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IMPROVED EXTRACELLULAR VESICLE DETECTION AND CHARACTERIZATION

Ph.D. thesis

Xabier Osteikoetxea

Doctoral School of Molecular Medicine

Semmelweis University

Supervisor: Edit I Buzás MD, D.Sc

Official reviewers: Dr. Mihály Józsi Ph.D.

Zoltán Jakus MD, Ph.D.

Head of the Final Examination Committee: Dr. Éva Szökő, D.Sc

Members of the Final Examination Committee: Zoltán Benyó MD, D.Sc

Dr. Zsuzsanna Bajtay, D.Sc

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1. ABBREVIATIONS

% CV	Percentage coefficient variation
% Error	Percentage error
16K PRL	16 kDa N-terminal prolactin fragment
A	Ampere
Ab/AM	Antibiotic Antimycotic Solution
AC8	Anti-cholesterol antibody
ACD	Acid-citrate dextrose
ADP	Adenosine diphosphate
Alix	Alg-2 interacting protein X
ANOVA	Analysis of Variance
APC	Allophycocyanin
APO	Apoptotic body
ARF6	ADP-ribosylation factor 6
BBB	Blood–brain barrier
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
CTX	Cholera toxin
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DEX	Dendritic cell exosome
DMEM	Dulbecco's Modified Eagle's Medium
DRF3/Dia2	Diaphanous Related Formin 3
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EM	Electron microscopy
EMT	Epithelial-mesenchymal transition
ESCRT	Endosomal sorting complex required for transport
EV	Extracellular vesicle
EXO	Exosome
FBS	Fetal bovine serum
FITC	Fluorescein Isothiocyanate
FSC	Forward Scatter
g	*g*-Force, equivalent to RCF

GM-CSF	Granulocyte-macrophage colony-stimulating factor
GP	General polarization
GTPase	Guanosine triphosphate phosphohydrolase
HGFR	Hepatocyte growth factor receptor
HIV	Human immunodeficiency virus
hr	Hour
HSV	Herpes simplex virus
IL-6	Interleukin-6
ISEV	International Society for Extracellular Vesicles
ISTH	International Society on Thrombosis and Haemostasis
min	Minute
miRNA	microRNA
MMP	Matrix metalloproteinase
mRNA	messenger RNA
MV	Microvesicle
MVB	Multivesicular body
NK Cell	Natural killer cell
nSMase 2	Neutral sphingomyelinase 2
OMV	Outer membrane vesicle
OSE	Oxidation-specific epitopes
PBS	Phosphate Buffered Saline
PE	Phycoerytherin
PFP	Platelet-free plasma
P-gp	P-glycoprotein
PS	Phosphatidylserine
Rab	Ras-related proteins in brain
RCF	Relative Centrifugal Force, equivalent to *g*-Force
Refs	References
RPMI	Roswell Park Memorial Institute Medium
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error mean
SPV	Sulfophosphovanilin
SSC	Side Scatter
TEX	Tumor exosome
TF	Tissue factor
TfR	Transferrin receptor

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TRAIL	TNF-related apoptosis-inducing ligand	**TRPS**	Tunable resistive pulse sensing
Treg	Regulatory T cell	**V**	Volt
TrpC5	Transient receptor potential channel 5	**VEGF**	Vascular endothelial growth factor
		VPS	Vacuolar protein sorting

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2. INTRODUCTION

Extracellular vesicles (EVs) are a heterogeneous group of lipid bilayer enclosed particles found to be released by most, if not all, cells. To date EVs have been found to be present in most biological fluids as well as in the environment around us. These vesicles can efficiently carry and protect from degradation different biological molecules such as functionally active proteins, lipids, and nucleic acids. By transferring such molecules between cells, different EVs participate in a diversity of biological processes in health and disease such as inflammation, immune suppression, antigen presentation, tumor development, as well as in the transfer of genetic information, morphogens and signaling molecules.

Given their ability to participate in the above biological processes and to transfer molecules among cells, EVs have become increasingly attractive to researchers from various disciplines for the development of novel diagnostic and therapeutic tools. As a consequence, the field of EV research has seen tremendous growth and an exponential increase in number of publications year after year which has profoundly impacted our understanding of intercellular communication, tumor and stem cell biology, inflammation, virology, circulating extracellular RNA and DNA research among other fields

Despite of the fast growth experienced in the field of EVs there are still remain many questions to address about the fundamental characteristics of distinct EV subpopulations. One important limitation for the field is that there are still no universally accepted molecular markers with which to characterize EVs. Additionally, there are no gold standard EV isolation techniques. Because of these reasons some disparity exists in the literature in regards to the different isolation and characterization techniques used for various studies. Consequently, even if there is ample evidence clearly supporting the effects of EVs in many biological processes there are still some areas where the evidence is ambiguous due to dissimilar findings.

Considering these important limitations in the field, the present work focused on improving existing EV characterization and detection techniques by introducing to the field new approaches based on lipid properties with which to quantify and characterize EV preparations.

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2.1 Theoretical Background

2.1.1 Extracellular vesicles and their classification

Before the full realization of their biological significance, classification, or functions, different observations provided evidence for the existence of EVs. Some early evidence for these vesicles was provided in 1946 by the findings of procoagulant platelet-derived particles in healthy human plasma (1). Later, these particles were termed "platelet dust" (2) and in the ensuing decades more reports surfaced of such particles found in other biological settings including rectal adenomas (3) and tumor tissues (4), during bone calcification (5, 6), in human semen (7, 8), in bovine sera (9) and human cell cultures (10). Only until 1983 did more evidence emerge about how some of these vesicles were generated when detailed electron microscopic studies revealed that these particles were released upon fusion of inner multi-vesicular bodies (MVBs) with the cell membrane during the differentiation of immature reticulocytes (11-13).

Following these discoveries more research attention was brought unto EVs with further reports paving the way for a rapidly emerging interest. One early report showed that EVs released by B cells were capable of inducing T cell responses (14). Later, other reports showed that the vesicles contain and can horizontally transfer functional RNA between cells (15, 16). Recently more reports have expanded the known functions and biological roles of EVs impacting our understanding of many fields of biology such as immunology (17, 18), virology (19), neuroscience (20, 21), cardiovascular (22) and reproductive (23) biology, parasitology (24), and bacteriology (25, 26). With this rapid growth in the amount of reports about EV biology some classification and nomenclature of different subsets of EVs has emerged. Although there is still some debate over the nomenclature used, most in the field utilize the classification of different EVs based on their biogenesis and size ranges as exosomes (EXOs) of approximately 100 nm in size, microvesicles (MVs) of 100 to 1000 nm in size, and apoptotic bodies (APOs) of more than 1000 nm in size. These three important subsets are depicted in Figure 1 and some of their characteristics are summarized in Table 1 and further discussed in the following sections but it is also important to note that other terms may also be encountered in EV literature. Some of the other used terms for EVs will be described later but in most cases they simply represent alternative nomenclature that actually corresponds to one of these previously described subtypes with size ranges similar to EXOs, MVs, or APOs.

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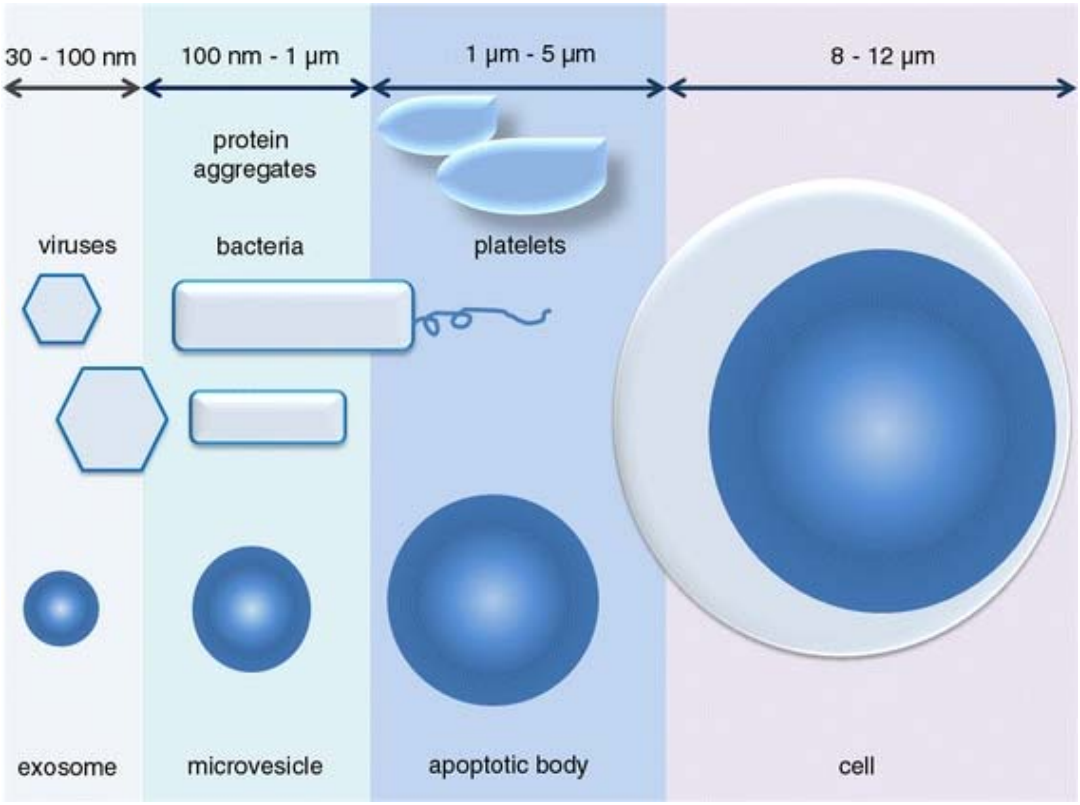

Figure 1. Size ranges of different EV subtypes (27).

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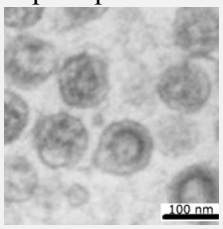
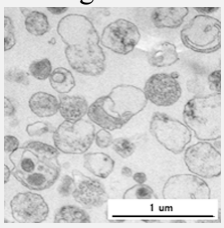
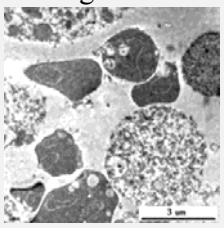
Table 1. Characteristic parameters of different EV subtypes

Parameter	Exosomes	Microvesicles	Apoptotic bodies	Refs
Size	50–120 nm	100–1000 nm	≥1000 nm	(18, 27-30)
Homogeneity	relatively uniform	heterogeneous	≥2 subpopulations	(31, 32)
Nucleic acid content	miRNA, mRNA, DNA	diverse RNAs	rRNAs, DNA	(15, 32-36)
Lipid content	high cholesterol, high glyco-sphingolipids,	phosphatidyl-serine	phosphatidyl-serine	(37-40)
Approximate density	1.13–1.19 g/mL	1.03–1.20 g/mL	1.16–1.28 g/mL	(14, 18, 41-43)
EM morphology	cup shaped 	heterogeneous 	heterogeneous 	(14, 18, 27, 29, 32)

In the following sections the basic characteristics of the different EV subtypes are discussed in further detail with special emphasis on their defining parameters and mechanisms of biogenesis. Focus on their functions will be discussed in later sections.

2.1.1.1 Exosomes

Exosomes comprise the smallest particles among the EV subpopulations with an approximate size of 100 nm and currently attract the most research interest. Importantly, this vesicle subtype is not only distinguished from the other subtypes based solely on size but also based on having a separate molecular mechanism of formation. In contrast to MVs and APOs which may bud directly from the plasma membrane, the biogenesis of EXOs occurs intracellularly within the endosomal network when intraluminal vesicles are formed inside multi-vesicular bodies which later fuse with the plasma membrane for subsequent release instead of fusing with lysosomes for degradation (44). This unique

type of vesicular formation was originally described in studies about the maturation steps of rat reticulocytes when these cells need to discard their membrane bound transferrin receptors (TfR). After an initial endocytosis of TfRs into multivesicular endosomes it was observed that, instead of an expected fusion with lysosomes for degradation of TfR, these endosomes later fused with the plasma membrane for subsequent release of TfR-containing intraluminal vesicles (12, 45).

Given their endosomal origin, EXO secretion is coordinated by several members of the Rab GTPase family of proteins which are essential regulators of intracellular vesicle transport between different compartments (46). Early work showed that among this large family of more than 60 GTPases, RAB4 and RAB5 were enriched in EXOs (47). Further studies later found that RAB11 is important for EXO release pathway since its inhibition decreased EXO secretion (48). More recent studies have further expanded the members of this family of proteins known to have functions in the EXO release pathway to include RAB2B, RAB5A, RAB9A, RAB27A/B (49), as well as RAB35 (50). In addition to Rab GTPases, other proteins have also been found to mediate EXO secretion and include elements of the endosomal sorting complex required for transport (ESCRT) pathway such as ALIX and VPS4 as well as syndecan and syntenin (51).

Similarly to the other vesicle subpopulations, EXOs may be released constitutively or upon activation and contain various proteins, lipids, and nucleic acids including RNAs. Although there are some differences in cargo depending on the releasing cell and its activation state, some molecules may be commonly found in most EXOs. Among these molecules EXOs contain high amounts of cholesterol and glycosphingolipids (37-40), small RNAs including mRNA and miRNA (15, 52), as well as different transmembrane and cytosolic proteins (53, 54). A few reports have also found DNA to be associated with EXOs (36, 55). In recent past, different candidates have emerged for markers of EXOs including CD63, CD81, CD9, TSG101, and externalized phosphatidylserine (PS). However, although these molecules are indeed found in most EXOs, one problem is that they may also be found in other EV subpopulations and thus may not be considered markers of EXOs but instead of EVs. EXOs have also been characterized to have a cup shaped morphology by electron microscopy (14), although there is much debate about whether this is an artifact of the preparation needed to take electron microscopic images. An important parameter for EV isolation and characterization is the density of the vesicles which allows to separate them from denser

protein aggregates. In this regard EXOs have and approximate buoyant density of 1.13 to 1.19 g/mL (14, 41).

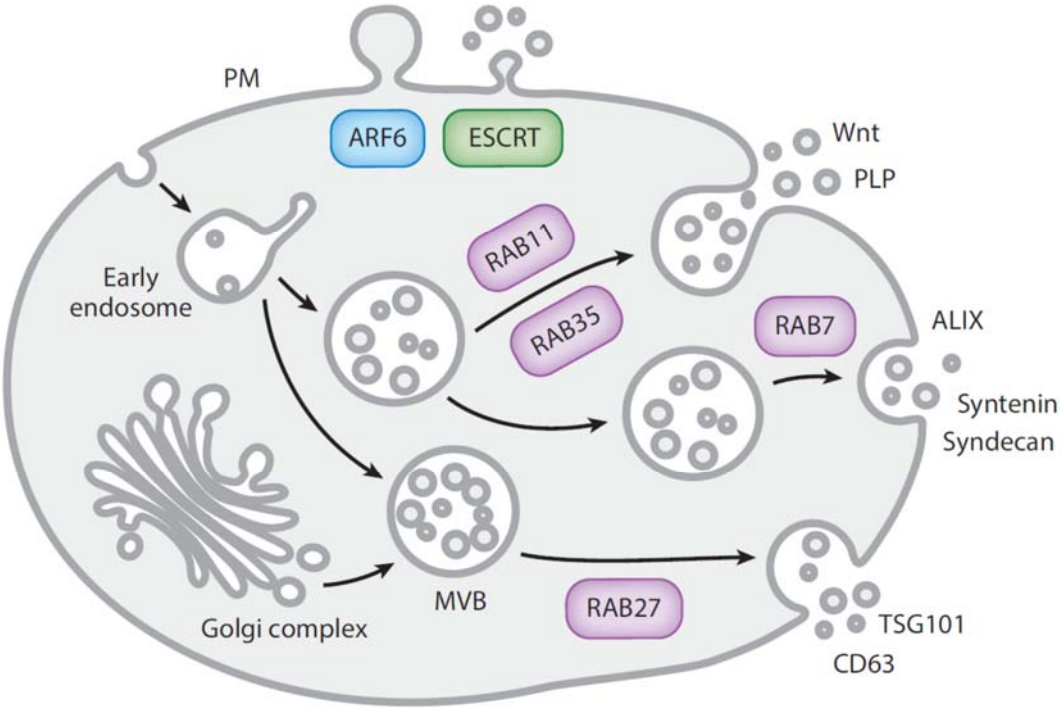


Figure 2. Schematic representation of selected genes involved in the molecular machinery of exosome and microvesicle secretion (44).

2.1.1.2 Microvesicles

Microvesicles comprise the intermediate particles among the EV subpopulations with an approximate size range of 100 to 1000 nm. Not only are these particles larger but also more heterogeneous in terms of sizes and morphology which reflects in their wider buoyant density range of 1.03 to 1.20 g/mL (18, 42, 52). Initially many reports characterized these vesicles in blood and termed them microparticles and most were found to be secreted by platelets, red blood cells, and endothelial cells. However, by now many reports have found that MVs are secreted constitutively by most if not all cells or upon activation.

Similarly to APOs and in contrast to EXOs, MVs are secreted by budding at the cells' plasma membranes. The release of MVs occurs by the budding of small cytoplasmic protrusions which then detach from the plasma membrane (56, 57). There is evidence from several reports that this budding process is regulated and induced by plasma

membrane remodeling and a rise in intracellular calcium. An early report with platelets showed the importance of intracellular calcium in activating the calpain protease which remodels the cytoskeleton by cleaving cytoskeletal proteins and thus induces MV release (58). Later the induction of MV release by a rise in intracellular calcium was also observed in other studies with different cells including microglia and dendritic cells (59, 60). In addition to intracellular calcium levels, other plasma membrane remodeling mechanisms have been found to play key roles for MV release. Normally, the asymmetry of lipid composition between the two leaflets of the plasma membrane bilayer is maintained by several transmembrane lipid transporter proteins including inward-directed flippases which internalize mostly PS and phosphatidylethanolamine, outward-directed floppases, and scramblases which mediate unspecific bidirectional redistribution of lipids across the bilayer (61). However, modulation of the levels of these enzymes leads to increase membrane budding and ultimately MV release. One of the most prominent features of the lipid bilayers of MVs compared to that of releasing cells is their loss of membrane asymmetry (62). More recent reports also show that in addition to plasma membrane remodeling, changes in the cell cytoskeleton towards an amoeboid phenotype can also induce MV release either following small GTPase ADP-ribosylation factor 6 (ARF6) mediated actin depolymerization (63) or loss of the actin-nucleating protein Diaphanous Related Formin 3 (DRF3/Dia2) (64). Lastly, different lipid-metabolism enzymes such as phospholipase D2 (65, 66) and sphingomyelinases (67) have also been involved in budding of MVs.

However, some of the above mechanisms responsible for MV budding such as ARF6, lipases, and also some proteins of the ESCRT pathway are not exclusively implicated in MVs biogenesis as they play similar roles in the inward budding of intraluminal vesicles in MVBs during EXO formation so their modulation may regulate secretion of both EV subpopulations (44). Likewise, RAB22A, a member of the Rab family of proteins implicated in EXO biogenesis, has been recently implicated in MV formation (68) as well highlighting that some of the mechanisms behind membrane budding may affect more than one secretion pathway by simultaneously acting at the plasma membrane or in intracellular compartments.

The molecular cargo of MVs also includes various proteins, lipids, and nucleic acids including RNAs. Although the releasing cell type and its activation state may

influence their cargo most MVs contain externalized PS, heterogenous RNAs, integrins, selectins, and CD40 ligand, CD63, CD81 and CD9 (62, 69).

2.1.1.3 Apoptotic vesicles

Apoptotic bodies comprise the largest particles among the EV subpopulations with an approximate size range of more than 1000 nm. Unlike EXOs and MVs, there are not so many reports about APOs and for this reason it is not entirely clear whether APOs are only secreted as membrane surrounded fragments of cells undergoing apoptosis (70, 71) or if they may also be secreted constitutively by different cells as has been reported more recently with amoeboid type of tumor cells (64, 72). Although there are not many reports about the biogenesis of APOs, given their common plasma membrane origin it probably shares many similarities to the biogenesis of MVs. Among the important molecular players discussed for MVs, those related to cytoskeletal remodeling and changes towards amoeboid phenotype have already been implicated in the formation of very large EVs (64, 72).

However, regardless of whether APOs are produced constitutively or not, they can be detected and isolated from most biofluids and cell culture supernatants due to the low level of apoptosis found in different cells and tissues or due to their constitutive secretion by non-apoptotic or tumor cells, but most likely due to a combination of both since they can be detected even in proliferating cell cultures with less than 5% apoptosis. Isolated APOs have also been found to be heterogeneous in size and morphology with some evidence showing that there might be different subpopulations of APOs depending on whether they are derived from the apoptotic cells' endoplasmic reticulum (ER) or plasma membrane (73). So far the reported buoyant density of APOs is higher than other EVs at 1.16 to 1.28 g/mL but only one study has looked into their density (41).

Similarly to other EV subtypes, the molecular cargo of APOs also includes various proteins, lipids, and nucleic acids including RNAs. Additionally, since APOs can be secreted following apoptosis, any cellular component may be packed within them (70) resulting in a higher diversity of molecular cargo than MVs or EXOs. Notably, APOs may contain DNA fragments (74) and other nuclear components in addition to externalized PS, RNAs, CD63, CD81 and CD9.

