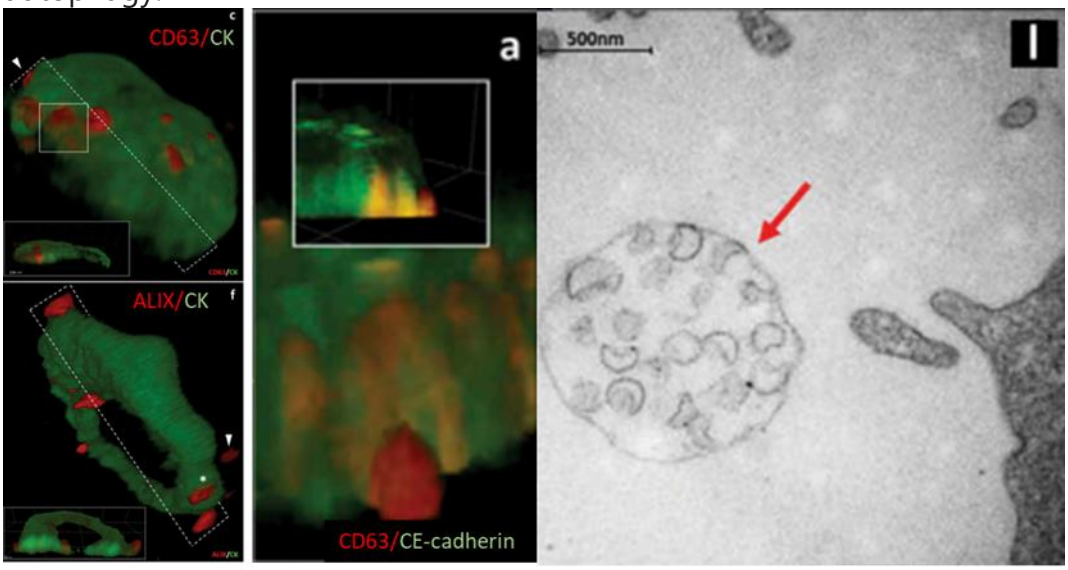
Given that the phospholipid membrane shell is a defining component of all EVs, it appeared logical to develop an assay which characterizes the lipid content of the EV preparations. Our laboratory has suggested earlier the sulfo-phospho-vanillin (SPV) lipid assay for EV analysis (PMID: 25798862), however, its sensitivity was rather low. In this project, we set to optimize this assay by eliminating organic solvents from the reaction mixture to abolish the background color that interfered with the assay. Also, we substantially improved the sensitivity of the lipid assay by optimizing the concentration of the vanillin reagent. Thus, overall, we enhanced the sensitivity of the assay with approximately one order of magnitude. We consider that this optimized simple lipid assay gives a valuable complementation to the available nanoparticle tracking or protein detection EV platforms. Our laboratory has used this SPV lipid assay routinely for EV characterization throughout this closing OTKA project.



An improved 96 well plate format lipid quantification assay for standardisation of experiments with extracellular vesicles. Visnovitz T, Osteikoetxea X, Sódar BW, Mihály J, Lőrincz P, Vukman KV, Tóth EÁ, Koncz A, Székács I, Horváth R, Varga Z, Buzás EI. J Extracell Vesicles. 2019;8:1565263. Impact factor: 14.20

Identification of a novel type of large EVs

We first investigated the heterogeneity of large EVs (> 1000 nm). These very large vesicles currently attract surprisingly low attention, and very little information is available about their heterogeneity. Most available data are related to apoptotic bodies and large oncosomes.
In our project, we identified a novel type of large EVs. We found that in formalin fixed, paraffin embedded sections of human colorectal patients, migrating cancer cells released very large, multivesicular body (MVB) like structures. For their analysis, we used confocal microscopy with 3D reconstructions and HyVolution as well as STED imaging. The *en bloc* released MVB-like small EV clusters showed positivity for exosomal markers such as ALIX and CD63 and were surrounded by a Rab7 positive membrane. The *en bloc* released MVB-like structures either protruded from the surface of the migrating tumor cells or were found in the close vicinity of the cells. We also analyzed *in vitro* the HT29 colorectal cancer cell line. By using immunohistochemistry and immune electron microscopy, we confirmed the emission of the *en bloc* released MVB-like small EV clusters into the conditioned medium. Importantly, we detected LC3 positivity of the novel type of EVs (*en bloc* released small EV clusters) suggesting that they were released by a process related to autophagy.