2.1.1.4 Other vesicle types

In addition to the three previously described EV subtypes there are also other vesicles terms to be found in the literature. Some simply represent alternative nomenclature that actually corresponds to one of the previously described EV subtypes. Such is the case for example of microparticles, ectosomes, or membrane particles which are all released directly from the plasma membrane and can be viewed as alternative terms for MVs. Other types of EVs are named after their sources such as tumor exosomes (TEXs), dendritic cell exosomes (DEXs), oncosomes derived from tumors, and prostasomes which are released by the prostate and found in seminal fluid, among others.

More interestingly, there are EVs found to be secreted by gram positive and negative as well as archaeal species of bacteria (25, 26). The term OMV emerged because initially most reports of bacterial EVs found these vesicles to be secreted by gram negative bacteria which have an outer membrane from where the vesicles were secreted (75-78) but now we know that also other bacteria without outer membranes can secrete vesicles too (25, 26, 79-81). Since the mechanisms for OMV formation are not completely understood and since they fall within the size ranges of previously described EV subtypes, it is still debated whether bacterial derived EVs should be considered part of the previously described EV subtypes or if they should instead be viewed as a completely separate subtype of EVs.

2.1.2 Extracellular vesicles and their functions

In the last decades, EVs have been found to exert many different biological functions in both physiology and pathology explaining the rapid growth of interest in this research field. These functions can be explained due to their unique ability to carry and protect from degradation various biologically active molecules between different cells, tissues, or even across species. In the following sections will be discussed several of the biological roles in which EVs have been involved. In discussing these roles the collective term EV is chosen for all cases where the studied particles are not thoroughly characterized or supported by the described methodology to belong to a particular EV subtype. Nevertheless, the originally reported EV subtype is retained for all other cases in which the described methodology and characterization matches with those widely utilized and accepted for the described subtype.

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2.1.2.1 Extracellular vesicles in health

Many of the early reports about EVs described their roles in normal physiological processes such as coagulation (1, 2) reticulocyte maturation (12, 45), and antigen presentation (14). To date EVs have been found in most biofluids and thus many new functions have been unraveled for their roles in different physiological processes. In this section are described a few of the roles of EVs in physiological processes.

Since EVs were first described to participate in coagulation many reports have further elucidated their roles in the process. Due to the externalized PS, which is a negatively charged phospholipid, EVs can promote coagulation by binding to several coagulation factors in the presence of calcium (1, 2). Additional support for the procoagulant properties of EVs emerged when the key initiator of the coagulation cascade tissue factor (TF), formerly known as thromboplastin, was discovered to be present in EVs shed by different cells (82, 83). More recent studies have shown that blood contains large amounts of procoagulant EVs derived mainly from platelets and that they are important players in normal hemostasis at their baseline levels. However different disease states may also be presented with increased levels of EVs which promote pathological thrombosis (84-86).

Following the early report on the capacity of B cell derived EVs to carry both antigenic material and peptide–MHC complexes (14) lots of research interest was sparked in the field of immunology about the potential roles of vesicles. Since then many different reports have been published further clarifying how EVs participate in various immunological processes in health and EVs derived from many cell types and biofluids have been found to be capable of antigen presentation and immune regulation. By now different studies have reported that in addition to B cells other cell types can carry preformed antigen-MHC complexes capable of activating T Cells (87-90) and EVs isolated from malignant effusions have also been found to induce the activation of antigen-specific T cells *in vitro* (91). In addition to their antigen presentation roles, EVs have been found to have participate in immunoregulation in antigen-independent manners. One example is that EVs derived from thymic cells have been found to induce the development of Foxp3+ regulatory T cells (Treg) in the lung and liver as well as inducing the conversion of CD4+CD25- T cells into Tregs. These EV-induced Tregs were capable of suppressing the proliferation of CD4+CD25- T cells *in vitro* and *in vivo* (92).

EVs secreted by other cells have also been found to have immunoregulatory effects such as those derived from neutrophils and erythrocytes which can restrain inflammation by inhibiting macrophages from releasing pro-inflammatory tumor necrosis factor (TNF) and IL-8 (93). Likewise, EVs isolated from different biofluids such as milk (94), pregnant women plasma (95), or serum and bronchoalveolar fluid (96) have also been found to have immunosuppressive effects.

In contrast to the above reports, EV derived from other sources have been found to have immunostimulatory effects. EVs secreted from activated platelets have been reported to carry CD40L and activate B cells through interaction with their CD40 costimulatory proteins (97). EXOs derived from DCs were also described to be capable of activating Natural Killer cells (NK Cells) by delivering ligands for IL-15Rα and Natural Killer Group 2 member D (NKG2D) dependent proliferation and activation in humans and mice (98). Another example of immunostimulation is encountered in a study where T cell proliferation was synergistically increased when these cells were exposed to a combination of EVs and IL-2 (99).

Interestingly, EVs have also been implicated with important roles in non-synaptic communication among various cells of the nervous system including neurons and glia. Importantly, EVs are able to cross the blood–brain barrier (BBB) in both directions and thus can play a role in transport to the brain of various molecules including folate which plays an important role in neuronal development (100, 101). Moreover, EV release is also found in the normal synaptic activity between neurons. EVs secreted during glutamatergic synaptic activity carry away neurotransmitter receptor subunits, modulating synaptic strength by eliminating part of these receptors from the post-synaptic membranes (102). EVs can also facilitate the communication between two different types of cells of the nervous system. In one report, EVs from microglia were found to act at the presynaptic site and stimulate the synaptic activity of receiving neurons by enhancing their sphingolipid metabolism (103). More reports have demonstrated that microglia derived EVs modulate neurotransmission through lipid-signalling (104, 105). Lastly, Schwann cell and oligodendrocyte derived EVs mediate neuroprotection through pro-survival signalling pathways and modulation of gene expression in target neurons by making them more resistant to stress and enhancing axonal regeneration (106, 107).

In healthy pregnancy EVs have been reported to carry different roles in the immunological communication between the fetus and the mother. Several reports have shown that trophoblast derived EVs are present in maternal blood and interact with both immune and endothelial cells. It is now known that trophoblast derived EVs are involved in the mechanisms of fetal tolerance and can elicit pregnancy-specific immunosuppression of activated T cells by delivery of Fas ligand and Programmed death-ligand 1 (PD-L1) (108-110).

Finally, by transferring different molecules such as RNAs and proteins, EVs have been described to play important roles in tissue homeostasis and repair. Thus far, EVs have been implicated with roles in lung repair (111), cardiovascular protection (112-114), kidney repair (115, 116), hepatic regeneration (117), as well as bone calcification and remodeling (118-120).

2.1.2.2 Extracellular vesicles in cancer

So far many studies have reported on various functions of EVs in cancer making it one of the most intensely studied areas within the field of EV research. EVs have been found to be involved with tumor development, survival, progression, and metastasis.

One important mechanism by which EVs are implicated with tumor biology is by their ability to transfer pro-oncogenic molecules to other cells and tissues. For example EVs can transfer functional pro-oncogenic proteins such as epidermal growth factor receptor, EGFRvIII, to other cells and impart them more aggressive oncogenic phenotypes (121). In a similar manner different studies have found that drug resistance can be transmitted from resistant to non-resistant cells by EVs. EVs carrying P-glycoprotein (P-gp) can transfer this drug transport membrane protein to cells lacking drug resistance (122). Transient receptor potential channel 5 (TrpC5) (123) and Akt/mTOR protein complexes (124) are other proteins that have been found in tumor derived EVs that can transfer drug resistance to sensitive cells. In addition to proteins EVs can also transfer miRNAs such as associated with chemoresitance to drug sensitive cells and make them drug resistant (125, 126). Other pro-oncogenic phenotypes aside from drug resistance may also be transmitted by EV associated RNA and DNA. In one report EVs released by metastatic tumor cell lines contained miR-200 and transferred the metastatic capacity to non-metastatic cells (127). Another study showed that transcript

mRNA for the pro-oncogenic EGFRvIII can be found in serum EVs of glioblastoma patients (33). Moreover, genomic DNA can also be transferred between cells by EV, with one study finding that the oncogenic BCR-ABL fusion gene can be transferred to recipient cells (128).

Given their ability to transfer various molecules, tumor derived EVs have different pro-oncogenic effects on surrounding cells and tissues. Several studies have found that tumor derived EVs can promote tumor migration by remodeling of the extracellular matrix (ECM). Indeed several members of the matrix metalloproteinase (MMP) family of proteins have been found in tumor derived EVs including MT1-MMP, MMP9, MMP2, and A disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) (63, 72, 129, 130). Consequently, these MMPs may remodel the surrounding ECM and promote tumor progression as has been shown in a study that identified tumor cell derived EVs with functionally active MT1-MMP (131).

Another mechanism by which EVs have been found to promote tumor development is by activation and reprograming of fibroblasts toward the phenotype of cancer associated fibroblast (CAF) which support tumor growth and establishment of favorable tumoral microenvironment (130, 132). In turn, CAF derived EVs can increase recipient tumor cell motility and invasiveness as well as promoting epithelial-mesenchymal transition (EMT) (133-135).

Yet another important role of tumor derived EVs in cancer is related to their pro-angiogenic functions. It is well known that formation of new vasculature within tumors for the supply of oxygen and nutrients and the removal of waste products is a critical step for cancer cell growth and later metastasis (136). Cancer cell derived EVs have been found to be able to promote this process by delivering pro-angiogenic interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) (33, 137), EGFR (138), miR-9 (139) and miR-210 (140), as well as neutral sphingomyelinase 2 (nSMase 2) (141).

In more recent studies, tumor derived EVs have been described to play an important role in preconditioning the future metastatic sites. In one milestone study, EVs derived from highly metastatic melanoma cell lines were found to localize *in vivo* at the site of future metastasis and to educate the site towards a pro-metastatic phenotype inducing vascular leakiness via upregulation of hepatocyte growth factor receptor (HGFR) also called MET (142). Similarly, an earlier study had found that EVs derived

from human renal cell carcinoma could induce pro-metastatic phenotype changes by delivery of pro-angiogenic mRNAs and microRNAs at the site of future metastasis in the lung (143).

One last mechanism by which tumor derived EVs can support cancer progression is by inducing tolerogenic immune responses to evade recognition and elimination by the immune system. Different studies have found tumor derived EVs to be immunosuppresive and to induce apoptosis of T cells by delivery of FasL and TNF-related apoptosis-inducing ligand (TRAIL) (144-146).

2.1.2.3 Extracellular vesicles in cardiovascular diseases

The field of cardiovascular research has also been impacted with new understandings of the roles of EVs in the development of disease. Different studies now show that EVs can contribute to cardiovascular disease progression. In one report, following induction with the peripartum cardiomyopathy factor 16 kDa N-terminal prolactin fragment (16K PRL), endothelial cells secrete EXOs that transfer miR-146a to cardiomyocytes and inhibit their metabolic activity and contractile function (147).

Under different conditions, several reports have shown that cardiomyocyte-derived EVs transfer functional proteins and nucleic acids which have an adverse effect on other cardiac cells. Extracellular heat shock protein 60 (exHSP60) has been shown to induce apoptosis in a TLR4-dependent manner (148) and one report found that it was mostly found in EXOs released by either non-stressed or mildly stressed cardiomyocytes while it was absent from EXO-depleted fractions (149). Another study has found that EXOs derived from hypoxic cardiomyocytes contained functional TNF-α and were capable of inducing apoptosis when administered to normal cardiomyocytes (150). EXOs derived from cardiomyocytes of a type 2 diabetes rat model were shown to contain and transfer functional miR-320 to cardiac endothelial cells inhibiting their proliferation and decreasing angiogenesis (151).

In addition to cardiomyocytes, other cells can secrete EVs that contribute to cardiovascular disease. For example, EXOs secreted by cardiac fibroblasts induce a hypertrophic phenotype in cardiomyocytes by transferring miR-21 (152). Similarly, different reports show that EXOs derived from platelets of septic patients contribute to

cardiomyopathy by inducing apoptosis in endothelial cells and cardiomyocytes leading to vascular and cardiac dysfunctions as well as coagulation (153-155).

Until now there has been growing evidence that many cardiovascular diseases are associated with an increased oxidative stress (156, 157). Correspondingly, MVs from myocardial infarction patients carry oxidation-specific epitopes (OSE) and are increased at the site of coronary occlusion. Importantly, these MVs were found to be able to induce pro-inflammatory IL-8 secretion by monocytes in an OSE-dependent manner (158).

Finally, different studies have implicated EVs with promoting atherogenesis and atherosclerotic plaque formation by enhancing recruitement of inflammatory cells (159, 160).

2.1.2.4 Extracellular vesicles in other diseases

In addition to the previously described conditions, EVs have been found to play important roles in many other diseases. In this section some of the important findings are described for different conditions but the list of other diseases in which EVs play roles will surely expand in the future.

Different studies have shown that EVs are key mediators in the progression of neurodegenerative diseases such as Creutzfeldt–Jakob, Parkinson's, Alzheimer's, Gerstmann–Straüssler–Scheinker syndrome, and amyotrophic lateral sclerosis (ALS) by transferring the prions (161-164) or pathogenic proteins (165-170) responsible for each of these conditions.

In parasitic infections EVs can function in immunosuppresion of the host and in functionally preparing for the dissemination of the parasites. To date EVs have been described to be secreted by most parasitic protozoa and helminthes. EVs derived from these parasites can delivery various immunomodulatory signals to the host immune system by transferring small RNAs and proteins (171-177). Additionally, parasite derived EVs can modulate the adhesion of parasites to host cells (178).

Similarly to parasites, other pathogens release EVs that can be uptaken by the host and are involved in the development of pathology and different studies have found bacterial EVs in clinical samples (179-182). To date, several reports have shown that bacterial derived EVs are readily uptaken by host epithelial and immune cells (183-185).

One of the most important mechanisms by which bacterial EVs can contribute to pathogenesis is by the transfer of various biologically active toxins to host cells (79, 184, 186-188). Interestingly, in some cases delivery of equal amounts of toxins, either in soluble form or in association with EVs, produced different effects on host cells showing that EVs do not serve as mere transporters but also can have modulate the effects of the toxins (189, 190). Another important mechanism by which bacterial EVs interact with the host is by modulation of the immune system and induction of inflammation. In many studies upregulation of proinflammatory cytokines such as IL-6 and IL-8 has been observed following exposure to EVs derived from many pathogenic bacteria, including *Helicobacter pylori* (191), *Klebsiella pneumonia* (192), *Salmonella typhimurium* (193), *Pseudomonas aeruginosa* (194, 195), enterotoxigenic *Escherichia coli* (189), and *Neisseria meningitidis* (196, 197). Lastly, bacterial EVs play important roles in communication not only with the host but also with other bacteria and this has been found to be especially relevant for the formation and maintenance of biolfilms (198, 199).

Finally, viruses are another class of pathogens in which EVs have been found to play important roles. Different reports have shown that several viruses, including herpes simplex virus (HSV), human immunodeficiency virus (HIV), and Epstein-Barr virus (EBV), can exploit EV pathways for their own assembly and release (200-204). Furthermore, EVs released from infected cells can contain and transfer viral particles, proteins, and miRNA to non-infected cells and therefore allow propagation to cells that may not have been permissive for entry of the conventional EV free virus while also avoiding recognition by the immune system (205-209). One more important mechanism by which EVs can assist viruses is by immunoregulation of the host (210-214).

2.1.3 Clinical applications of extracellular vesicles

There currently is much interest in developing clinical applications for EVs considering the vast amount of findings showing a wide diversity of EV molecular cargos and biological roles in health and disease. Since EVs released by tissues and cells in pathological states have been found in several biofluids, one area of promise is to detect and isolate these EVs for diagnostic purposes as disease biomarkers earlier than it would be feasible to detect the affected releasing cells and tissues. Furthermore, EVs also offer great potential for therapeutics given their ability to protect from degradation and transfer

molecules. Some approaches are based on disrupting or removing pathological EVs so that they do not contribute to disease. Another interesting approach is to use unmodified or engineered EVs to deliver signaling molecules or therapeutics of choice to different cells or tissues while protecting them from degradation or immune detection. In the following section will be highlighted some of the important studies that attempt to translate some of the novel findings in EV biology into clinical applications for diagnostics and therapeutics.

2.1.3.1 Extracellular vesicles as diagnostic tools

So far many studies have reported on the release of EVs by cells implicated in various diseases and their molecular cargos. Since EVs have been detected in many biofluids such as saliva, urine, blood, ascites, breast milk, and cerebrospinal fluid there is great potential in the search for disease derived EVs for diagnosis from these sources (30). However, an important aspect to consider is that the majority of EVs found in circulation are derived from platelets and other blood cells (30, 158). Thus, in some conditions it may be technically unfeasible to reach the required sensitivity to detect the few EVs derived from the tissue or cells of interest, even if their secretion is strongly upregulated, within the large background of mostly blood derived EVs. For this reason different studies also assess the origin of EV used for diagnosis with specific cell or tissue markers.

Different approaches may be used independently or in combination when assessing EVs for diagnostic purposes. One approach is based on measuring the concentration of EVs in a given biofluid and assesing how different diseases may have changes in EV concentration compared to that found in normal healthy conditions. Such approach can be found in studies of EV as diagnostic tools for severe hypertension (215), preeclampsia (216), and heart diseases (158, 217, 218) as well as ovarian (219), lung (220), and gastric (221, 222) cancers.

Another promising approach with EVs for diagnosis of various conditions is to look at their cargo for disease specific molecules including proteins or nucleic acids. This approach is especially interesting considering that EVs pack and protect from degradation molecular cargo that reflects the composition of the secreting cells and thus unique disease associated molecules can be identified in circulation or at distant sites from the originating cells or tissues. With this approach several studies have shown promise for

diagnosis of many conditions with EVs based on analysis of their proteins (33, 146, 219, 221, 223, 224), lipids (225-228), RNA (33, 220, 229-232), and DNA (36, 55).

2.1.3.2 Extracellular vesicles as therapeutic tools

Considering their ability to transfer and protect various molecules from degradation EVs have been exploited in various studies for therapeutic purposes. To date several approaches have been employed to use EVs for anti-tumor therapy, vaccination, drug or nucleic acid delivery, and regenerative therapy as well.

So far different clinical trials have attempted to use EVs in anti-tumor therapies. One trial utilized ascites-derived EXOs in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) for colorectal cancer immunotherapy finding beneficial anti-tumor cytotoxic T cell responses (233). Other clinical trials used EV secreted by DCs, pulsed with antigenic peptides from patient tumors, for anti-tumor therapy against metastatic melanoma (234) and non-small cell lung cancer (235).

Other reports have also shown promise in use of EVs for vaccination. Similarly to some of the anti-tumor therapy clinical trials, EVs secreted by DCs pulsed with pathogenic antigens have shown promise for vaccination. One example is found with several reports showing that EVs released by DCs pulsed with *Toxoplasma gondii* antigens can act as an effective vaccine conferring protection against infection (236-238). Alternatively, EVs released directly by the pathogens have also been used for vaccination with a notable example of using EVs secreted by *Neisseria meningitidis* in a Novartis FDA-approved vaccine against meningococcal diseases in children named Bexsero® (239, 240).

One more important application of EVs in therapeutics is to modify them for delivery of drugs or nucleic acids. Use of EVs for drug or nucleic acid delivery is especially attractive since they can overcome several important barriers of other existing delivery systems, including liposomes and viruses, because of their lower immunogenicity and toxicity, increased stability in circulation and tissue, and intrinsic homing abilities. Among the potential therapeutic cargos, already several reports have successfully shown EV mediated delivery of various drugs (241-244) and nucleic acids such as small RNAs (245-247).

Lastly, EVs derived from various cells, including stem cells and immune cells, have shown promise in regenerative medicine. There is emerging evidence that in various cell based therapies EVs play an important role in mediating the therapeutic effects of such cells. Originally cell based regenerative therapies were envisioned to function mostly by replacing the diseased cell and tissues with therapeutic cells. However, in many cases the therapeutic cells are rarely found among the treated tissues leading the increasingly accepted conclusion that instead of directly engrafting the therapeutic cells were exerting beneficial effects through soluble mediators including EVs (248, 249). Many reports have shown by now that EVs derived from immune or pluripotent stem cells can be used for therapeutics in various diseases including acute kidney failure (116, 250-254), neurological disorders such as ischemic stroke and multiple sclerosis (255-260), lung (261, 262) and liver injury (117, 263, 264), cardiovascular conditions such as myocardial ischemia (112, 265, 266), and graft versus host disease (GVHD) (267).

2.1.4 Extracellular vesicle isolation and characterization techniques

In this section the commonly used methods for EV isolation and characterization are summarized with emphasis on their recommended applications and some of their advantages and disadvantages over other used methods.

2.1.4.1 Extracellular vesicle isolation techniques

Currently used EV isolation techniques are summarized with their strengths and best suited applications in Table 2. Traditionally, EVs have been isolated using differential centrifugation which also includes high speed ultracentrifugation (15, 32, 268). This common technique, encountered in many studies, allows to separate size-based EV subpopulations with different centrifugation speeds and to process high volumes of sample. Several other techniques for EV isolation have also emerged to complement or replace differential centrifugation. Some techniques such as density gradient isolation (sucrose or iodixanol) are well suited to obtain EV preparations of higher purity than differential centrifugation alone (14, 41-43, 268-270). Other isolation techniques which may be either faster or less dependent on instrumentation include precipitation techniques (261, 271, 272), microfluidic devices (115, 273-277), affinity capture (37, 278-280), size-exclusion chromatography (281-284), and field-flow fractionation (285, 286). The type

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of isolation procedure best suited for a given experiment is dependent on the origin of the sample (e.g. biological fluid or cell conditioned medium), its volume, equipment availability, and the subsequent type of analysis. These different techniques for isolation of EVs are currently used due to their relative strengths. However, it is conceivable that future technical advances in isolation methods may bring about protocols and techniques that can be applicable to most type of experiments and samples with equal efficiency.

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Table 2. Comparison of available EV isolation techniques

Technique Examples	**Advantages**	**Disadvantages**	**Suggested choice for**	**Refs**
Differential centrifugation - sequential centrifugation with or without gravity size filtration	- most widely used - separates EV subpopulations - processes large sample volume (20~200 ml)	- >3 hrs - requires ultracentrifuge	- large sample volumes (e.g. conditioned cell media) - no prior knowledge of EV markers - large scale EV isolation	(268)
Density gradient separation - sucrose density gradient - iodixanol (Optiprep™) density gradient - PureExo exosome isolation kit™	- high EV purity	- >10hrs - requires ultracentrifuge - low yields - small sample volumes (0.5-3 ml) - doesn't fractionate EV subpopulations as they share density	- purification of EV isolates - small sample volume	(14, 41-43, 268-270)
Precipitation - polyethylene glycol - acetate - ammonium sulphate - Total Exosome Isolation™ - Exoquick™ - Exo-spin™	- commercial kits available - fast - no specialized equipment - simple operation	- low purity (co-precipitation of non-vesicular material) - possible interference of precipitation reagents with downstream applications	- small volume (e.g. limited biological samples) - fast processing time	(261, 271, 272)
Microfluidic Devices - sieving - trapping - immunological separation - nanoshearing	- simple operation - single-step isolation - on-chip EV characterization - easy standardization and higher reproducibility	- currently not readily available - small sample volume (<0.5 ml) - to date has only been applied to EXOs and MVs	- small volume (e.g. limited biological samples)	(115, 273-277)

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Table 2 (Continued). Comparison of available EV isolation

Technique Examples	**Advantages**	**Disadvantages**	**Suggested choice for**	**Refs**
Affinity Capture - antibody coated magnetic beads - antibody coated latex beads - heparin affinity	- relatively high EV purity - some commercial kits available - simple operation	- biased by the choice of affinity reagent (only EVs with the corresponding ligand will be purified) - 0.5–3 ml sample volume - >2 hrs - difficulty in recovering EVs after capture for other applications	- small volume (e.g. limited biological samples) with known EV markers	(37, 278-280)
Size-Exclusion Chromatography - Sepharose gel - Sephadex gel - Bio-gel A™ - Izon qEV™ column	- ~15 mins - inexpensive - no specialized equipment required - simple operation	- 0.5–1.5 ml sample volume - doesn't fractionate EV subpopulations as they are all excluded from the gels - further dilutes EVs from the sample	- purification of EV isolates - small volume (e.g. limited biological samples) - no need for prior assumption of EV markers - fast processing time	(281-284)
Field-flow fractionation - flow field-flow - asymmetrical flow field-flow	- separates EVs with lower applied forces than used with centrifugation - achieves good size based separation of small EVs	- not readily available - mostly custom made - 1–2 ml sample volume - to date has only been applied to EXOs and MVs	- small volume (e.g. limited biological samples) - size-based separation of EV subpopulations	(285, 286)

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2.1.4.2 Extracellular vesicle characterization techniques

Similarly to EV isolation techniques, there is also a wide diversity of detection and characterization techniques currently available for EV studies. Two common techniques used for molecular characterization of EVs are Western blotting (14, 268) and flow cytometry (268, 287-289). Western blotting is routinely used for bulk molecular characterization of all EVs present in an isolate. After initial setup and bead or liposome based gating for EV detection, fluorescent flow cytometry allows for the molecular characterization of larger sized EVs such as MVs (100–1000 nm) and APOs (≥1 μm). Furthermore, flow cytometry enables bulk molecular characterization of bead-bound exosomes (EXOs, 50–100 nm). The coupling of EXOs to beads for flow cytometry is necessitated since the sizes of EXOs fall below the limit of detection of most instruments. EVs may be bound to beads with antibodies against specific markers or by unspecific adsorption of vesicular molecules to chemically modified surfaces such as sulfate aldehyde. Using antibodies for capture of EVs unto beads can result in highly specific binding and limited adsorption of other non-vesicular proteins, but this approach may also bias the measurement since EVs lacking a specific marker will be excluded. On the other hand, unspecific adsorption to chemically modified surfaces does not bias the measurements towards only those vesicles bearing a specific marker but other contaminating non-vesicular proteins may also bind. High resolution flow cytometry proves capable of circumventing the need for bead coupling allowing for direct molecular characterization of individual EXOs by employing optimized setups with improved fluidics, lasers, and detectors.

To assess EV particle size and concentration, tunable resistive pulse sensing (289, 290), nanoparticle tracking analysis (282, 289, 291), and dynamic light scattering (266, 282, 286, 292) may be used. For fast quantification of EVs, protein (268) content can be measured. Additionally, several microscopy methods are routinely used for EV size and morphology determination including transmission electron microscopy, scanning electron microscopy, and cryo-electron microscopy (29, 32, 268, 293) as well as atomic force microscopy (292, 294). Many new studies are also showing the applicability of label-free techniques such as grating coupled interferometry, surface plasmon resonance, as well as Raman, infrared, and electrochemical impedance spectroscopy for characterization EVs (295-299). Lastly, different “omics” techniques such as proteomics, lipidomics, and genomics among others have been applied to determine the precise

molecular composition of EVs (15, 33-40, 300, 301). All of these techniques are summarized in Table 3.

The International Society for Extracellular Vesicles (ISEV) has outlined minimal experimental requirement guidelines for the characterization of EV preparations that may be used for reference when deciding which characterization techniques to use (302). Some of the minimal experimental requirements include characterizing EV size ranges and if possible also morphology by at least 2 different techniques as well as showing the presence of various EV associated proteins in the isolates as well as the absence of proteins not expected to be found associated with EVs. As long as these guidelines are met, the best characterization technique for EV analysis is dependent on the particular experimental aims and instrument availability. Furthermore, as long as appropriate controls such as EV-depleted negative controls or detergent lysis are used to exclude the interference of non-vesicular components of the sample, the type of method used for characterization should not have a major impact on EV characterization and the biological functions observed.

Table 3. Comparison of available EV characterization techniques

Technique Examples	**Advantages**	**Disadvantages**	**Suggested choice for**	**Refs**
Flow Cytometry - scattering/ fluorescence flow cytometry - impedance flow cytometry -high resolution flow cytometry - imaging flow cytometry	- fast phenotyping of large number of EVs - may be used for determination of EV concentration in combination with counting beads -compatible with differential detergent lysis	- except for high resolution flow cytometry, it cannot detect EXOs without binding to larger beads - substantial initial setup and gating required to analyze EVs - swarm detection may hinder measurements	- individual characterization of EV molecular markers (also for EXOs with high resolution flow cytometry)	(268, 287-289, 292)
Western Blotting	- well established for EV marker determination	- time consuming - high amounts of EVs needed	- bulk characterization of EV molecular markers	(268)

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Table 3 (Continued). Comparison of available EV characterization techniques

Technique Examples	**Advantages**	**Disadvantages**	**Suggested choice for**	**Refs**
Tunable Resistive Pulse Sensing - Izon qNano	- ~50 µl sample volume - resolves multimodal particle distributions - compatible with differential detergent lysis	- requires substantial user training and experience	- determination of EV size and concentration	(289, 290)
Nanoparticle Tracking Analysis - Malvern Nanosight - Zetaview®	- fast (5~15 mins) - simple operation	- requires operational skills for the adjustment all software settings	- determination of EV size and concentration	(282, 289, 291)
Dynamic Light Scattering	- ~50 µl sample volume	- not optimal for polydisperse samples	- determination of EV size and relative concentrations	(266, 282, 286, 292)
Protein quantification - bicinchoninic acid (BCA) assay - Bradford assay - Lowry	- simple bench top assays - commercial reagent kits available - 1 hr reaction time	- prone to EV overestimation in case of protein contamination	- fast quantification of EV proteins - EV quality control when combined with lipid quantification	(268)
Electron Microscopy - transmission EM - scanning EM - cryo-EM	- visualization of EV morphology and size - identification of markers with immune-labelling techniques	- sample preparation can generate artifacts (e.g. cup shape morphology) - needs substantial user training and experience	- visualization of EV morphology and size - direct visualization of EXOs	(14, 29, 32, 268, 289, 293)

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Table 3 (Continued). Comparison of available EV characterization techniques

Technique Examples	**Advantages**	**Disadvantages**	**Suggested choice for**	**Refs**
Atomic Force Microscopy	- visualization of EV morphology and size	- needs substantial user training and experience	- visualization of EV morphology and size including EXOs	(292, 294)
Label free techniques - grating coupled interferometry - surface plasmon resonance - Raman, infrared, or electrochemical impedance spectroscopy	- determination of EV markers - determination of EV concentration - detection of EV binding - label free study of EV surface interactions	- methodology still in development for EV applications - needs substantial user training and experience	- dynamic study of molecular interaction with EVs	(295-299)
"OMICS" techniques - proteomics - lipidomics - genomics - transcriptomics - glycomics	- complete molecular characterization of EVs	- relatively high amounts of EV sample needed	- determination of EV molecular composition	(15, 33-40, 300, 301)

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3. OBJECTIVES

In the previous sections have been described many of the novel biological functions that have been discovered so far for EVs as well as their potential applications in the clinic. The field of EV research has truly emerged as one of the fastest growing areas due to all these recent findings and more scientists from other areas continue to get interested and start exploring the functions of EVs in their respective fields. However, despite this rapid growth one concern is that in regards to some biological processes it seems that different reports have found discordant roles for EVs. Therefore, it might seem puzzling whether EVs are in fact beneficial or detrimental in various biological processes in health and disease.

Some of the observed discordance in biological roles reported for EVs can be explained by the inherent differences among various subtypes of EVs simultaneously found in the body which can also be released by different cells possibly in different activation states. Furthermore, the EVs and their cargo can also undergo various post-synthetic modifications or combine with other EVs or soluble molecules which can alter their biological effects. Thus, when comparing different studies showing effects of EVs in any biological process all these inherent biological differences should be accounted for as they may explain discordant results if each report is analyzing completely different EVs.

Even if it may be that many of the discordances can be explained by biological differences among the studies EVs there are still some examples where various reports supposedly investigating the same particles do not obtain comparable results. One such example can be found with reports of EVs released during remote ischemic preconditioning (RIPC) which were observed to either reduce or to have no effect in infarct sizes (114, 303, 304). Based on this problem, we believe that in such a rapidly growing field it is also very important to have sound methodology and controls with which to characterize the material that is being studied so that researchers can easily compare their results to each other. As the field currently stands that is not always the case as many groups utilize different isolation and characterization methods and there are still no universally accepted protocols and molecular markers for EVs.

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For these reasons, the work undertaken during this Ph.D. had the following objectives.

I. Improve existing EV methodology.
II. Introduce novel assays for EV analysis.
III. Develop a workflow for the quantification of EV lipid content.
IV. Investigate the protein to lipid ratio of the different EV subpopulations.
V. Characterize the membrane lipid order of the different EV subpopulations.
VI. Establish the detergent lysis sensitivity of EVs.

By pursuing these objectives we attempt to develop methods which we believe will expand the existing toolkit for EV research and hopefully will allow researchers to better characterize the material under study.

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4. MATERIALS AND METHODS

In the following sections the methods used for EV isolation and characterization as well as for cell culturing and performing all experiments are described in detail.

4.1 Cell line cultures

Jurkat (TIB-152) human T cell lymphoma, THP-1 (TIB202) human acute monocytic leukemia, and U937 (CRL-1593.2) human histiocytic lymphoma cell lines were all obtained from ATCC (Manassas, VA). The BV-2 murine microglia cell line was a generous gift of Prof. Rosario Donato (Università degli Studi di Perugia, Perugia, Italy) (305). The MiaPaCa-2 (MiaPaCa) pancreatic cancer cells were kindly provided by Dr. Klaus Felix, (Universität Heidelberg, Heidelberg, Germany) and the MH-S murine alveolar macrophage cell line was kindly provided by Dr. Dolores Solis (Instituto de Química Física Rocasolano, Madrid, Spain). Both MiaPaCa and MH-S cell lines were originally obtained from ATCC.

All cell lines were cultured as indicated by the suppliers in RPMI-1640 or DMEM medium containing 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine, and 1% (v/v) Antibiotic Antimycotic Solution (Ab/AM) (all from Sigma-Aldrich, St Louis, MO), at 37 ^{o}C in 5% CO_2/air. The cell lines were regularly tested for Mycoplasma contamination by fluorescence microscopy using DAPI staining (Molecular Probes Life Technologies, Carlsbad, CA). Cells used for the production of EVs were first washed three times with phosphate buffered saline (PBS) and placed in FBS-free medium to avoid contamination of the preparations with EVs present in FBS. Cells were grown in this condition for 24 hrs at concentrations of 0.3–1 x 10^6/mL depending on the optimal density indicated by the supplier of each cell line. Cell viability was always monitored by flow cytometry to be >90–95% as confirmed by staining with Annexin V FITC and propidium iodide (both from BD Biosciences, Franklin Lakes, NJ). Prior to isolation, EV-containing conditioned FBS-free media was first submitted to 300 *g* centrifugation for 10 mins followed by gravity driven filtration through a 5 μm filter (Millipore, Billerica, MA) to deplete cells.

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4.2 Human blood, platelet, and red blood cell concentrate collection and processing

The use of human blood samples was approved by the Scientific Ethics Committee of the Hungarian Health Scientific Council (ETT TUKEB), and human blood donors provided written informed consent.

For EV isolation from platelets, acid-citrate dextrose (ACD) anti-coagulated plasma samples were centrifuged at 400 *g* for 15mins at room temperature (RT) to remove most of the blood cells, and then the platelet containing supernatant was diluted 4x in TRIS-citrate buffer. Next, the platelet-rich plasma was again centrifuged at 600 *g* for 20 mins at RT, and the pellet was resuspended in 3 mL PBS. Platelets were then incubated at 37 ^{o}C for an hour in the presence of 10 μM adenosine diphosphate (ADP) (Sigma-Aldrich). Next, 4 mL of PBS was added to reduce the viscosity of the samples, and centrifuged at 800 *g* for 20 mins at RT. For EV isolation from red blood cell concentrates, samples were first diluted 2x in PBS to reduce the viscosity.

To obtain EVs from human blood plasma, we collected 30–40 mL of blood from healthy adult donors in ACD-A tubes (Greiner Bio-One, Kremsmünster, Austria) to prevent ex vivo vesiculation related to blood sample handling and storage (306). The samples were processed following the International Society on Thrombosis and Hemostasis (ISTH) protocol for preparation of platelet-free plasma (PFP) (307, 308). Briefly, the ACD anti-coagulated blood was centrifuged twice at 2,500 *g* for 15 mins at RT to remove platelets using a HermLe Z206A bench top centrifuge (HermLe Labortechnik GmbH, Wehingen, Germany).

4.3 Extracellular vesicle isolation

Three different EV subpopulations were isolated including APOs, MVs, and EXOs by the combination of gravity driven filtration and differential centrifugation. Initially cells were removed by centrifugation at 300 *g* for 10 mins, and then the supernatant was filtered by gravity through a 5 μm filter (Millipore) and submitted to a 2,000 *g* centrifugation for 10 mins at RT to pellet APOs. The supernatant was next filtered by gravity through a 0.8 μm filter (Millipore) and centrifuged at 12,600 *g* for 30 mins at RT to pellet MVs. Finally, the supernatant was ultracentrifuged in an Optima MAX-XP

bench top ultracentrifuge with MLA-55 rotor (Beckman Coulter Inc, Brea, CA) at 100,000 *g* for 70 mins at 4 °C to pellet EXOs. To remove co-isolated materials and attain higher purity, each EV pellet was resuspended once in PBS and recentrifuged under the same conditions that were originally used for pelleting. Up to five consecutive washes were performed in the case of blood derived EVs. Notably, cells used for the production of EVs including APOs were not specifically induced for apoptosis. However, due to the inherent low level of apoptosis found in different cells and tissues APOs can be detected and isolated from most biofluids and cell culture supernatants even when no apoptosis is induced, as well as in physiological conditions. A simplified schematic representation of the EV isolation workflow is shown in the below Figure 3.

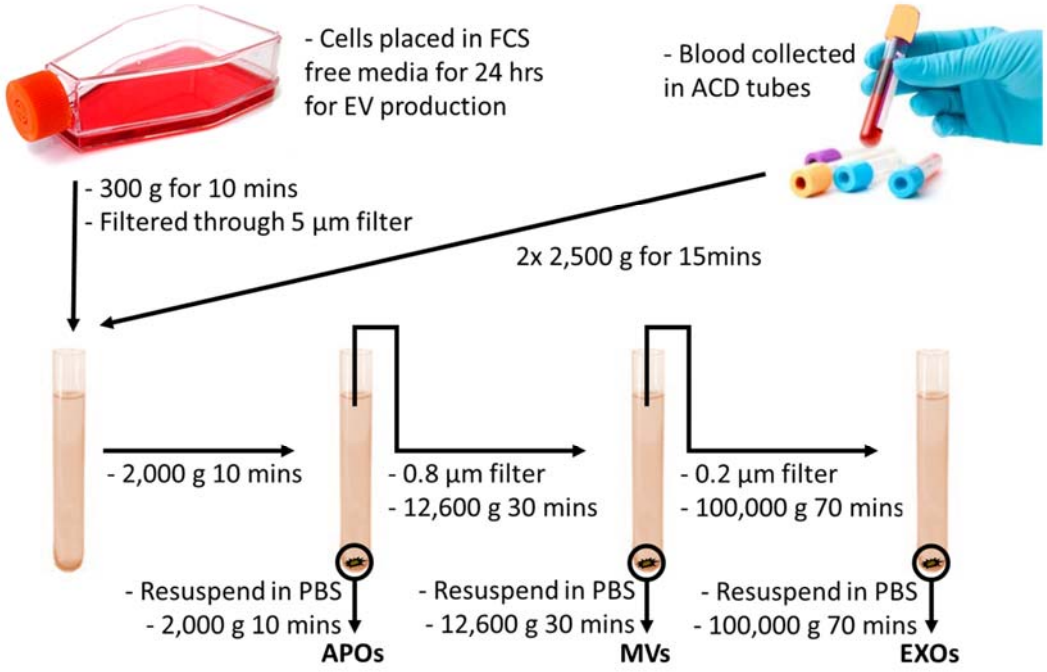

Figure 3. Schematic summary of the EV isolation workflow

4.4 Extracellular vesicle concentration and size

EV preparations or EV-containing cell-depleted conditioned media were submitted to tunable resistive pulse sensing (TRPS) analysis using a qNano instrument (IZON Science, New Zealand). At least 500 particles were counted except when no EVs were present or lysis of EVs was observed, in which case one minute recording time was used regardless of particle counts. NP100, NP400, NP800 and NP2000 nanopore

membranes stretched between 43–47 mm were used. Voltage was set in between 0.04–0.7 V to achieve a stable current between 115–145 nA. Particle-size histograms were recorded when root mean square noise was below 12 pA, and particle rate in time was linear using calibration beads CPC100B, CPC400E, CPC800D and CPC2000D (mode diameters 110 nm, 340 nm, 740 nm and 1900 nm, respectively) (all from IZON). For detergent lysis experiments shown in Section 5.2, the calibration beads were also measured at every detergent concentration step at least twice in order to detect any possible differences in their modal size and particle rate possibly due to the presence of detergent micelles. For none of the detergents used in Section 5.2 this was found to be the case.

4.5 Transmission electron microscopy of extracellular vesicle preparations

In order to characterize the morphology and size of the different EV preparations, pellets were fixed with 4% paraformaldehyde in 0.01M PBS for 60mins at RT. Following washing with PBS, the preparations were postfixed in 1% OsO_4 (Taab, Aldermaston, Berks, UK) for 30 mins. After rinsing the intact fixed pellets within the centrifugation tubes with distilled water, the pellets were dehydrated in graded ethanol, including block staining with 1% uranyl-acetate in 50% ethanol for 30 mins, and were subsequently embedded in Taab 812 (Taab). Overnight polymerization of samples at 60 °C was followed by sectioning, and the ultrathin sections were analyzed using a Hitachi 7100 electron microscope (Hitachi Ltd., Japan) equipped with a Megaview II (lower resolution, Soft Imaging System, Germany) digital camera.