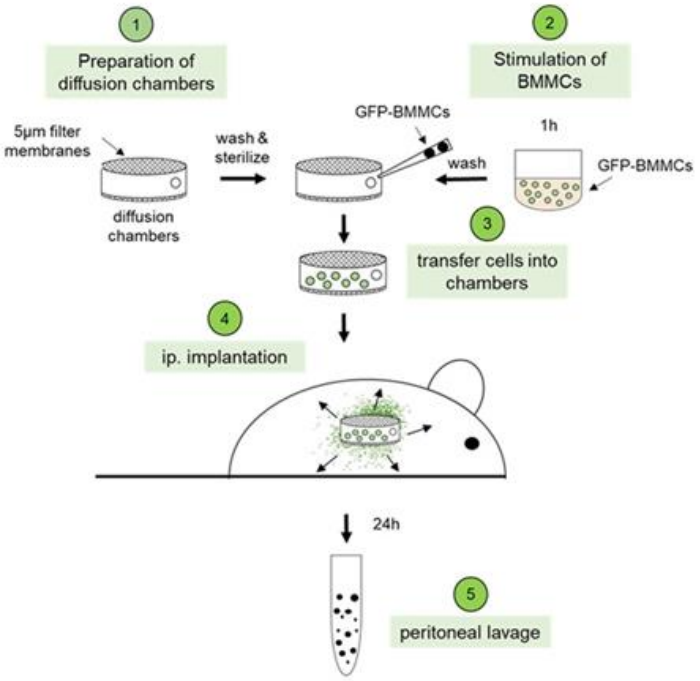
En bloc release of MVB-like small extracellular vesicle clusters by colorectal carcinoma cells. Valcz G*, Buzás EI*, Kittel Á, Krenács T, Visnovitz T, Spisák S, Török G, Homolya L, Zsigrai S, Kiszler G, Antalffy G, Pálóczi K, Szállási Z, Szabó

V, Sebestyén A, Solymosi N, Kalmár A, Dede K, Lőrincz P, Tulassay Z, Igaz P, Molnár B.J Extracell Vesicles. 2019 8:1596668. Impact factor: 14.20

Characterization of medium sized and small EVs released by murine mast cells

In the project, we characterized the functional ability of murine mast cell-derived EV subpopulations to induce a pro-inflammatory response in recipient cells. *In vitro*, we exposed primary mast cells from GFP transgenic and wild type mice to lipopolysaccharide (LPS) stimulation. We found that the released medium and small sized EVs were readily taken up by other, LPS-naïve mast cells, and these EVs induced TNF-alpha secretion by the recipient mast cells in a TLR4, JNK and P38 MAPK dependent manner.

Next, we wanted to investigate the functions of mast cells derived EVs *in vivo* as well. To this end, we implanted diffusion chambers into the peritoneal cavity of mice. The diffusion chambers enabled the release of GFP positive mast cell-derived EVs into the peritoneal cavity. After 24 hours, peritoneal lavage cells were assessed for the uptake of GFP positive EVs and for TNF-α production. With this i*n vivo system*, using the implanted diffusion chambers, we confirmed the release and transmission of mast cell-derived EVs to other mast cells with subsequent induction of TNF-α expression. These data show an EV-mediated spreading of pro-inflammatory response between mast cells, and provide the first *in vivo* evidence for the biological role of mast cell-derived EVs.

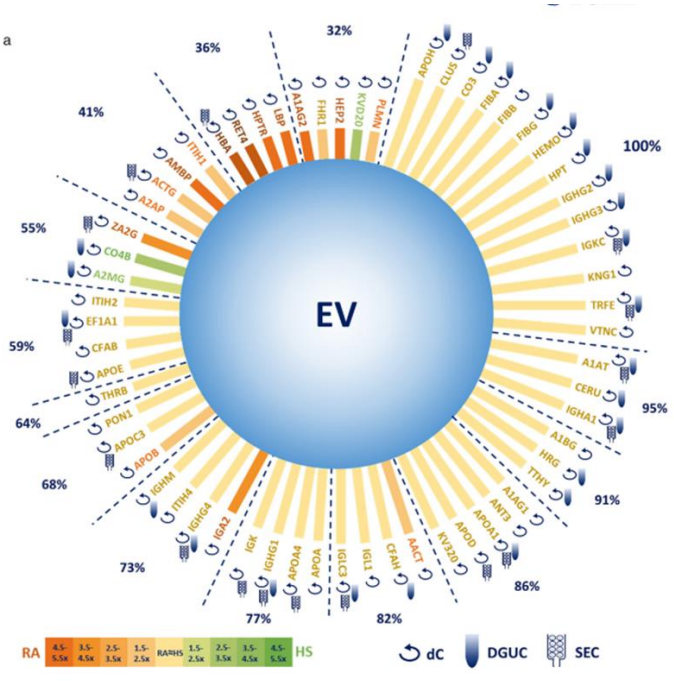


.An implanted device enables in vivo monitoring of extracellular vesicle-mediated spread of pro-inflammatory mast cell response in mice. Vukman KV, Ferencz A, Fehér D, Juhos K, Lőrincz P, Visnovitz T, Koncz A, Pálóczi K, Seregélyes G, Försönits A, Khamari D, Galinsoga A, Drahos L, Buzás EI. J Extracell Vesicles. 2020;10:e12023. Impact factor: 25.841