4.6 Flow cytometry of cells and extracellular vesicles

For flow cytometric measurements, EV preparations were incubated for 30mins at RT in the dark with different fluorochrome conjugated antibodies and affinity reagents. These included cholera toxin (CTX) subunit B-Alexa Fluor647 and di-4-ANEPPDHQ which were purchased from Life Technologies, an anti-cholesterol antibody (AC8) which was produced at Eötvös Loránd University (Budapest, Hungary) (309) and conjugated to CF488A fluorophore according to the Mix-n-Stain protocol (Biotium, Sigma-Aldrich) as

well as annexin V FITC or APC conjugated (in annexin binding buffer from BD Biosciences), anti-CD9 FITC and anti-CD63 PE-conjugated antibodies (all from BD Biosciences). Isotype controls (all from BD Biosciences) were used for samples stained with fluorochrome-conjugated antibodies, whereas autofluorescence was detected in the absence of either annexin V or CTX. To verify the vesicular nature of MVs and APOs, and to exclude the presence of antibody aggregates, we added Triton X-100 to 0.05% final concentration to the samples. This step resulted in prompt disappearance of fluorescent event counts from the MV and APO gates suggesting the presence of membranous structures within these gates. EXOs were resuspended in a total volume 50 µL of PBS, and aliquots of 5µL were incubated with 5 µL of 4 µm aldehyde/sulfate latex beads (Life Technologies), followed by an incubation of 5 mins at RT. Then 20 µL of PBS was added, and incubation was continued for another 15 mins at RT. Thirty µL of 2% bovine serum albumin (BSA) in PBS was added and samples were blocked for 2 hrs at RT. Then the Eppendorf tubes were filled with PBS, and were centrifuged at 2,700 *g* for 3 mins at RT. The supernatant was discarded, and the pellet was resuspended in 200 µL of 100 mM glycine in PBS, and was incubated for 30 mins at RT. The samples were washed with PBS, and centrifuged at 2,700 *g* for 3 mins at RT. Then the supernatant was discarded, and the pellet was resuspended in 100 µL of PBS for staining as described above. EV preparations were analyzed by using FACSAria III and FACSCalibur flow cytometers (both from BD Biosciences). Events were counted for 1min from equal sample volumes at medium flow rate and data were analyzed by FlowJo software (Treestar, Ashland, USA).

In case of APOs and MVs, instrument settings and gates were set as recommended by the International Society on Thrombosis and Haemostasis (ISTH) (307, 308) using Megamix-Plus SSC beads (BioCytex, Marseille, France) and further optimized with 1 µm Silica Beads Fluo-Green (Kisker, Steinfurt, Germany). Megamix-Plus SSC beads are a mix of fluorescent polystyrene beads of different diameters covering the MV size range (0.16 µm, 0.20 µm, 0.24 µm, and 0.5 µm). Since the forward scatter (FSC) of most flow cytometers is not sensitive enough to differentiate within the size range of EVs we used these beads to calibrate the settings of the instrument to be able to resolve each of the distinct sized beads by their side scatter (SSC) (Figure 4). However, one important consideration is that side scatter is not only due to the diameter of the particles but also their refractive index and thus EV size estimation based on SSC after calibration with

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polystyrene beads alone leads to underestimation of their size. For this reason silica beads of 1µm were also utilized as their refractive index matches closer that of EVs and thus serves as a better estimate for their size. As shown in Figure 4 Megamix-Plus SSC beads and Silica beads of known sizes were measured separately and in combination to calibrate the instrument settings and create a gate for EVs.

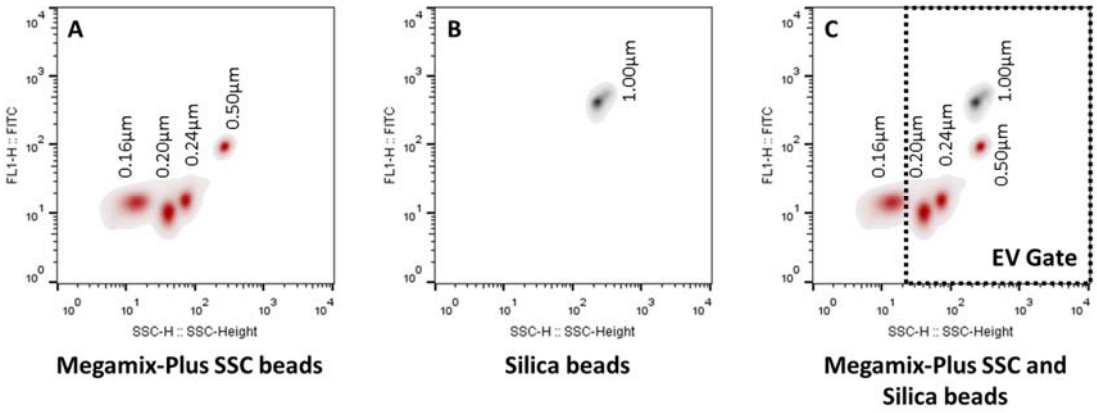

Figure 4. Bead based calibration of instrument settings and gating for flow cytometric measurement of EVs

4.7 Protein and lipid determination of extracellular vesicle preparations

Total protein content of EV preparations was determined using the commercially available bicinchoninic acid assay (BCA), Micro BCA Protein Assay Reagent Kit (Thermo Scientific, Rockford, IL), following the manufacturer's specifications and using bovine serum albumin (BSA) (Thermo Scientific) as a standard.

To measure total lipid content of EVs, the colorimetric reaction of sulfuric acid and phosphovanilin with lipids (310) was adapted for use in a 96 well plate format sulfophosphovanilin (SPV) assay. Lipid standard solutions (2 µg/µL) were prepared from menhaden fish oil and cholesterol in chloroform (all from Sigma-Aldrich). Different volumes of the lipid standard solutions in chloroform were pipetted into Eppendorf tubes to result in 0 to 140 µg of lipid per tube, and chloroform was added to each tube up to a final volume of 70 µL. Chloroform (70 µL) was also pipetted onto either dry EV pellets or empty Eppendorf tubes to be used later for EV containing suspensions in PBS. The chloroform was then evaporated by incubating the tubes at 90 ^{o}C for 10 mins in a heater block (Techne DB-2D, Bibby Scientific Ltd, Staffordshire, UK). All steps involving chloroform handling were performed under a fume hood with lids open for all tubes. For

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EV suspensions, instead of pellets, 50 µL (with at least 10–30 µg/mL of protein depending on the EV type) was added to the empty chloroform-pretreated Eppendorf tubes and 50µL of PBS was also added to each tube containing the lipid standards. The pre-treatment with equal volumes of chloroform, and PBS in the case of EV suspensions, ensured the same background color development in all Eppendorf tubes. As a following step, 250 µL of 96% sulfuric acid was added to the tubes followed by incubation with open lids at 90 ^{o}C for 20 min in a heater block. Next, 220 µL of samples and standards in sulfuric acid were transferred into a 96-well polystyrene plate (Nunc, Thermo Scientific), and allowed to cool down to RT. Finally, 110 µL of 0.2 mg/mL vanillin in 17% phosphoric acid (both from Sigma-Aldrich) was added to each well, and the plate was incubated for 10 mins at RT. Absorbance was measured at 540 nm by an MS Reader (Multiskan MS; Labsystems, Helsinki, Finland). Figure 5 shows a simplified schematic summary of the total lipid determination protocol.

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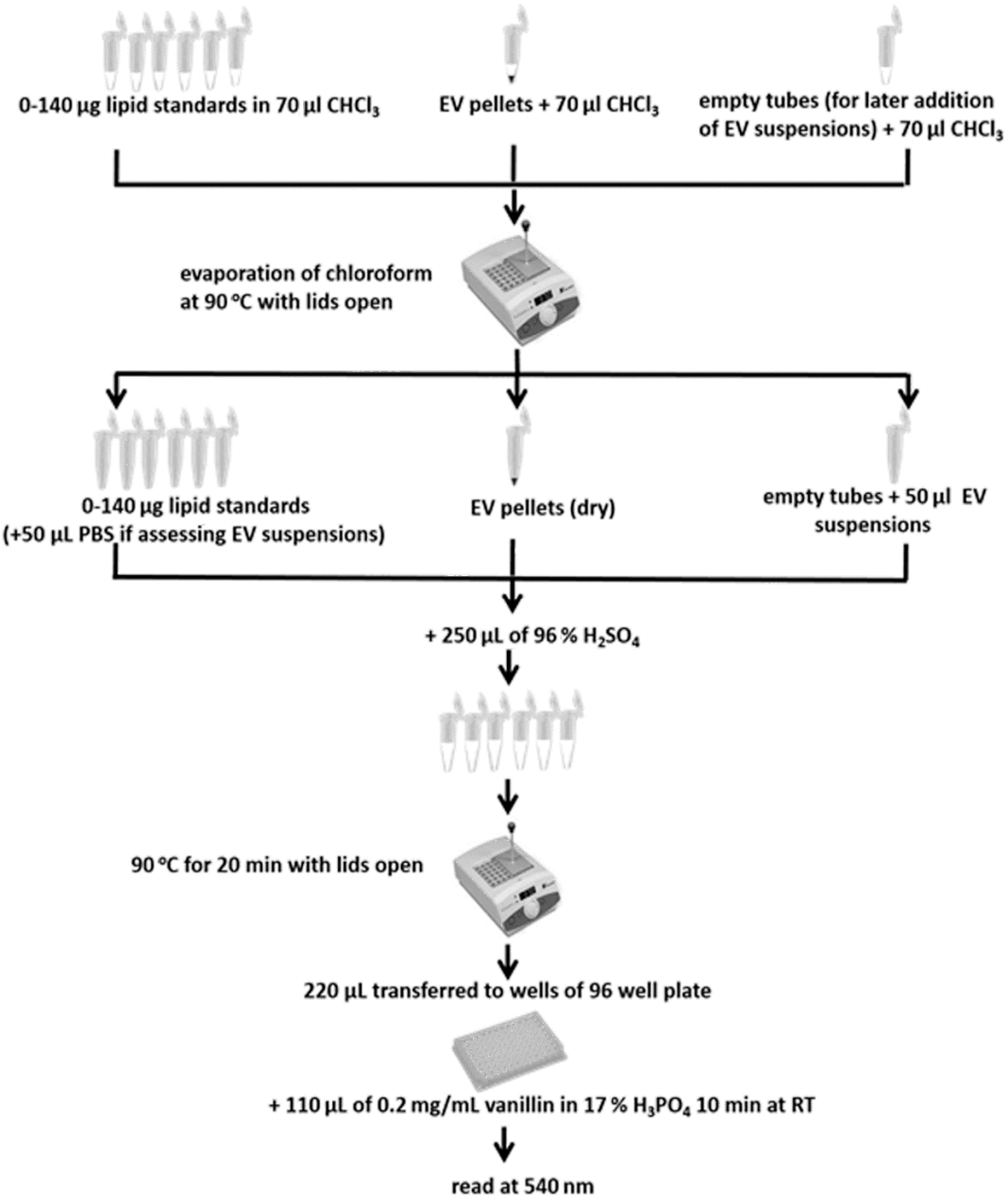

Figure 5. Schematic summary of the total lipid determination protocol for EV preparations

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4.8 Membrane lipid bilayer order of extracellular vesicle preparations

In order to investigate the membrane lipid bilayer order of EV preparations, EVs were stained with the membrane probe Di-4-ANEPPDHQ (Life Technologies) at 5 μM final concentration for 30 mins at 37 ^{o}C as described previously (311). EV subpopulations, immobilized on poly-L-lysine (Sigma-Aldrich) coated cover slips, were analyzed by confocal microscopy (IX81 inverted microscope based Fluoview500 laser scanning confocal system and software; Olympus Europe, Hamburg, Germany) at 60x magnification. Suspensions of APOs, MVs as well as 4-μm latex bead-bound EXOs were also assessed by flow cytometry. The Di-4-ANEPPDHQ probe exhibits a 60 nm spectral shift between the liquid-ordered and liquid-disordered phases. Ratiometric measurement of the fluorescence intensity by confocal microscopy was performed in the 560–600 nm and the >660 nm emission channels, defined by appropriate band pass and cut off filters, respectively. Background-corrected fluorescence intensities of EV samples in each channel were calculated by ImageJ software (http://imagej.nih.gov/ij/). General polarization (GP) values were determined as follows: GP = (I560–600– I>660)/(I560–600+I>660). In flow cytometry, PE (585±21 nm) and PE-Texas Red (616±12 nm) filters were used to define the corresponding two emission channels and GP values were calculated in similar way.

4.9 Detergent lysis of extracellular vesicles

Sodium dodecyl sulphate (SDS), Triton X-100, Tween 20 and sodium deoxycholate were all purchased from Sigma-Aldrich. Detergents were added to EV-containing samples to different final concentrations in the range of 0.005–15% (w/v for SDS and deoxycholate and v/v for Triton X-100 and Tween 20), vortexed for 30 seconds, and analyzed by TRPS or flow cytometry. All steps of detergent lysis were carried out at RT. These conditions were established based on preliminary experiments using different incubation times of EVs with detergents and showing essentially no difference in lysis efficiency with increasing time.

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4.10 Statistical analyses

For data analysis GraphPad Prism (GraphPad Software, La Jolla, USA) was used. For comparison of different parameters of EV subpopulations, Wilcoxon matched-pairs signed rank test was used. For membrane lipid order data obtained by confocal microscopy, 1-way ANOVA was used followed by Dunnetts post-hoc test. For analysis of separate cell line-derived EV counts two-way ANOVA was used. For the analysis of combined EVs counts for all cells and all detergents, one-way ANOVA was used, and for parameters not showing normal distribution, Kruskal-Wallis non-parametric test was applied. P values <0.05 were considered statistically significant (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

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5. RESULTS

5.1 Improved characterization of extracellular vesicle preparations based on protein to lipid ratios and lipid properties

Given the important need for better tools for EV research, the goal of this first study was to find improved parameters to characterize EV preparations. Here a simple 96 well plate-based total lipid assay was introduced for the determination of lipid content as well as protein to lipid ratios of EV preparations from various myeloid and lymphoid cell lines as well as blood plasma. These preparations included APOs, MVs, and EXOs isolated by differential centrifugation and gravity filtration. Furthermore, membrane lipid bilayer order of EV subpopulations was measured with the Di-4-ANEPPDHQ lipid probe and their lipid composition was assessed with affinity reagents to clustered cholesterol and ganglioside GM1. Different protein to lipid ratios were found to be characteristic for the investigated EV subpopulations and these were substantially altered in the case of vesicular damage or protein contamination. Spectral ratiometric imaging and flow cytometric analysis also revealed marked differences between the various vesicle populations in regards to their lipid order and their clustered membrane cholesterol and GM1 content. Thus, this study introduced for the first time a simple and readily available lipid assay to complement the widely used protein assays in order to better characterize EV preparations. Besides differentiating extracellular vesicle subpopulations, the novel parameters introduced in this work (protein to lipid ratio, lipid bilayer order, and lipid composition), may prove useful for quality control of EV related basic and clinical studies.

5.1.1 Optimization of the sulfophosphovanilin total lipid assay for EV studies

Given the unmet need in the EV research field for a simple and inexpensive lipid assay with the sensitivity to detect EVs, we first attempted to adapt the colorimetric SPV assay for total lipid determination of EV subpopulations (310, 312). One very important limitation when working with EVs is that typically the yields are much smaller than most other biological samples. Therefore, prior to any further experimentation it was crucial to first assess whether the SPV reaction had the necessary sensitivity to detect the few micrograms of lipids commonly found in most EV preparations. Thus we evaluated

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whether typical EV preparations could be detected in a 96 well plate adaptation of the SPV reaction using menhaden fish oil as a lipid standard and found that under these optimized conditions the assay sensitivity is adequate for EV studies measuring samples containing as little as 0.5μg EVs either in dry pellet or in up to 50μL volume.

5.1.2 Interference of different compounds with lipid and protein assays

Once we established that the SPV assay had the necessary sensitivity to measure EV preparations we next determined the possible interference with the SPV and BCA assays of different compounds that may be commonly found in cell lines supernatants or biofluids from which EVs are isolated (Figure 6). In addition to these common molecules, interference of lipids with the BCA assay and proteins with the SPV assay was also investigated (Figure 6). In order to assess the possible interference of different substances with the SPV and BCA assays, standard curves of 0μg to 20μg of tryptophan, leucine, serine, BSA, menhaden fish oil, low molecular weight heparin, glucose (all from Sigma-Aldrich), as well as human U937 cell-derived total DNA and RNA were subjected to lipid and protein determination as described above. Since interference was found only for tryptophan in the case of BCA and glucose in the case of both assays we could determine that these assays can be suitable for measurement of EV samples containing both proteins and lipids as well as small amounts of other contaminating compounds.

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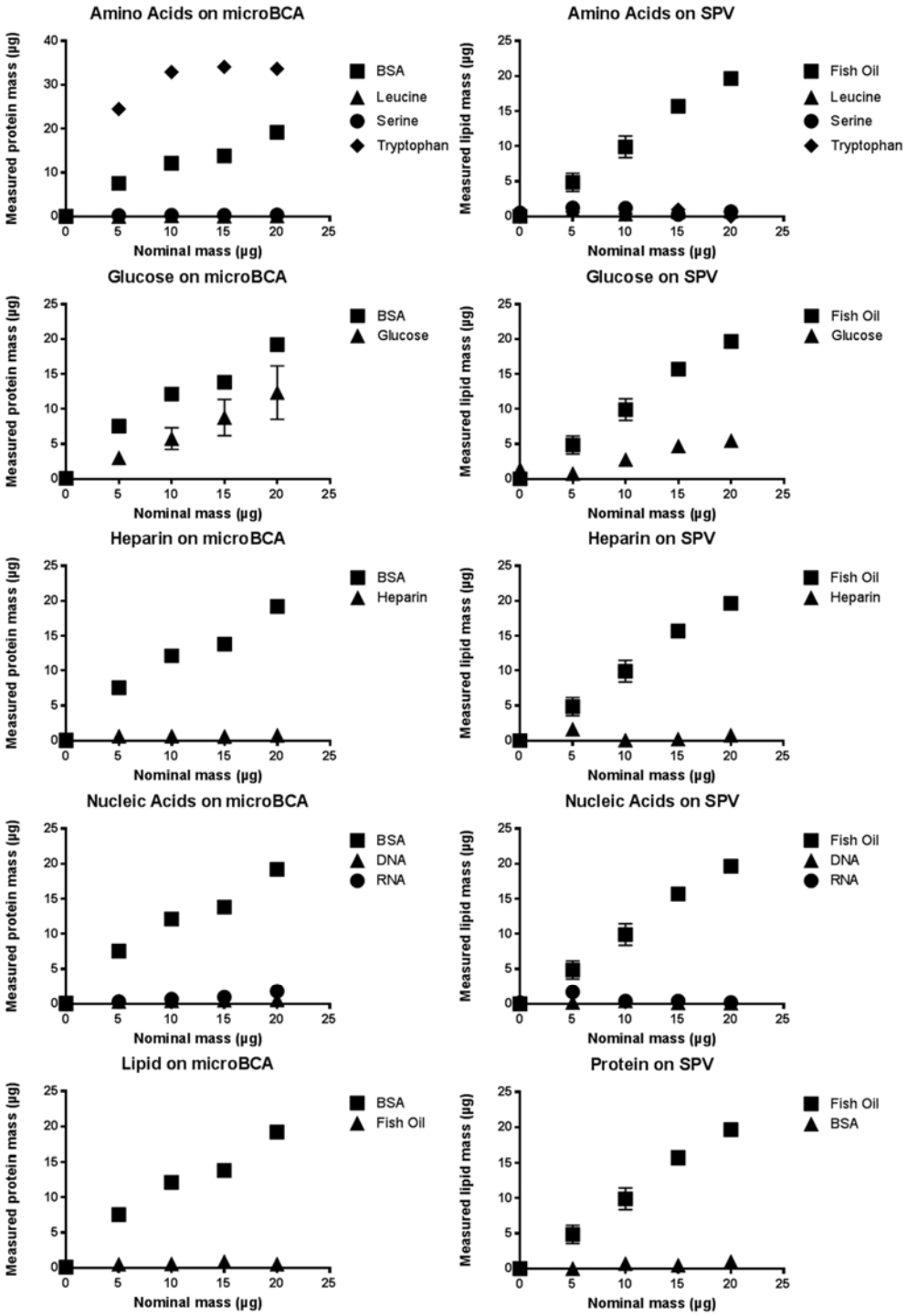

Figure 6. Interference of various compounds with BCA and SPV assays

Interference of glucose with the SPV assay while the BCA assay reacts with both tryptophan and glucose. All other molecules did not interfere. Data of n=3 independent experiments are shown. The scale of the Y axis the amino acid interference on the BCA assay is different from the rest.

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5.1.3 Accuracy and variability of lipid and protein assays

After observing that the SPV assay had adequate sensitivity for EV research and lesser interference by other compounds than the BCA assay we next assessed how these two assays compared in regards to accuracy and variability. To this end, equal concentrations of menhaden fish oil and BSA (both from Sigma-Aldrich) were subjected to SPV and BCA assays respectively in 6 parallel technical repeats of 0, 10, 20, 30, 40, 50, 100, and 200 μg/mL concentrations in three independent experiments. Accuracy was assessed by calculating the percentage error (% Error) as follows: (|measured value - nominal value|/nominal value) x 100. Variability was assessed by determining the intra-assay variation calculated as percentage coefficient variation (% CV) using the following formula: standard deviation (SD) of measured values/measured values x 100. The results are shown in Figure 7.

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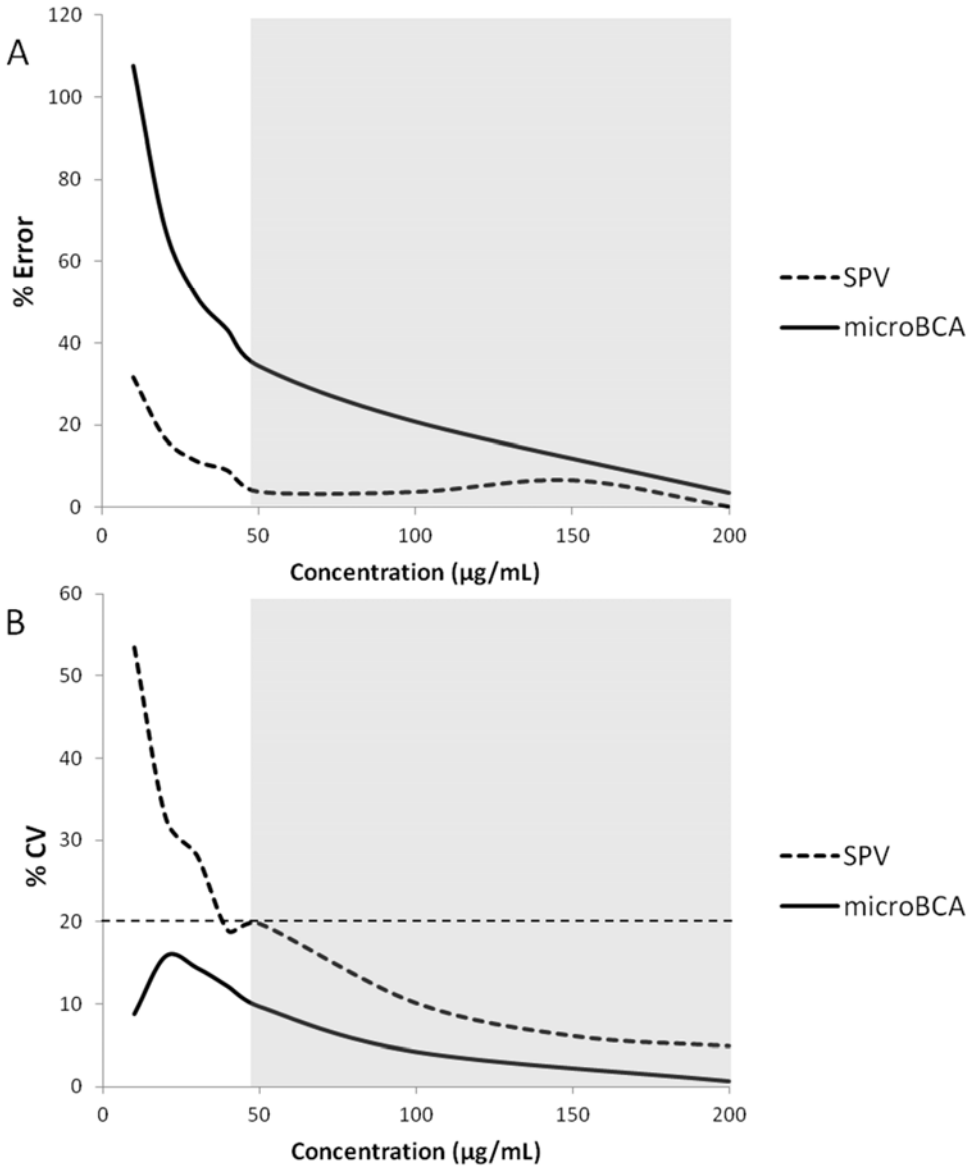

Figure 7. Working concentration ranges and coefficients of variations of the BCA and SPV assays

Intra-assay % Error is shown for the two assays reflecting higher accuracy of SPV. 7B shows a precision profile as defined earlier (313) indicating the working concentration range by % CV plotted against concentration for both SPV and BCA assays. The suggested working range (gray box) is defined as the range of concentrations for which the % CV < 20%. The scales of the Y axis are not the same. Inter-assay variation was also determined as follows: average of 10 µg/mL (lowest) and 200 µg/mL (highest) % CVs, and were found to be 11.4 and 3.1 for the SPV and BCA assays, respectively. n = 3.

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5.1.4 Characterization of different EV subpopulations

Separate EV subpopulations (APOs, MVs, and EXOs) were isolated from the supernatants of the cell lines THP-1, BV-2, MH-S, and Jurkat as described in earlier work form our research group and others (32, 268, 293). The vesicles from the BV-2 cell line were characterized by tunable resistive pulse sensing (TRPS) and transmission electron microscopy. EVs from the three subpopulations were found to have different size and morphology (Figure 8). The APO preparation predominantly contained vesicles between 1250–2500 nm in diameter (1627.05 nm mode diameter). In contrast, most vesicles in the MV preparation had approximately 200 nm diameter (208.32 nm mode diameter). The most uniform population was the one of EXOs with a peak size around 100 nm (98.76 nm mode diameter). In electron microphotographs some of the APOs contained highly electron dense intravesicular content presumably corresponding to fragmented DNA. MVs were somewhat variable in shape, size and electron density, whereas EXOs had uniform size and cup shaped morphology (Figure 8).

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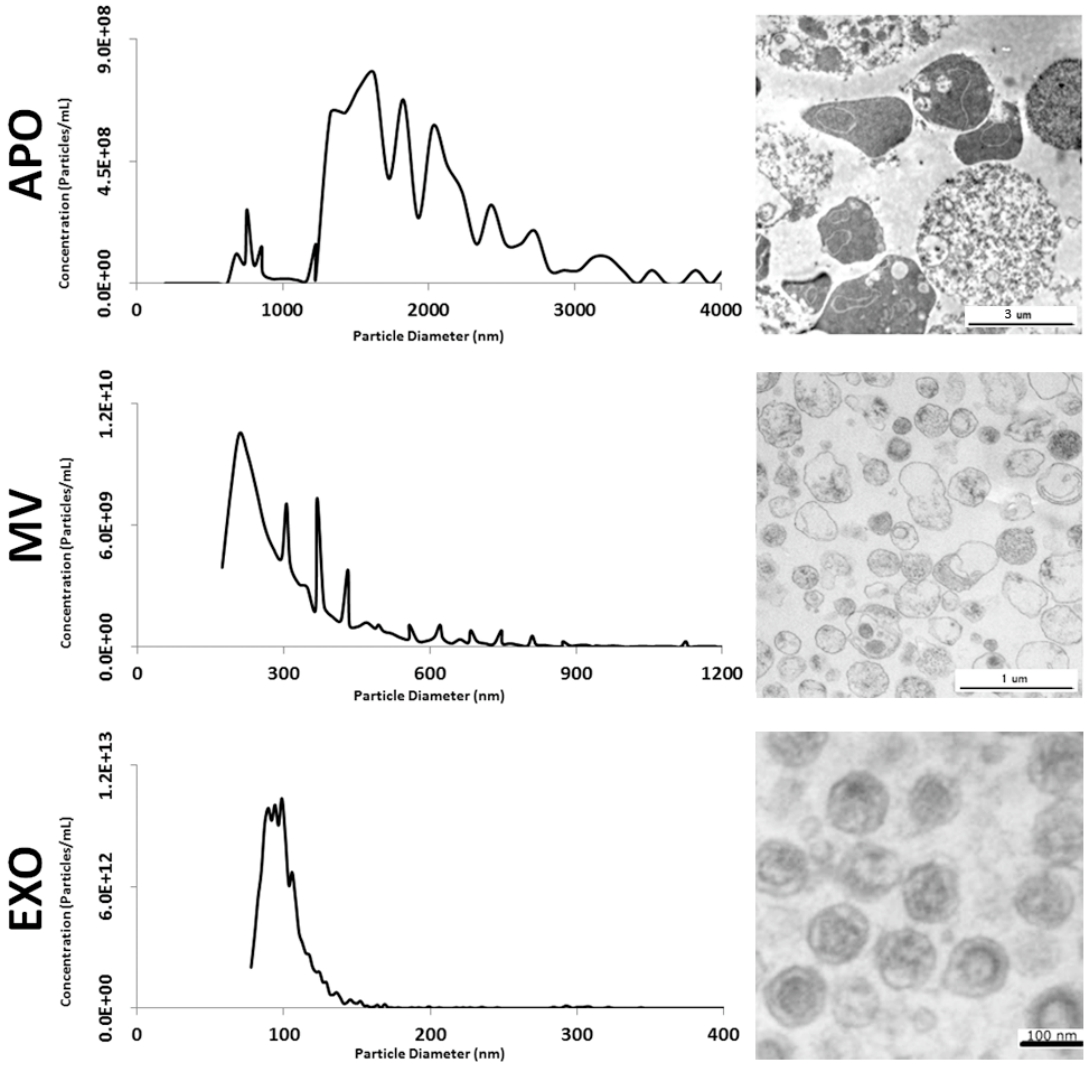

Figure 8. Characterization of EV subpopulation size and morphology

Apoptotic bodies (APO), microvesicles (MV) and exosomes (EXO) isolated from conditioned tissue culture supernatant of BV-2 cells. Size distributions of the different subpopulations were assessed by tunable resistive pulse sensing (qNano) using three different membranes (NP200, NP400, and NP2000). Obtained particle concentrations were merged into single histograms for each EV subpopulation (left panels). Electron microscopic images of respective EV subpopulation pellets are shown (right panels).

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EVs were further characterized with flow cytometry by their staining with fluorochrome labeled annexin V, antibodies, and cholera toxin (Figure 9). Annexin V staining was found positive for all three EV subpopulations reflecting phosphatidylserine externalization and also revealed high and low binding subpopulations within the APO fraction. While APOs exhibited minimal staining with anti-CD9, both MVs and EXOs showed strong positivity. CD63 was found to be uniformly present on both MVs and EXOs, whilst APOs showed heterogeneity in their staining (Figure 9). The AC8 antibody that recognizes clustered cholesterol found preferentially in lipid rafts showed relatively low binding to all EV subpopulations with EXOs showing the strongest staining (Figure 9). When staining EV subpopulations with fluorochrome-conjugated cholera toxin B (CTX) known to bind to ganglioside GM1 enriched in lipid rafts (314), we found the strongest binding to MVs while the weakest one to EXOs. Interestingly, APOs exhibited two different CTX binding subpopulations (Figure 9).

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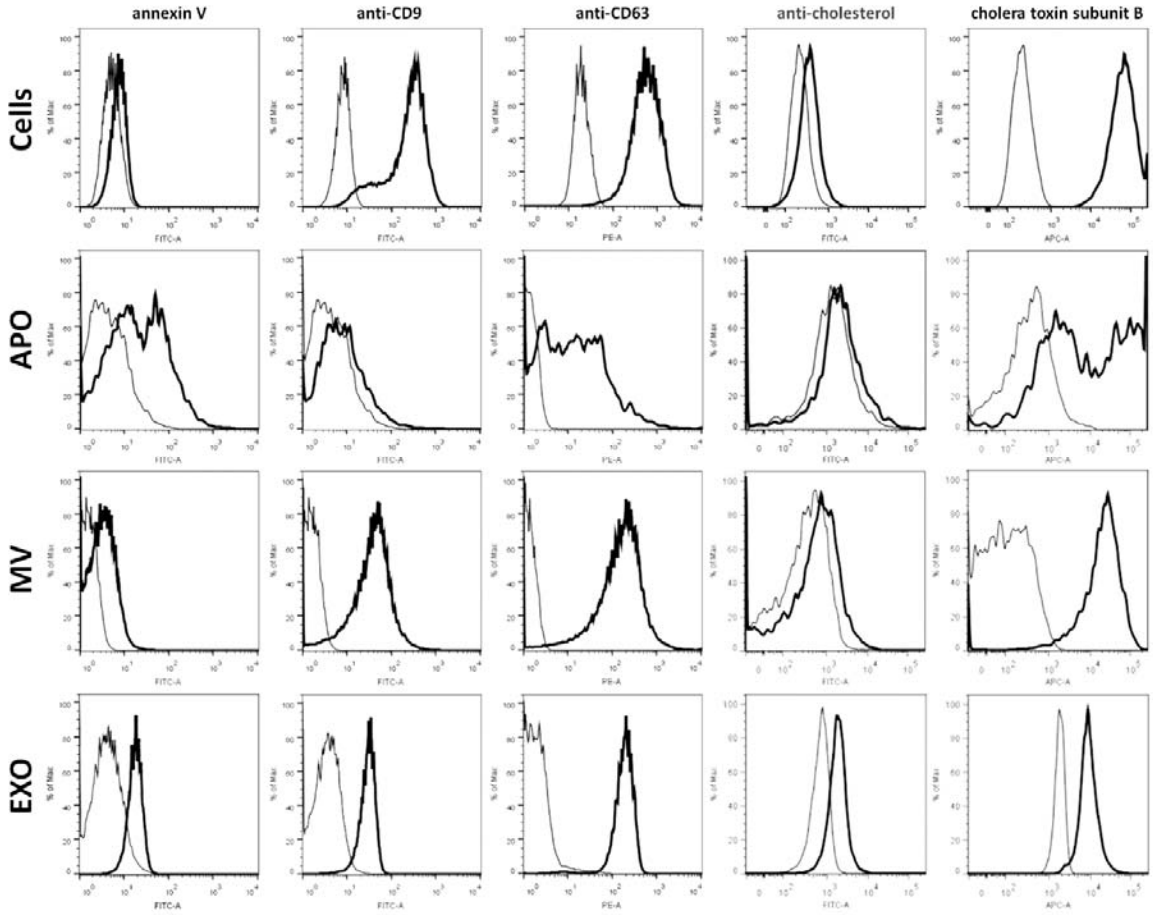

Figure 9. Flow cytometric characterization of cells and EV subpopulations

Isolated EVs and secreting cells were measured by flow cytometry either directly (MVs and APOs) or after coupling to latex beads (EXOs) using annexin V FITC, as well as anti-CD9 FITC, anti-CD63 PE and anti-cholesterol CF488-conjugated antibodies, and Alexa Fluor647-conjugated cholera toxin (CTX) (all marked with thick black lines), and were compared to respective negative controls (thin black lines). Isotype controls were used for samples stained with fluorochrome-conjugated antibodies, whereas autofluorescence was detected in the absence of either annexin V or CTX. Images and figures are representatives of at least three independent experiments.

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5.1.5 Characterization of protein to lipid ratios of the EV subtypes

Following the characterization of distinct EV subpopulations obtained by our isolation methods we next determined the lipid content of the EV preparations using menhaden fish oil as a lipid standard. In parallel with the total lipid determination, aliquots of the same EV preparations were also submitted to protein determination by BCA assay. Total protein to total lipid ratios of EV preparations from three different cell lines (Figure 10A-C). Protein to lipid ratios of EV subpopulations measured independently for all tested cell lines and human blood plasma were combined into Figure 10D to show that they are characteristic of the respective EV subpopulations regardless of their cell origin. APOs were consistently characterized by the highest protein to lipid ratio followed by an intermediate ratio for MVs and the lowest ratio was in all cases found for EXOs (Figure 10). As shown in Figure 10E, we found differences in the combined protein concentrations of EV subpopulations of different cellular origin with MVs showing the lowest protein concentration. Interestingly a striking elevation in lipid concentrations of the combined EXOs, was observed as compared to the combined MVs or APOs (Figure 10F).

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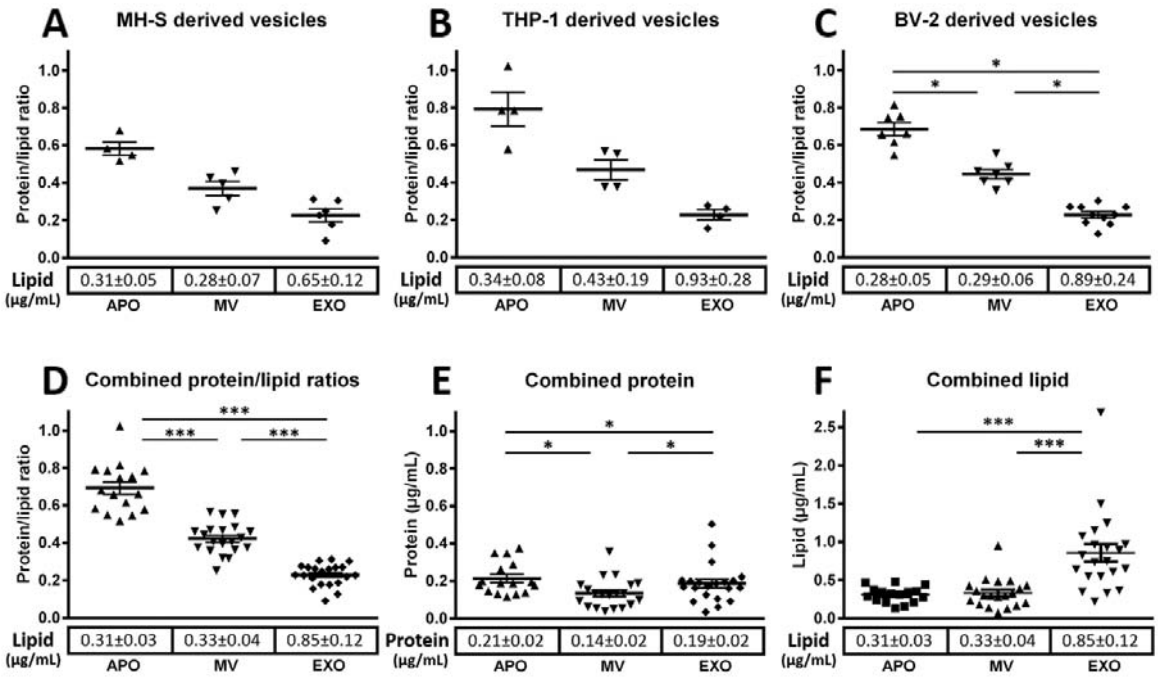

Figure 10. Protein to lipid ratios of EV subpopulations

Protein to lipid ratios are shown for APOs, MVs, and EXOs isolated from MH-S (A), THP-1 (B), and BV-2 (C) cell lines ($n \geq 3$ independent experiments for each EV type from all cell lines). Combined protein to lipid ratios obtained using EVs derived from MH-S, THP-1, BV-2, Jurkat, U937 as well as from human blood plasma are shown (D) (results of ≥ 12 independent experiments each EV type). Combined protein (E) and lipid concentrations (F) for the above cell line derived and blood plasma derived EVs are also shown. Mean values are represented by horizontal lines, and standard error means (SEMs) are indicated by error bars. The mean values ± SEM of lipid and protein concentrations (µg/mL) of conditioned media are reported below each respective EV subpopulation.

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5.1.6 Protein to lipid ratios of damaged or contaminated EV samples

To show the applicability of protein to lipid ratio as a quality control parameter of EV preparations, we tested EVs from different conditions. Firstly, platelets were incubated with ADP for one hour in PBS, and the supernatant was subsequently submitted for EV isolation. The low speed (20,000 *g*) MV pellet was characterized by the expected protein to lipid ratio (Figure 11A) and the presence of MVs was confirmed by using transmission electron microscopy (Figure 11B). However, the protein to lipid ratio of the high speed "EXO" (100,000 *g*) pellet was found to be abnormally elevated (Figure 11A). In accordance with this value, electron microscopy showed the lack of any vesicular structures in the 100,000 *g* pellet (Figure 11C), suggesting that under these conditions the pellet was mostly composed of proteins. This is in line with the previous finding that ADP is a poor inducer of EXO production (315).

Secondly, we exposed freshly isolated MVs to overnight incubation at 37 ºC, a condition that we have found earlier to damage MV preparations. Protein to lipid ratio was determined in the low speed MV pellet before and after overnight incubation. The overnight incubated and re-pelleted MV preparation was characterized by an abnormal protein to lipid ratio compared to that of freshly isolated MVs (Figure 11D). This was in agreement with the electron microscopy showing vesicular damage following the overnight incubation (Figure 11E and F). Finally MV preparations derived from red blood cell concentrates were compared after 2, 3, 4 or 5 consecutive washes. As shown in Figure 11G, the 3rd washing step resulted in a shift from elevated protein to lipid ratio to the ratio expected for MVs suggesting the removal of contaminating proteins, and this ratio remained stable upon further washes.