A previously unrecognized source of EV heterogeneity: a personalized protein corona formation around medium sized EVs in blood plasma

Our recent work has shed light to a new factor determining EV heterogeneity: the adsorbed proteins forming a corona around EVs. The formation of a protein corona around artificial nanoparticles has been long known and has been well characterized. Identification of corona proteins by mass spectrometry was a straight forward approach given that all nanoparticle-associated proteins were constituents of the corona. Similarly, when determining the composition of a viral protein corona, virus proteins could be subtracted from the human proteins. However, determination of the corona components in humans proved to be a lot more challenging, given that both EV proteins and the corona components are of the same species.

We chose the experimental strategy to isolate nascent medium sized THP1 cell-derived EVs. The nascent EVs are freshly released *in vitro* into a serum free medium. We co-incubated the nascent EVs with EV-depleted plasma from either healthy subjects or from patients with rheumatoid arthritis. After extensive washing, the EVs were re-isolated by i) differential centrifugation, ii) size exclusion chromatography (SEC) or iii) Optiprep density gradient ultracentrifugation (DGUC). This was followed by the mass spectrometry analysis of nascent and plasma-incubated, re-isolated EV preparations. We identified 61 protein as corona components, which were present in EV preparations after incubation with blood plasma, but not in the nascent EVs.