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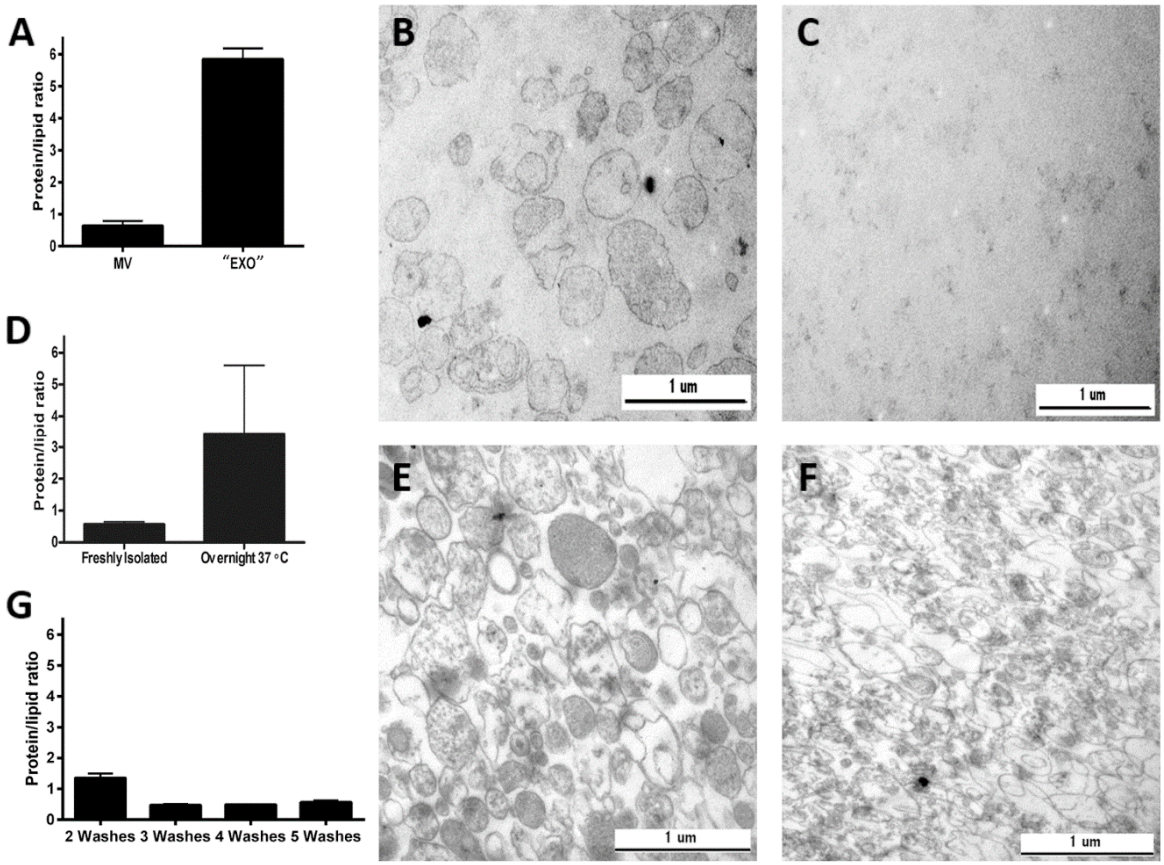

Figure 11. Protein to lipid ratio as a quality control parameter of EV preparations

A-C panels show the results of 4 independent experiments where there were visible pellets for both the 20,000g (MV) and 100,000g ("EXO") preparations. While the normal protein to lipid ratio of MVs reflected a true vesicular pellet as also demonstrated by electron microscopy (B), a strongly elevated protein to lipid ratio suggested the absence of EXOs as was later confirmed by electron microscopy (C).

In another experiment protein to lipid ratios obtained before and after overnight incubation at 37 °C of EVs (n = 8) are shown (D). This incubation resulted in an increase in the protein to lipid ratio of MVs. As compared to the freshly isolated MVs (E), a strong deterioration of vesicular morphology was observed after overnight incubation at 37 °C and repelleting of the vesicles (F) in parallel with the increased protein to lipid ratio (D). MV preparations derived from red blood cell concentrates were compared after 2, 3, 4 or 5 washes (G).

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5.1.7 Lipid membrane order of the EV subtypes

Finally, in order to determine if membrane lipid order could be a distinguishing feature of different EV subpopulations, we stained EVs with the fluorescent polarity-sensitive lipid probe di-4-ANEPPDHQ. Using confocal microscopy and ImageJ software, we determined the general polarization (GP) values for APO, MV and EXO preparations, respectively, and found significant differences between subpopulations of EVs (one-way ANOVA, $p<0.001$). The GP value was calculated from the fluorescence intensities at 560–600 nm and 660 nm resulting in values between -1 and +1 with higher GP values reflecting higher membrane lipid order (low liquid disordered, high liquid ordered structure). APOs and MVs were characterized by intermediate and partially overlapping lipid orders with GP values of -0.15 ± 0.15 and 0.00 ± 0.21 (median ± SEM), respectively. In contrast, EXOs displayed a GP value of 0.36 ± 0.29 (median ± SEM), reflecting a remarkably higher degree of lipid order than either MVs or APOs (Figure 12). The observed GP values were also assessed by flow cytometry and the results were close to those of the confocal microscope measurement. The GP values measured by flow cytometry were -0.14, -0.14, and 0.25 for APOs, MVs, and EXOs, respectively (Figure 13). The differences between the GP values measured by confocal microscopy and flow cytometry could be ascribed to the small differences in the available filters in each instrument (560–600 nm and >660 nm in the confocal microscope as opposed to 585 ± 21 nm and 616 ± 12 in the flow cytometer). However, the pattern of higher order in EXOs was observed by both instruments.

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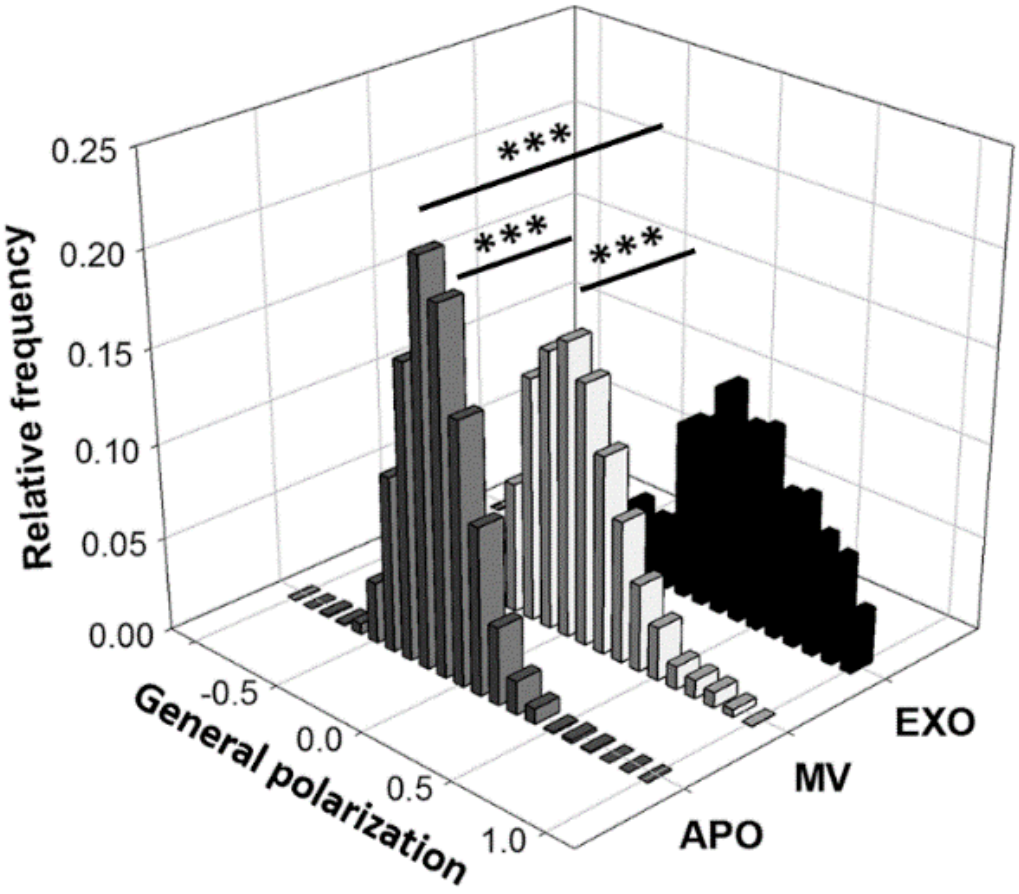

Figure 12. Confocal microscopy determination of EV membrane lipid order

Quantitative assessment of membrane lipid order in subpopulations of EVs secreted by BV-2 cells is shown (≥3 independent experiments each EV type). Ratiometric measurements of the fluorescent intensities at 560–600 nm and at >660 nm were carried out by confocal microscopy, and results are expressed as general polarization (GP) values. The higher GP value reflects a higher membrane lipid order. EXOs showed the highest order, while APOs and MVs were characterized by partially overlapping, intermediate order.

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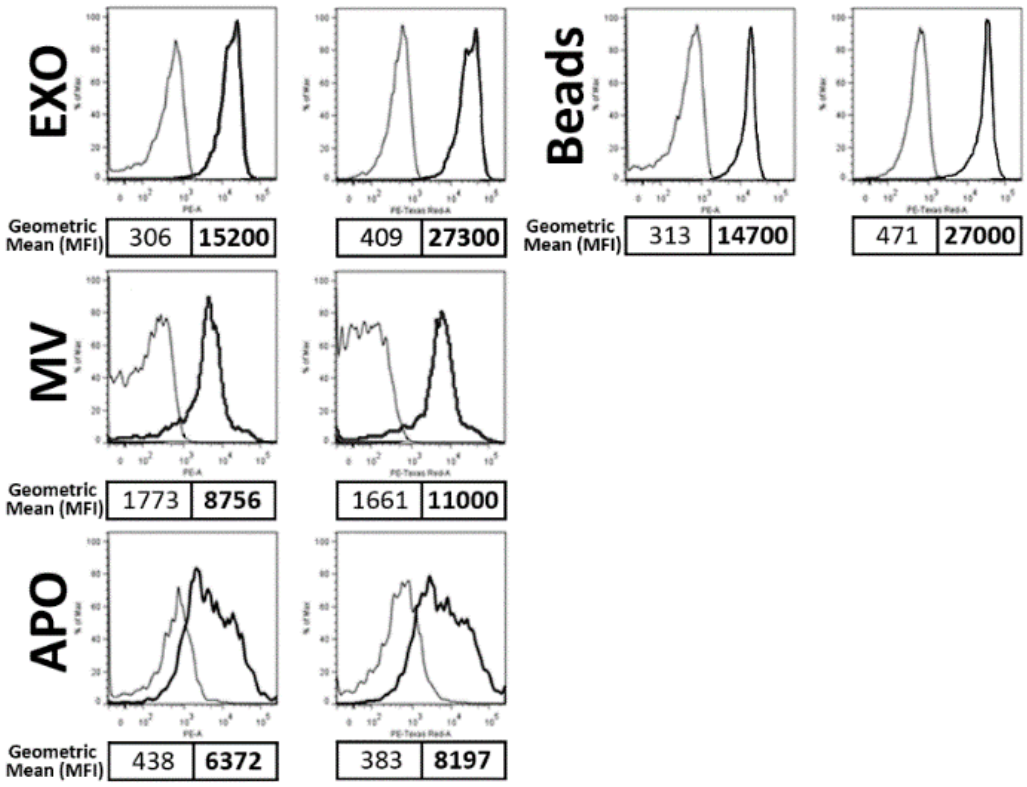

Figure 13. Flow cytometric determination of EV membrane lipid order

Flow cytometric determination of di-4-ANEPPDHQ staining of EV subpopulations secreted by BV-2 cells. Representative results of one out of n=3 independent experiments. Left panels represent the fluorescence at 585±21 nm, while right panels represent fluorescence at 616±12 nm of unstained and stained vesicles and beads (thin and thick lines, respectively). Geometric mean fluorescent intensities (MFI) of unstained and stained APOs, MVs, EXOs coupled to latex beads, and beads without EXOs are shown (regular and bold text, respectively).

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5.2 Differential detergent sensitivity of extracellular vesicle subpopulations

Based on previous findings of our group (292), in this study we carried out a comprehensive analysis of the effect of detergents on various EV subpopulations. Here we investigated the sensitivity of various subpopulations of EVs to different concentrations of detergents including sodium dodecyl sulphate (SDS), Triton X-100, Tween 20 and deoxycholate. We determined the required detergent concentration that lysed each of the vesicle subpopulations secreted by Jurkat, THP-1, MiaPaCa and U937 human cell lines.

5.2.1 Morphology and concentrations of EVs found in the serum-free conditioned media of various human cell lines

EV-containing conditioned media of Jurkat, THP-1, U937, and MiaPaCa cell lines were collected for analysis of their secreted EVs as described in Section 4. We found that all EV subtypes were simultaneously present in the cell line supernatants as shown with a representative transmission electron microscopic image of pelleted EVs from conditioned media collected from THP-1 cells in Figure 14. We also found that the concentration of EVs released by each cell type differed significantly from one another as shown in Figure 15. However there was no correlation between the original concentrations of EVs present in the supernatants and the concentrations of the detergents required for their lysis as seen in Figures 16-19.

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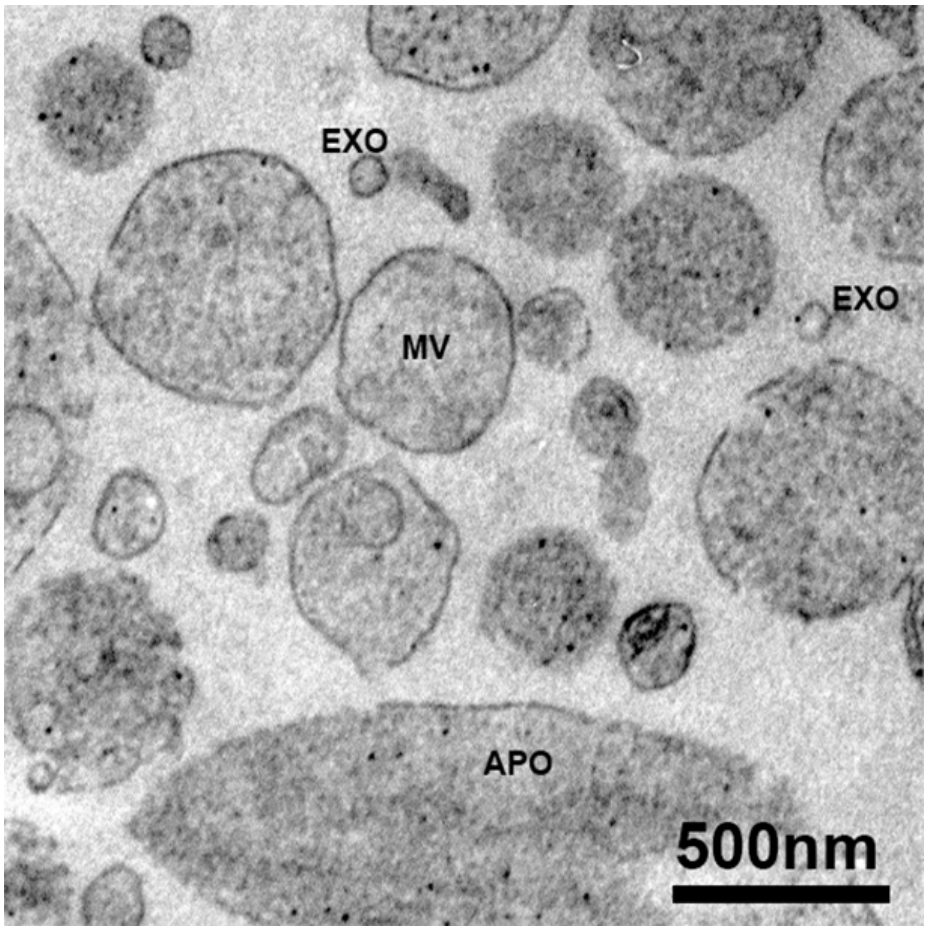

Figure 14. Transmission electron microscopy of EVs in conditioned media

A representative transmission electron microscopic image of pelleted EVs from conditioned media collected from THP-1 cells revealing that a combination of all three EV size-based subpopulations (APO, MV and EXO) can be found simultaneously present in pellets of the conditioned media. This image was obtained from serum-free, cell-depleted 24 hrs conditioned medium of THP-1 cells that was ultracentrifuged at 100,000 *g* 60 mins and submitted for electron microscopic imaging.

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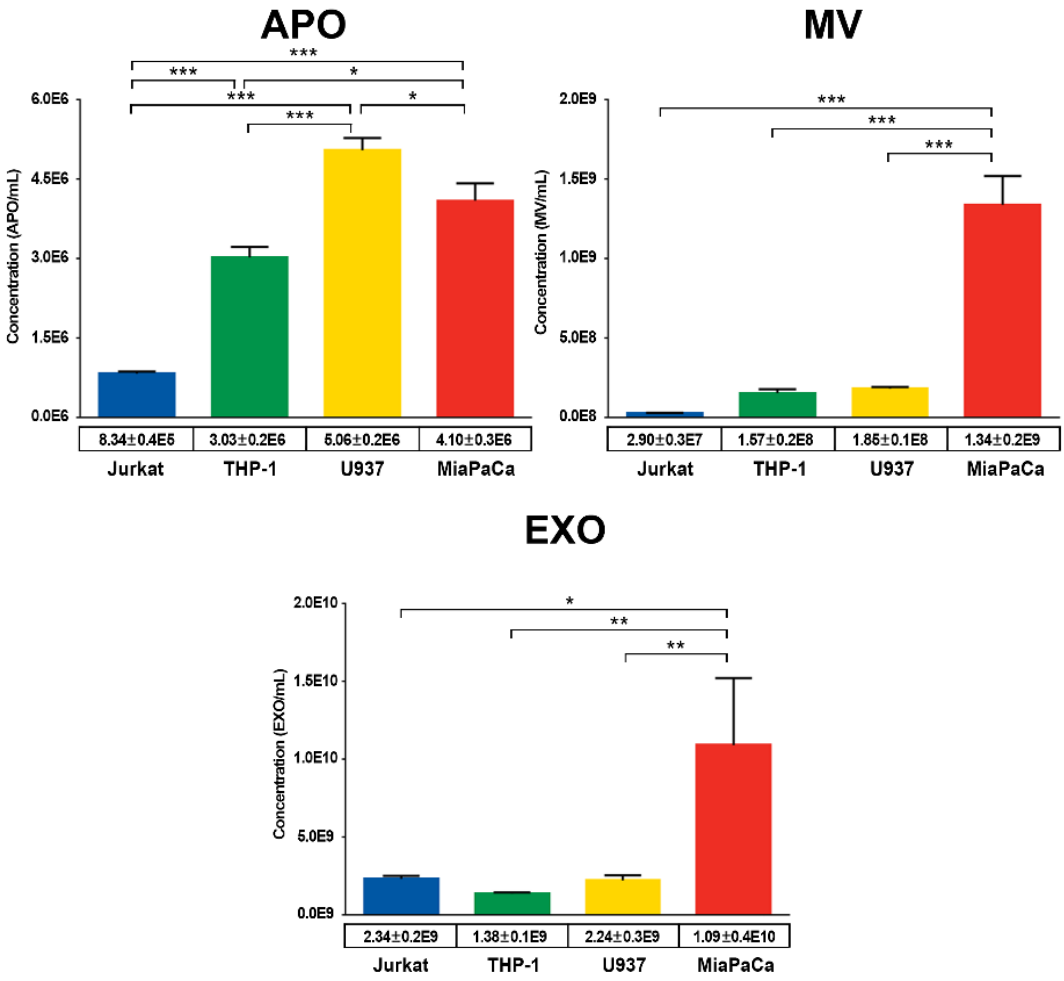

Figure 15. Concentration of EVs present in the supernatant of various cell lines

MiaPaCa cells produced significantly higher amounts of both MVs and EXOs as compared to the other three cell lines. In contrast, significantly different amounts of APOs were produced by the four cell lines with U937 producing the most. The mean values ± SEM of EV concentrations (EV/mL) of conditioned media are reported below each respective EV subpopulation for the different cell lines. Concentrations were compared by one-way ANOVA.

5.2.2 Detergent sensitivity of apoptotic bodies and microvesicles

Various size-based subpopulations of EVs derived from the four different cell lines were analyzed by different sized pores in the TRPS system before and after treatment with increasing concentrations of detergents. Prior to TRPS analysis, cell-depleted conditioned media were supplemented with detergents and were vortexed for 30 seconds. For the detection of APOs in the conditioned media a NP2000 nanopore

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membrane with a detection range of 1000–5000 nm was used. For the analysis of MVs a combination of NP400 and NP800 nanopore membranes with a detection range 150–1000 nm was used. In Figures 16 and 17 are shown representative size distribution histograms of APOs and MVs, respectively, as detected with increasing concentrations of SDS. For both EV subpopulations, 0.01% SDS resulted in strong decrease in the EV concentrations. Furthermore, the decrease in EV concentrations did not produce a shift in the modal size and size ranges of each subpopulation.

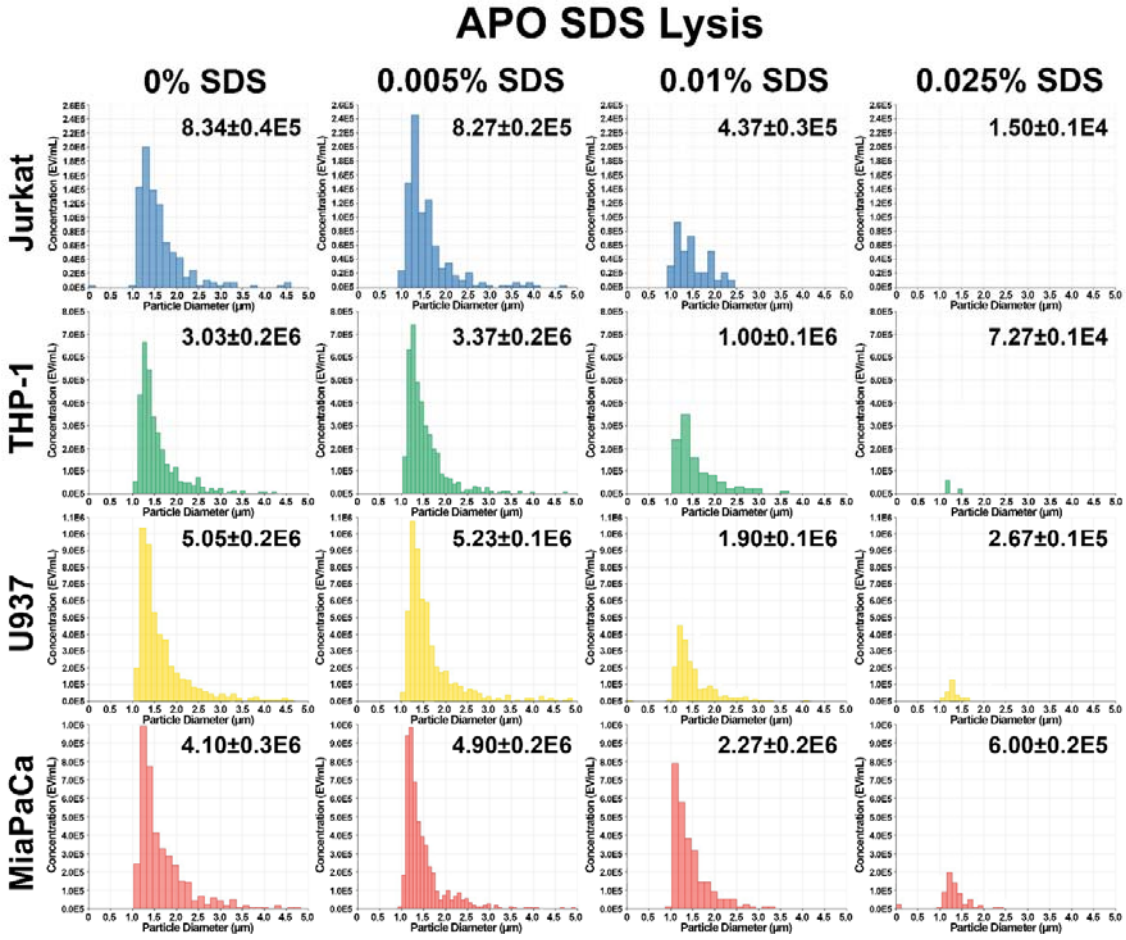

Figure 16. Size distributions of APOs in the presence of increasing concentrations of SDS

Representative TRPS-determined size distribution histograms of the APOs in 24h serum-free conditioned media of four different human cell lines in the absence or presence of increasing concentrations of SDS. Numbers in the size histograms indicate mean ± SD of total concentration of EVs/mL in cell-depleted conditioned media. $n \geq 3$

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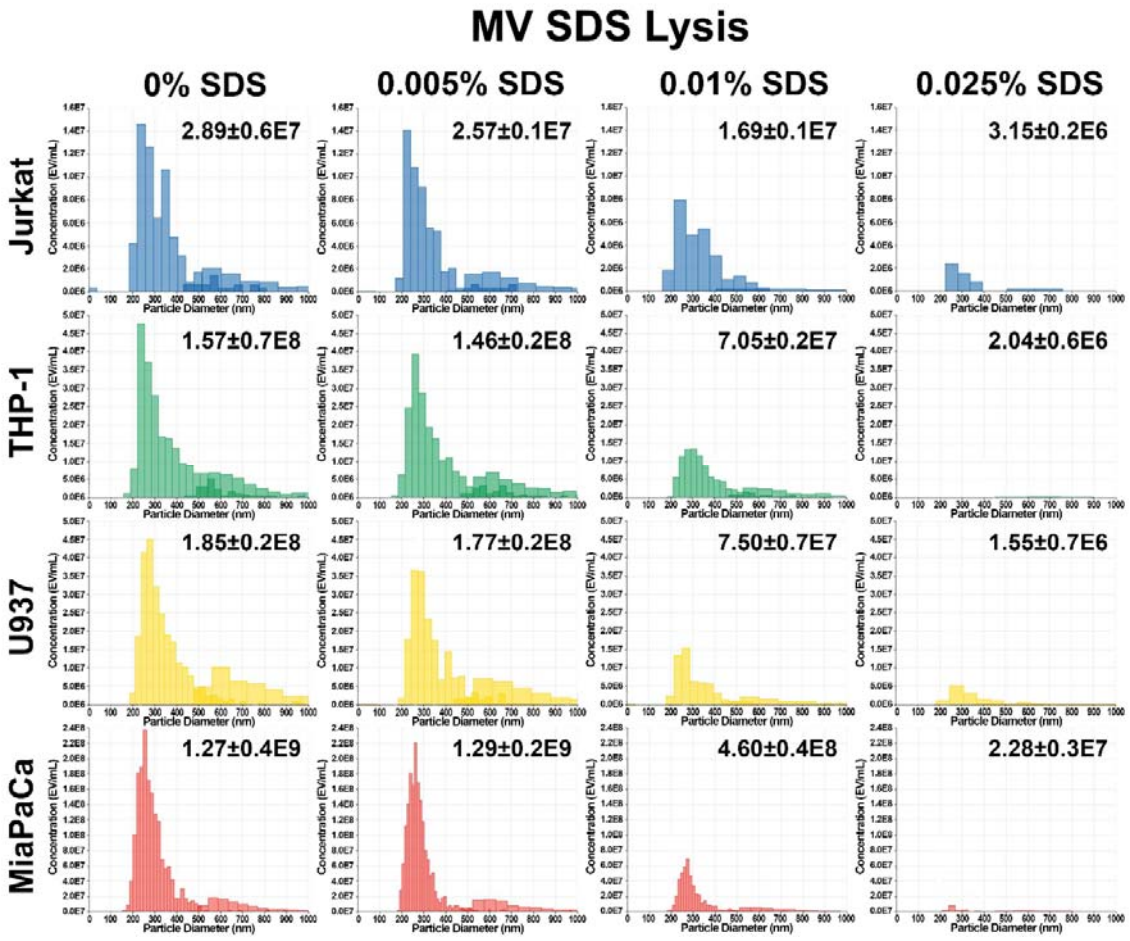

Figure 17. Size distributions of MVs in the presence of increasing concentrations of SDS

Representative TRPS-determined size distribution histograms of the MVs in 24h serum-free conditioned media of four different human cell lines in the absence or presence of increasing concentrations of SDS. Numbers in the size histograms indicate mean ± SD of total concentration of EVs/mL in cell-depleted conditioned media. $n \geq 3$

5.2.3 Higher detergent resistance of exosomes

Using the same detergent treatment than APOs and MVs, EXOs were also analyzed in the conditioned media using a NP100 nanopore membrane with a detection range of 60–300 nm. Figures 18 shows representative size distribution histograms of EXOs as detected with increasing concentrations of SDS. In contrast to APOs and MVs, EXOs were found to resist treatment with higher concentrations of detergents with a strong decrease in concentration observed at 0.125% SDS instead of 0.01%. Furthermore,

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similar to APOs and MVs the decrease in EXO concentrations was also not accompanied with a shift in the modal size and size ranges of each subpopulation.

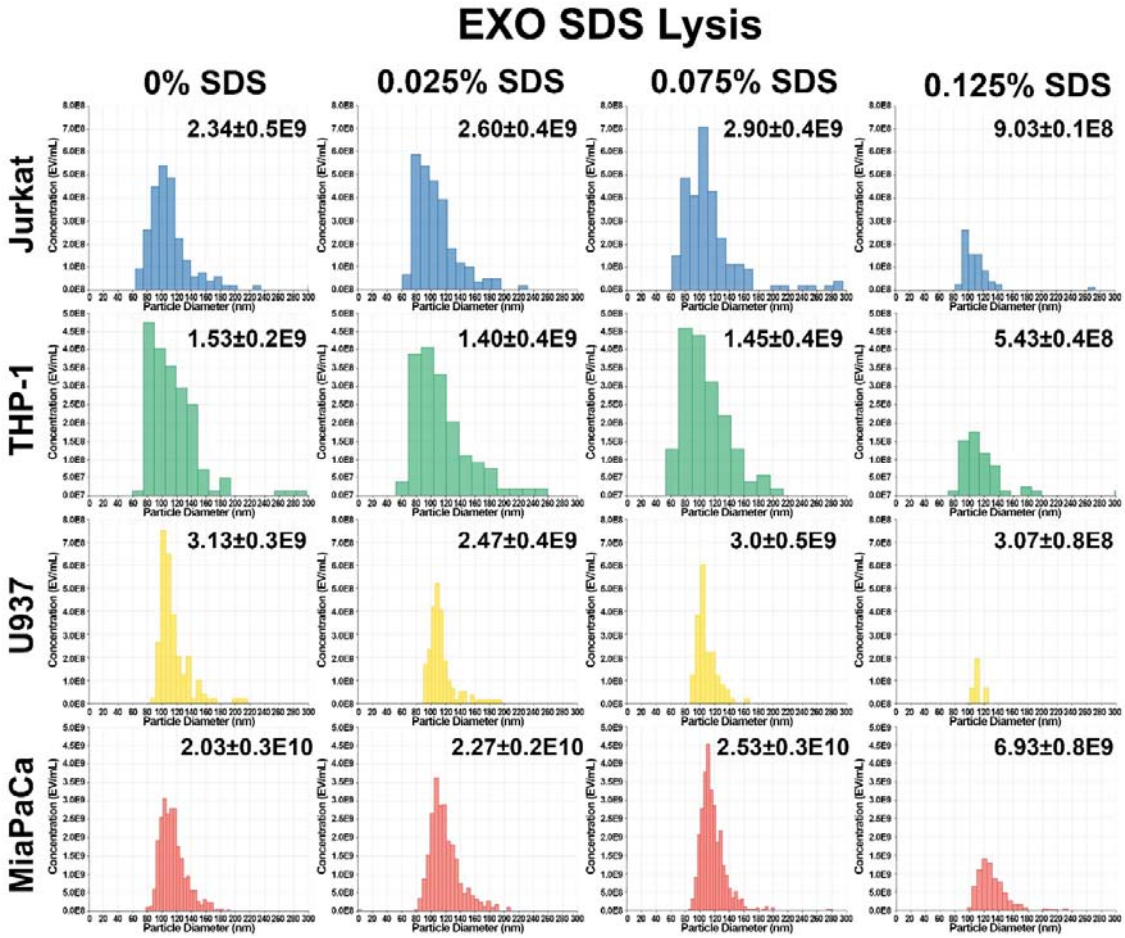

Figure 18. Size distributions of EXOs in the presence of increasing concentrations of SDS

Representative TRPS-determined size distribution histograms of the EXOs in 24h serum-free conditioned media of four different human cell lines in the absence or presence of increasing concentrations of SDS. Numbers in the size histograms indicate mean ± SD of total concentration of EVs/mL in cell-depleted conditioned media. $n \geq 3$

5.2.4 Comparison of detergent capacity to disrupt EVs

All EV subpopulations were also treated with Triton X-100, Tween 20 and deoxycholate in addition to SDS. All four detergents were found to lyse EVs albeit at different concentration ranges. Out of the four detergents, SDS was found to reduce APO and MV counts at the lowest concentrations (0.01%) while Triton X-100 lysed EXOs at

the lowest concentration (0.075%). In contrast, EVs were the most resistant to treatment with Tween 20 which was found to reduce EV counts at very high concentrations (10–15%) only. EVs derived from the four different cell lines showed similar detergent sensitivity within each size-based subpopulation regardless of the original concentration of the secreted EVs present in the conditioned media. We found no statistically significant decrease in EV counts until the suggested lysing concentrations for each detergent. For all detergents, at their lysing concentrations we found statistically significant reduction of the measured EV counts (two-way ANOVA) of each separate cell line: $p < 0.0001$ for APOs, $p < 0.05–0.0001$ for MVs (with the exception of SDS lysis of Jurkat MVs), and $p < 0.01–0.0001$ for EXOs. Clearly, EXOs were found to be the least sensitive to detergent lysis while APOs and MVs showed higher sensitivity to detergent lysis (and were similar to each other). Similarly to Triton X-100, the bile acid deoxycholate had moderate efficiency to disrupt all EV subpopulations. Interestingly, SDS lysed EXOs at least at an order of magnitude higher concentration (0.125%) than required for lysing MVs and APOs (0.01%). In contrast, all three EV subpopulations resisted concentrations of Tween 20 as high as 5%. Figure 19 and 20 summarizes these data on the differential ability of the four applied detergents to disrupt EV subpopulations.

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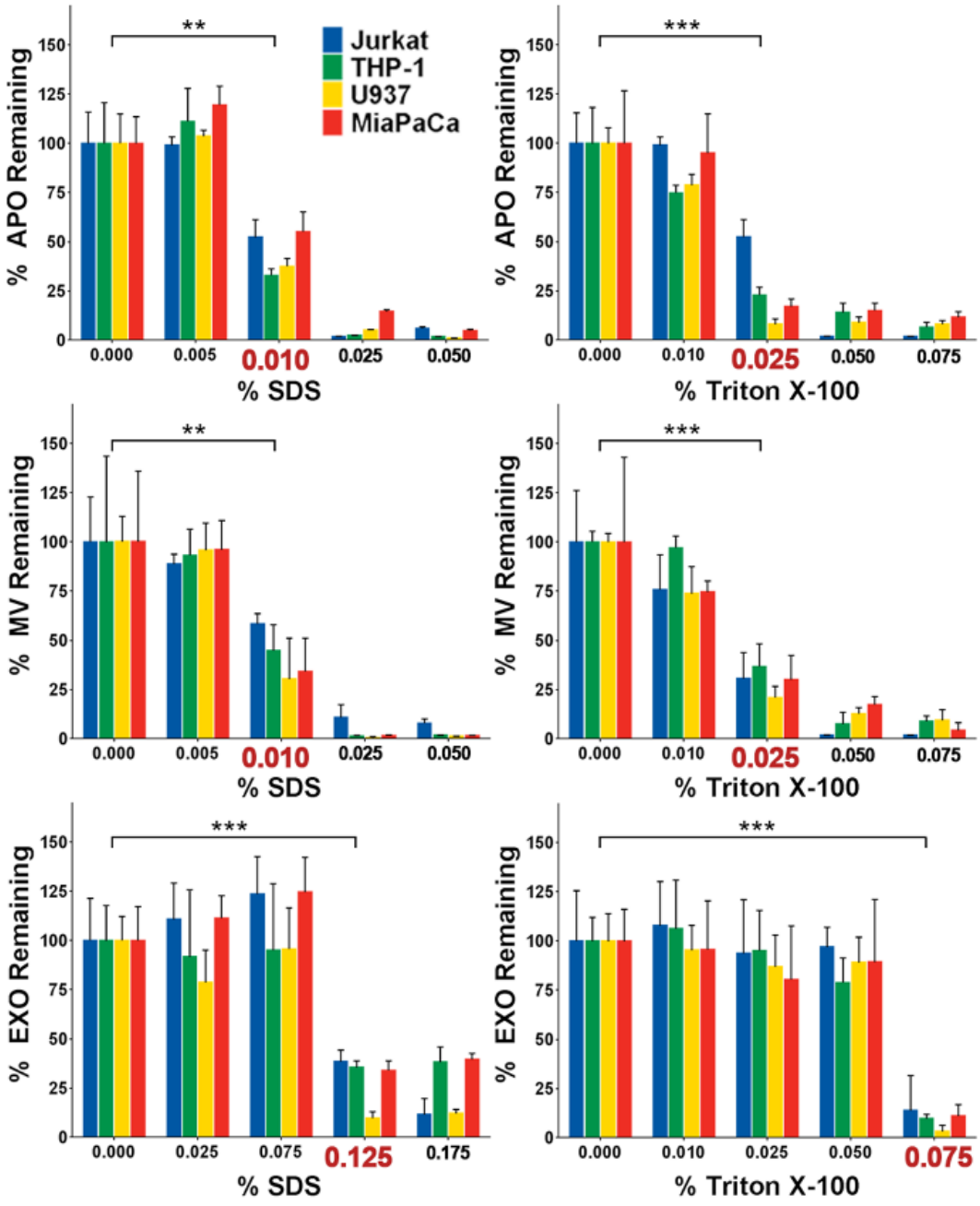

Figure 19. Comparison of detergent lysis sensitivity of EV subpopulations

Comparative sensitivity of APOs, MVs and EXOs secreted by different cell lines (Jurkat, THP-1, U937 and MiaPaCa) to increasing concentrations of detergents SDS and Triton X-100. EV concentration is shown as a percentage of the initial concentration prior to detergent treatment. Note that the detergent concentration ranges differ due to the large variation among detergents in working ranges for EV lysis. $n \geq 3$

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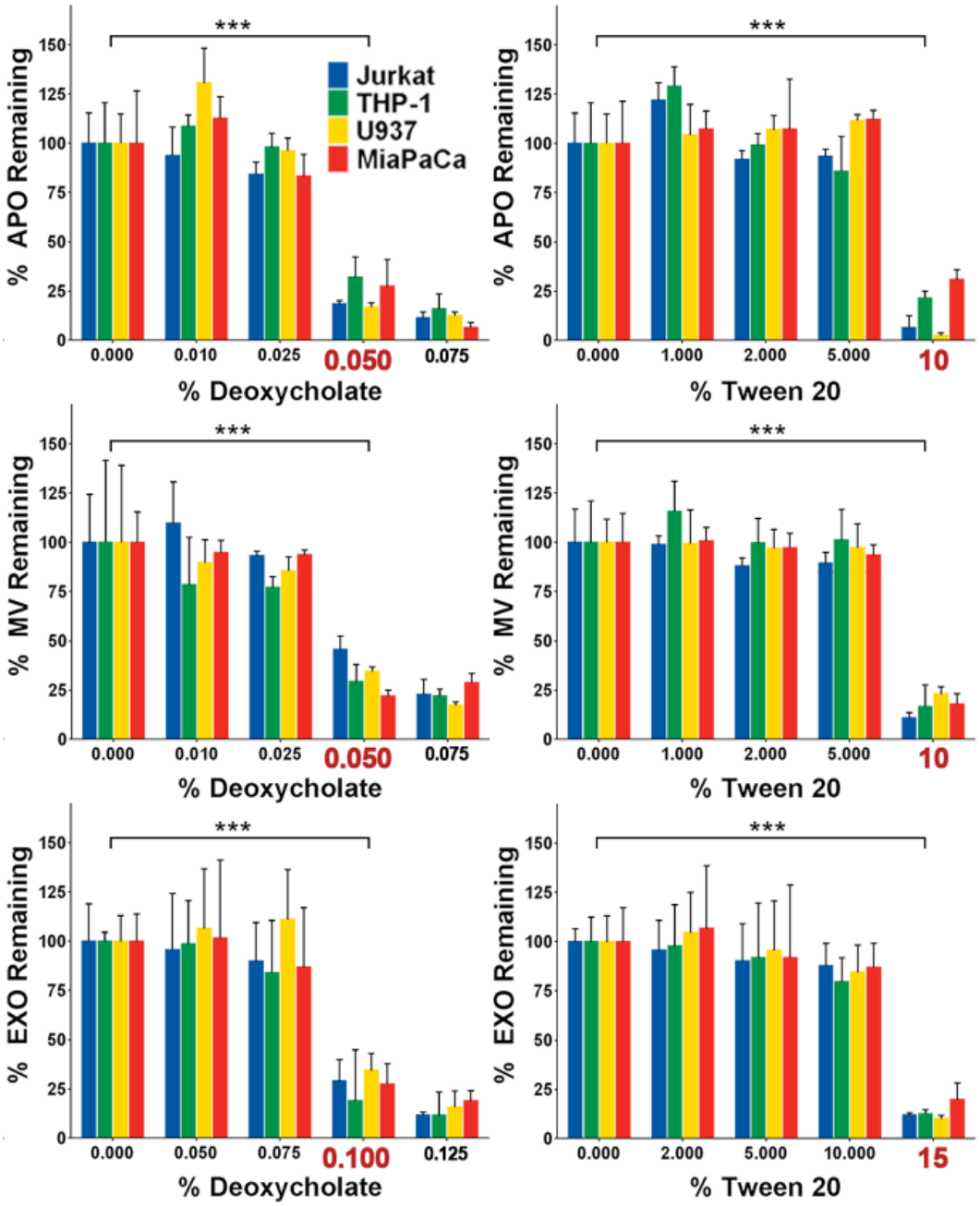

Figure 20. Comparison of detergent lysis sensitivity of EV subpopulations

Comparative sensitivity of APOs, MVs and EXOs secreted by different cell lines (Jurkat, THP-1, U937 and MiaPaCa) to increasing concentrations of detergents deoxycholate and Tween 20. EV concentration is shown as a percentage of the initial concentration prior to detergent treatment. Note that the detergent concentration ranges differ due to the large variation among detergents in working ranges for EV lysis. $n \geq 3$

These results demonstrate that SDS is the detergent that disrupts MVs and APOs at the lowest concentration which is in good accordance with the fact that ionic detergents, such as SDS, are extremely effective in the solubilisation of membrane proteins (316). Given the large difference in SDS concentration that lyses EXOs compared to MVs and APOs, the use of this detergent for differential lysis of the different EV subpopulations may appear superior to other detergents. However, for downstream applications it may be needed to also consider that in most instances ionic detergents are denaturing to some extent, while bile acids and non-ionic detergents are relatively mild and are usually non-denaturing (316). Thus, the choice of detergent for EV studies might dictated by the type of work to be carried out. Non-ionic detergents such as Triton X-100 and Tween 20 are known to break lipid–lipid interactions and lipid–protein interactions rather than protein–protein interactions, a feature that can enable them to differentiate EVs from protein aggregates (292, 317).