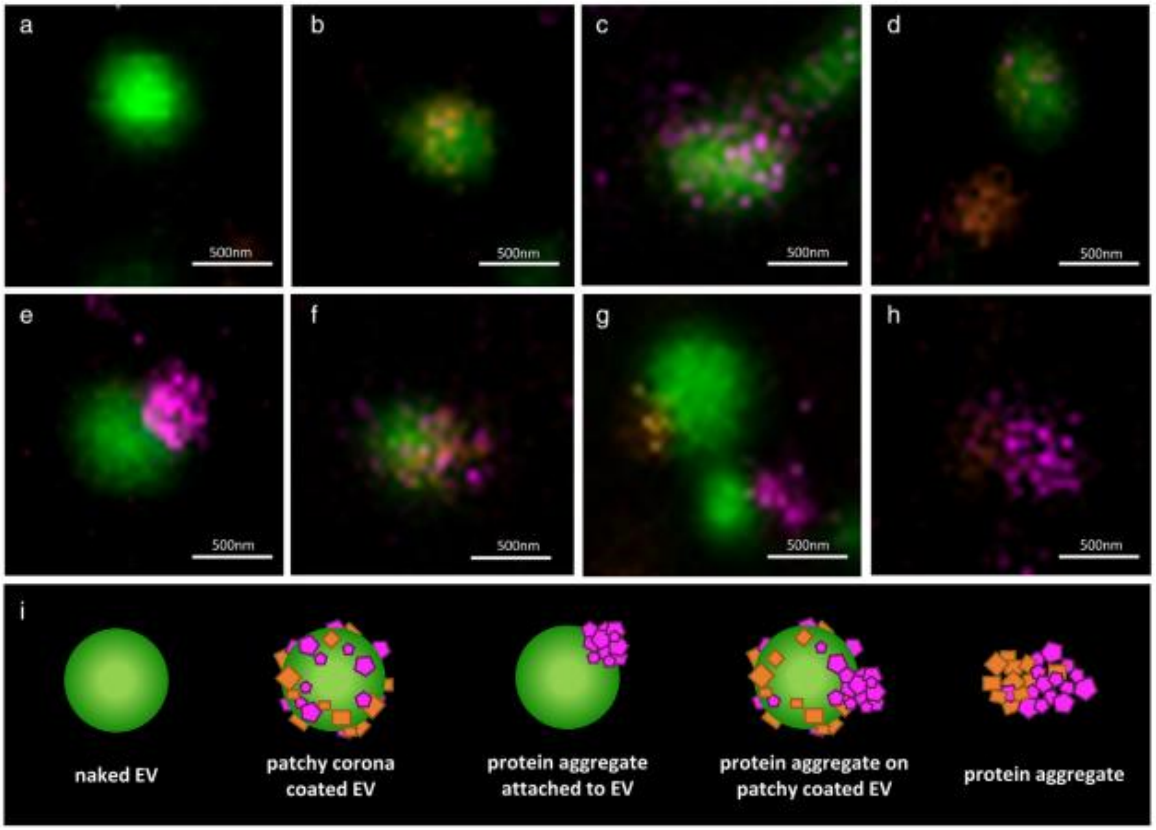


We found high inter-individual differences. Certain proteins related to inflammation were 4-5 fold more frequently detectable in the corona of rheumatoid arthritis patients than in healthy subjects (such as HBA, RET4, LBP, A1AG2, HEP2, IGA2, ZA2G HPTR). Of note, we also listed certain proteins as members of an extended corona protein list. These proteins (such as ALBU, AT1A1, FETUA and VTDB) were always present in the protein coronas of EVs, however, were also detectable in the nascent EVs. Corona proteins were detected in EVs isolated by either differential centrifugation, SEC and DGUC or by all these methods. We compared the floatation densities of the nascent and corona coated EVs, and as expected, the corona coated EVs had a significantly higher floatation density. We compared the corona components that we detected around medium sized THP1 EVs and platelet concentrate-derived mEVs, as well as published corona

components detected around respiratory syncytial virus, herpes simplex virus, as well as positively and negatively charged synthetic lipid nanoparticles.
To our surprise, this comparison resulted in a consensus set of 9 corona proteins. These included (ApoA1, ApoB, ApoC3, ApoE, complement factors 3 and 4B, fibrinogen α-chain, immunoglobulin heavy constant γ2 and γ4 chains).
To confirm that the above consensus „universal" corona proteins were indeed associated with EVs, we used a completely different approach of EV separation: affinity capture by Annexin V-coated beads. EVs separated this way were analyzed by capillary immuno assay. We confirmed that presence of the corona proteins in the affinity-isolated EV preparations.
We next addressed the question if the identified EV corona proteins were also present in the entries in the proteomic database Vesiclepedia. We found that some of the corona components (such as CLUS, CO3, FIBA, FIBB, FIBG, TRFE and VTNC) were detected in as many as approximately one or two hundreds of EV proteomic datasets. Unexpectedly, the vast majority of these EVs preparations were separated from samples other than blood plasma. Moreover, we found that while 72 out of the nascent EVs were among the top100 Vesiclepedia hits, 20 top hits were not detecetd in the nascent THP1 mEVs.
We also used confocal microscopy to directly investigate protein association with the surface of DIO labelled fluorescent EVs. With this approach, we found direct evidence for the presence of a diffuse, patchy corona around some EVs, while in other instances we detected the adsorption of large protein aggregates onto the surface of EVs. Also, by using immunogold electron microscopy, we provided direct evidence for the presence of corona proteins on the surface of EVs. When analyzing ultrathin sections of mEV pellets, we detected significantly higher frequency ($p<0.0001$) of thickened, fluffy contours of model protein (fibrinogen)-corona coated EVs as compared to nascent ones (which were characterized by a thin membrane lining). Using the STING database, we identified a high number of protein-protein interactions between the corona proteins and proteins with transmembrane domains. Interestingly, we also detected a very high number interactions between the corona components as well.
Next we wanted to assess if electrostatic interactions also played a role in adsorption of corona proteins to the EVs surface. To this end, we incubated nascent mEVs with FITC conjugated fibrinogen. We demonstrated that fibrinogen binding could be abolished by using an increasing concentration salt solution.
We had a real surprise when we looked at the proteomic composition of washed pellets from healthy EV-depleted blood plasma samples processed by differential centrifugation, SEC or GDUC. Unexpectedly we found a striking overlap between the proteomic compositions of the corona coated EVs and the pellets obtained by processing the EV-depleted plasma samples by the three different methods. We concluded that the pellets represented protein aggregates, which was confirmed by the detergent resistance of the pellets. We analyzed the protein aggregates by immune electron microscopy and laser confocal microscopy. Importantly, we showed that we

obtained protein aggregate containing pellets even after 6 consecutive centrifugations of the EV-depleted plasma samples.
Finally, we assessed if the protein corona had any functional impact on EVs. We exposed human monocyte-derived dendritic cells (DCs) to EVs, and found that corona coated EVs (but not protein aggregates from the same samples) induced expression of DC maturation markers (CD83, CD86 and HLA DR) as well as secretion of TNF-α and IL-6.

Taken together, this was the first report that provided direct experimental evidence for the spontaneous formation of a protein corona around EVs. Analysis of the corona may provide a biomarker platform whereas artificial modification of the proteins corona may provide us a tool for better targeting of EVs.
Because of its general relevance for the EV field, our study has already elicited substantial international scientific interest.



Tóth EÁ, Turiák L, Visnovitz T, Cserép C, Mázló A, Sódar BW, Försönits AI, Petővári G, Sebestyén A, Komlósi Z, Drahos L, Kittel Á, Nagy G, Bácsi A, Dénes Á, Gho YS, Szabó-Taylor KÉ, Buzás EI. J Extracell Vesicles. 2021;10:e12140. 25.841 Impact factor: **25.841**