These data also point to the similarity of the lipid membrane composition of APOs and MVs as opposed to EXOs in line with the results described earlier related to lipid membrane composition and membrane liquid order of different size-based EV subpopulations and falls in accordance with the findings that liquid ordered as opposed to liquid-disordered membranes are more resistant to detergents (318, 319).

5.2.5 Flow cytometric validation of detergent sensitivity of extracellular vesicles

Lastly, EVs were characterized with flow cytometry by their staining with fluorochrome labelled annexin V and anti-CD9 antibody and the results are shown in Figure 21. Annexin V and anti-CD9 stained all three EV subpopulations. Using SDS concentrations shown in the present study by TRPS to lyse vesicles, we could demonstrate the prompt disappearance of fluorescent events validating our prior TRPS observations.

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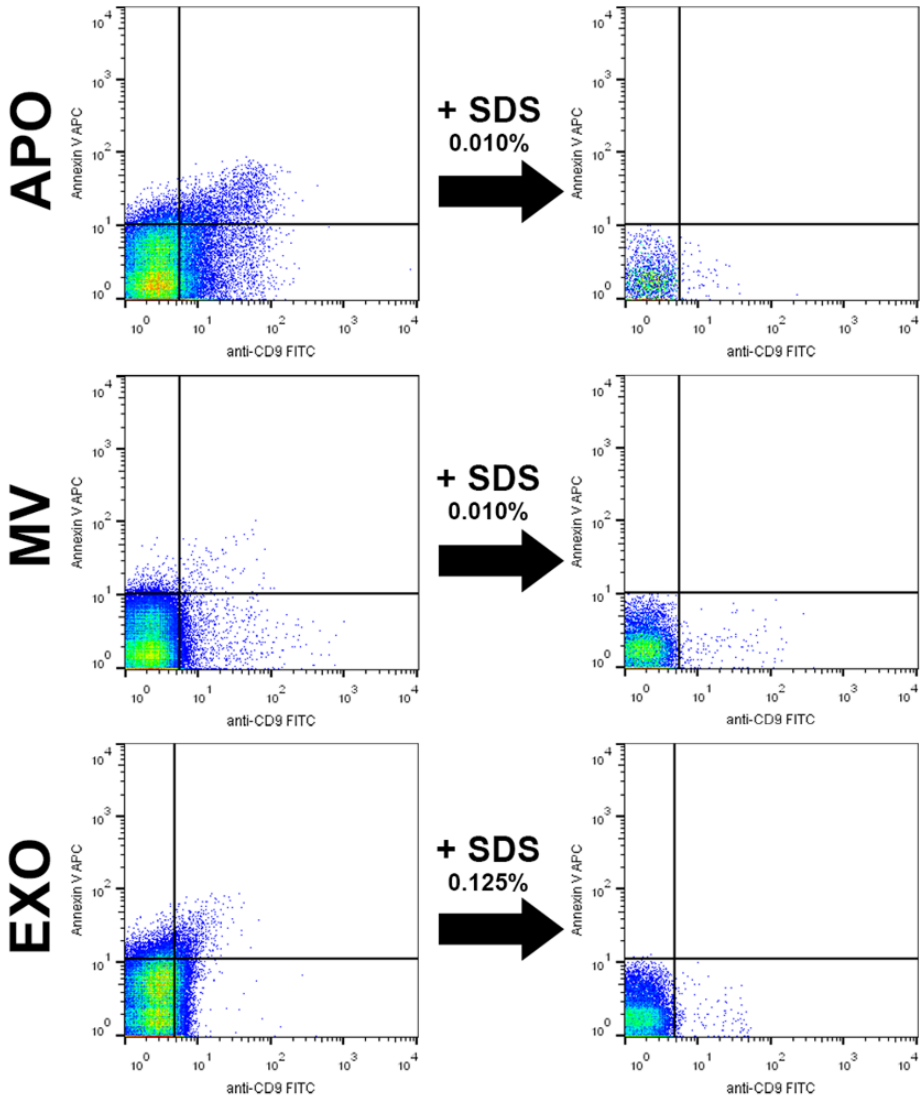

Figure 21. Flow cytometric validation of EV lysing concentration of SDS

Serum-free, cell-depleted 24h conditioned media of cell lines were submitted for APO, MV and EXO isolation using differential centrifugation/ultracentrifugation and gravity driven size filtration. EVs were stained with Annexin V APC and anti-CD9 FITC. APOs and MVs were analyzed directly, whereas EXOs were detected upon binding to latex beads. All fluorescent events promptly disappeared upon addition of the concentration of SDS found to lyse each EV subpopulation. The figure shows representative data for the lysis of THP-1 cell-derived EVs.

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6. DISCUSSION

In the past decades EV research emerged as a novel field of cell biology. Understanding of the roles of EVs has profoundly impacted our understanding of many biological processes. In the meantime, different methods have been adopted to quantify EVs. However, currently used assays do not discriminate between vesicular or non-vesicular particles (such as NTA and TRPS), or solely quantify EVs in a given preparation based on the total protein content. Since protein aggregates have been shown to be co-purified with EVs (292), and in some samples soluble proteins and protein aggregates are more abundant than lipid bilayer enclosed EVs, such approaches are error-prone. We hypothesized that as an alternative to protein-based EV quantification, simultaneous determination of both the protein and lipid content may lead to a better quantification of the EVs in a sample. In spite of the large body of published data on EVs, virtually none of the studies have used an assay to determine total lipids in EV preparations besides determining the protein content. Thus, there has been an unmet need in the field for a simple lipid assay to detect EVs. Furthermore, there have been no universally accepted molecular markers to distinguish among the subpopulations of EVs.

Thus the work of this Ph.D. dissertation was undertaken to establish for the first time a simple lipid assay for EVs and to find good quality control parameters to characterize EV preparations that might be utilized both in basic research and in clinical laboratory settings. To this end, we used and optimized the SPV total lipid assay for EV studies and combined this lipid assay with the conventional microBCA protein determination that is used widely in the EV field. This total lipid assay is simple and fast, requiring only 30 min of incubation, and 0.5µg protein containing EVs either in dry pellet or in up to 50µL volume. Furthermore, we showed if used under the optimized conditions described in this dissertation, the SPV assay sensitivity is adequate for EV studies and compares favorably to microBCA in terms of accuracy, although shows slightly higher intra and inter-assay variabilities. However, at concentrations >50 µg/mL lipid for SPV or protein for Micro BCA, both assays show low variability and good accuracy as shown earlier in Figure 7. Using these two assays, we introduced protein to lipid ratio as a novel parameter to characterize EV preparations enriched in EXOs, MVs, and APOs.

As expected, we found increasing protein to lipid ratios for EV subpopulations of increasing diameter. Our data are in line with results of an earlier study that determined

protein and lipid composition of platelet microparticle size fractions using mass spectrometry and a detection of lipid phosphorous with the method of Bartlett (320). We found that the protein to lipid values depended on the lipid standard used in the assay. Although the reference lipids (such as menhaden fish oil) that we used in this study may not exactly reflect the complex composition of EV lipid membranes, they are produced under standardized conditions, they are inexpensive and they are commercially available for the broad scientific community. More sophisticated lipid standards may be further developed as the lipid composition of EVs will be better understood. Regardless of the method used, if adhering to the same lipid standard consistently throughout the experiments, protein to lipid ratio determination may prove very useful for quality control of EV preparations. In addition to the SPV method introduced in this study, more sophisticated methods such as infrared spectroscopy could be alternatively used for the determination of protein and lipid content of EVs. However, the requirement for special equipment may prevent the use of infrared spectroscopy in many laboratories. Furthermore, cholesterol as well as other sterols do not have many useful infrared features by which to distinguish them from other molecules. The vibrational bands due to C–C or C-H bonds, which are commonly used to measure sterols with infrared spectroscopy, are not only very weak but are also shared by most biological molecules including proteins (321). Its reduced cholesterol sensitivity may thus, limit the wide use of infrared spectroscopy for EV studies.

In addition to EVs, we also determined the protein to lipid ratio of cells releasing EVs. We found that, as compared to the EVs, cells showed higher protein to lipid ratios (2.19) but with substantially higher variation (SEM = 0.95) probably reflecting differences in cell types and in cell cycle phases. Lipid sorting mechanisms (such as preferential sorting of cholesterol into EXOs) during the generation of different EV subpopulations have been reported (40, 322). Such sorting process may result in the enrichment of given lipids in different EV subpopulations which may contribute to the relatively low variation of protein to lipid ratios of the same EV subpopulation compared to the producing cells.

In an attempt to find novel parameters to characterize EV subpopulations, using flow cytometry we stained our EVs with an anti-cholesterol antibody, and found strongest staining for EXOs. Furthermore, we found staining of all EV subpopulations with CTX reflecting the presence of GM1 gangliosides in all EV subpopulations. GM1 gangliosides

have been previously detected in EXOs (323, 324). Here we found that GM1 was not only present in EXOs but also especially enriched in MVs and differentiated two subpopulations of APOs. Intriguingly, heterogeneity within APOs was not only suggested by bimodal CTX binding, but also by the annexin V and anti-CD63 staining. Transmission electron microscopy also revealed the presence of highly electron dense, possibly fragmented DNA containing APOs and less electron dense and more granular APOs possibly corresponding to the recently described different subpopulations of APOs (73).

The spectral ratiometric approach of this study provides evidence that EV subpopulations can be distinguished based on the difference in their membrane lipid order. This parameter reflects the degree of lipid packing and is one of the most important biophysical parameters of membranes (311). In low ordered membrane domains the probability of protein-protein interactions is decreased. Therefore membrane lipid order can impact signaling pathways. This is strongly supported by the findings that high membrane lipid order is typically found at the immunological synapse, sites of cell adhesion, viral entry and budding as well as in exosomes (the latter described for the first time in this work). Our data suggest that EXOs are characterized by highest membrane lipid order, while APOs and MVs showed low to intermediate, partially overlapping lipid order reflecting important differences in membrane lipid composition. A high degree of membrane lipid order and the relatively high content of cholesterol in EXOs may be important factors explaining their distinguished role in intercellular signaling.

Without universally accepted molecular markers reflecting different biogenic origin of EV subpopulations, most studies simply classify EVs based on their size. Such a size-based classification could be challenged by observations that exosome-sized EVs can also be shed from the plasma membrane (202). In an attempt to assess whether this kind of observations represent exceptions to the rule or if size-based classification is arbitrary, we looked beyond size, and compared lipid properties and protein to lipid ratios in EVs fractionated by size. We found differential binding of an anti-cholesterol antibody and CTX to different sized populations.

Additionally, our data show an increasing protein to lipid ratio with an increasing diameter for each EV subpopulation. This was as expected because with an increasing diameter, the amount of protein that may be contained inside an EV increases as a

function of the volume while the amount of lipid only increases as a function of the surface area. However our data also show that the increase in protein to lipid ratio is not perfectly proportional to the increased volume to surface area ratio. Instead, as shown in Table 4 we found a smaller increase in the protein to lipid ratios than what would be expected based solely on theoretical estimations derived from calculations of volume to surface area ratios. This observation may be due to different cargo packing densities such as a decrease in intravesicular protein concentration with an increasing diameter or because of differences in the molecular composition of EV subtypes with different abundance of other molecules such as nucleic acids which may diminish the cargo capacity for proteins.

Table 4. Comparison of protein to lipid ratios with calculated volume to surface ratios of different sized EV subpopulations

Parameter	Exosomes		Microvesicles		Apoptotic bodies	
Particle Size Range (nm)	50	100	100	1000	1000	5000
Volume (nm^3) = $4/3\pi(d/2)^3$	65450	523599	523599	$5.24x10^8$	$5.24x10^8$	$6.54x10^{10}$
Surface (nm^2) = $4\pi(d/2)^2$	7854	31416	31416	3141593	3141593	78539816
Volume/Surface = 1/6·d **(fold increase over EXO)**	8.33	16.67	16.67	166.67 **(20x)**	166.67 **(20x)**	833.33 **(100x)**
Mean Volume/Surface **(fold increase over EXO)**	12.5		91.67 **(7.33x)**		500 **(40x)**	
Observed mean Protein to Lipid Ratio **(fold increase over EXO)**	0.21		0.48 **(2.29x)**		0.64 **(3.05x)**	

Table 4 shows the calculated volumes to surface area ratios for the size ranges of different EV subpopulations in comparison with the observed protein to lipid ratios.

Together, these observations suggest that simple size-based classification enables distinguishing EVs with different membrane lipid properties. Until selective molecular markers become available, established protein to lipid ratios may serve as suitable parameters to characterize different EV subpopulations. In addition, protein to lipid ratio may also prove useful for routine quality control of EV preparations.

Based on previous findings of our group, we also carried out a comprehensive analysis of the effect of detergents on size-based EV subpopulations. This work provides a practical tool to confirm the vesicular nature of particles present in biological samples using either TRPS or flow cytometry. Given that both TRPS and flow cytometry are commonly used methods to characterize EVs, the use of detergent control in EV analysis may prove useful in a wide variety of experimental settings and may also be combined with other techniques of EV analysis such as those described previously in Table 3.

Results of the experiments with detergents clearly demonstrate that SDS disrupts MVs and APOs at the lowest concentration. This is in good accordance with the fact that ionic detergents, such as SDS, are extremely effective in the solubilization of membrane proteins (316). It was also found that there is an order of magnitude difference between the concentration of SDS that lyses both APOs and MVs and the one that lyses EXOs. Therefore the use of SDS for differential lysis of APOS, MVs and EXOs may appear superior to other detergents. However, for downstream applications it also has to be considered that in most instances ionic detergents are denaturing to some extent, while bile acids and non-ionic detergents are relatively mild and are usually non-denaturing (316). The choice of detergent for EV studies is thus, dictated by the type of work to be carried out. Non-ionic detergents are known to break lipid–lipid interactions and lipid–protein interactions rather than protein–protein interactions, a feature that enables them to differentiate EVs from protein aggregates (292, 317).

Interestingly, a non-statistically significant increase in EV concentrations was observed in the case of treatment with SDS and deoxycholate. This observation is probably due to a small decrease in instrumental noise due to the used of these detergents and thus slightly enhanced lower detection limit for smaller particles which would be below the noise threshold without detergent. Furthermore, it is also possible that a small

percentage of previously aggregated EVs might dissociate from each other at low detergent concentration and thus be counted as more individual particles instead of their aggregated form.

Taken together, our results point to the similarity of the lipid membrane composition of APOs and MVs as opposed to EXOs which were found to be more liquid ordered while both APOs and MVs had similar and lower membrane liquid orders. Accordingly, several studies show that liquid ordered as opposed to liquid-disordered membranes are resistant to detergents (318, 319).

Moreover, our findings show that using detergents at the suggested concentrations, vesicle enclosed cytokines, RNAs and metabolites may be released for detection. Also, analysis of vesicle cargo by Western blotting or mass spectrometry requires both the disruption of the vesicle membrane and the release of membrane proteins. Recently, particular attention follows RNA as a vesicular cargo. RNA extraction from EVs may involve the use of detergents (325). Results of our study also have important implications in other research methods such as in ELISAs where Tween 20 is used at lower concentrations (typically 0.05–0.1%) than what we found to lyse EVs. Thus this study introduced a practical tool for confirmation of the vesicular nature of particles present in biological samples using either TRPS or flow cytometry. Given that both TRPS and flow cytometry are commonly used methods to characterize EVs, the use of simple and fast detergent controls to differentiate between EXOs and other extracellular vesicle subpopulations as well as between vesicular and non-vesicular structures in EV analysis may prove useful in a wide variety of experimental settings.

In conclusion, for the first time our work introduced a simple lipid assay and novel EV characterization parameter as well as the appropriate concentrations of different detergents to lyse EVs. We demonstrated that different size-based subpopulations of EVs secreted by highly diverse human cell lines show similar protein to lipid ratios, membrane lipid orders, and detergent sensitivity patterns. Taken together, our data validate the use of the SPV lipid assay, membrane lipid order assessment, and detergent lysis as integral components of the EV detection toolbox.

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7. CONCLUSIONS

In conclusion, with this work the existing methodology of extracellular vesicle research has been improved with the introduction of a new assay as well as different parameters useful for the detection and characterization, as well as quality control and validation of extracellular vesicle preparations.

This was achieved by introducing a new assay for quantification of total lipids. Using this assay we found a novel parameter consisting of the protein to lipid ratio that is suitable for the characterization of the subtype as well as the purity and quality of extracellular vesicle preparations.

Furthermore we have characterized the membrane lipid order of different extracellular vesicle subpopulations finding a higher degree of order in exosomes lipid membranes as compared to microvesicles and apoptotic bodies. Interestingly we also found an increased detergent resistance with the increased membrane lipid order of exosomes.

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8. SUMMARY

Extracellular vesicles including exosomes, microvesicles, and apoptotic bodies have so far attracted rapidly increasing attention from various fields of biology due to their ability to carry complex information and act as autocrine, paracrine and even endocrine intercellular messengers. Despite this growth, there is still an important need to improve the methodology and controls with which to characterize extracellular vesicles.

For this reason the aim of this Ph.D. research was to introduce to the field of extracellular vesicles new and improved methods for detection and characterization.

Extracellular vesicles were isolated by differential centrifugation and gravity driven filtration from the conditioned media of cell lines as well as human blood plasma. Extracellular vesicles were characterized by using tunable resistive pulse sensing, transmission electron microscopy, and flow cytometry. Additionally, we introduced for the first time a simple bench top lipid assay for determination of lipid content of extracellular vesicles based on a 96 well plate adaptation of the sulfophosphovanilin colorimetric reaction.

Using this novel lipid assay and an available protein assay we found characteristic protein to lipid ratios for each of the investigated extracellular vesicle subpopulations. Moreover, these ratios were substantially altered in the case of vesicular membrane damage or protein contamination. We also found marked differences in membrane lipid order between the various vesicle subpopulations using ratiometric spectrophotometry. Lastly, we determined the sensitivity of extracellular vesicles subpopulations to different concentrations of various detergents. Interestingly, we found that microvesicles and apoptotic bodies had similar and higher sensitivity to detergent lysis than the smallest sized extracellular vesicles, exosomes.

Together, all these observations may contribute to a more comprehensive detection of extracellular vesicles. Protein to lipid ratio that we introduced to the field, may prove useful as a quality control parameter for extracellular vesicle preparations both in basic research and in clinical studies.

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9. ÖSSZEFOGLALÁS

Az extracelluláris vezikulák (mint pl. az exoszómák, mikrovezikulák és apoptotikus testek) az elmúlt években igen nagy érdeklődést váltottak ki a biológia számos területén amiatt, hogy komplex információhordozóként működnek, továbbá annak kapcsán, hogy autokrin, parakrin és endokrin intercelluláris hírvivőként képesek a sejtek működését szabályozni. Az extracelluláris vezikulákra irányuló egyre intenzívebb kutatások ellenére nagy szükség van a jellemzésükre alkalmazható alapmódszerek továbbfejlesztésére.

PhD munkám célja az extracelluláris vezikulák detektálására és karakterizálására alkalmas új alapvető módszerek és módszerkombinációk keresése.

Az extracelluláris vezikulákat sejtvonalakból felülúszójából és humán vérplazmából izoláltuk differenciálcentrifugálással és hidrosztatikus filtrációval. Tunable resistive puls sensing („hangolható rezisztív impulzus érzékelő”) módszerrel, transzmissziós elektron mikroszkópiával és áramlási citometriával jellemeztük őket. Elsőként alkalmaztuk lipid assay-t az extracelluláris vezikulák lipidtartalmának meghatározására. Az extracelluláris vezikulák lipidtartalmának mérésére adaptált 96 lyukú lemez alapú szulfo-foszfo-vanillin kolorimetriás assay gyors és egyszerű lehetőséget kínál az extracelluláris vezikulák jellemzésére. Az új lipid assay és a Micro BCA fehérje assay együttes használatával minden extracelluláris vezikula szubpopuláció esetében jellegzetes fehérje/lipid arányt tapasztaltunk. Igazoltuk, hogy az extracelluláris vezikulák membránjának károsodása vagy a vezikula preparátum fehérjeszennyeződése hatására ez az arány lényegesen megváltozik. Emellett sajátos különbséget találtunk a membrán lipid rendezettségben a különböző vezikula szubpopulációk között ratiometrikus spektrofotometriás vizsgálatokkal. Végül, a differenciál detergens lízisnek a tunable resistive pulse sensing módszerrel illetőleg áramlási citometriával való kombinációjával elsőként határoztuk meg az extracelluláris vezikula alpopulációk érzékenységét különböző detergensek általi lízisre. Igazoltuk, hogy a mikrovezikulák és apoptotikus testek egymáshoz igan hasonló módon érzékenyebbek a detergens lízisre mint a legkisebb méretű extracelluláris vezikulák, az exoszómák.

Eredményeink hozzájárulhatnak az extracelluláris vezikulák pontosabb detektálásához. A bevezetett protein/lipid hasznos lehet az extracelluláris vezikulák

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„quality control” paramétereként az extracelluláris vezikulákra irányuló alapkutatási és klinikai kutatási vizsgálatok során.

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