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Contributions of the author to each publication

Paper I: Developed the concept. Designed the experiments. Optimized and developed the methodology. Carried out experiments. Data collected and analyzed. Wrote the manuscript.

Paper II: Contributed to developing the concept. Optimized the methodology. Performed all experiments involving NTA and zeta potential (ZP) measurements. Data (NTA and ZP) collected and analyzed. Participated in the writing and reviewing of the manuscript.

Paper III: Developed the concept. Designed the experiments. Optimized and developed the methodology. Performed all experiments. Data collected and analyzed. Wrote the manuscript.

ABBREVATIONS

AFM	Atomic force microscopy
BFF	Bovine follicular fluid
CMG	CellMask® green
CCD	Charge-coupled device
CMC	Critical micelle concentration
DDS	Drug delivery system
DLS	Dynamic light scattering
EDL	Electrical double layer
EPM	Electrophoretic mobility
ESCRT	Endosomal-sorting complex required for transport
EC	European commission
EVs	Extracellular vesicles
FBS	Fetal bovine serum
FC	Flow cytometry
FL-NTA	Fluorescence nanoparticle tracking analysis
fl-NPs	fluorescent nanoparticles
HDL	High-density lipoprotein
ISEV	International society for extracellular vesicles
ILVs	Intraluminal vesicles
JAr	human choriocarcioma cells
LDL	Low-density lipoprotein
MVs	Microvesicles
MWCO	Molecular weight cut-off
MVBs	Multivesicular bodies
NMs	Nanomaterials
NPs	Nanoparticles
NTA	Nanoparticle tracking analysis
nSMase2	neutral sphingomyelinase 2
PS	Phosphatidylserine
PM	Plasma membrane
PDI	Polydispersity Index
PEG	Polyethylene glycol
PsV	Psoriasis vulgaris
PsA	Psoriatic arthritis
SEM	Scanning electron microscopy
SEC	Size-exclusion chromatography
TFF	Tangential flow filtration
t-NPs	total nanoparticles
TEM	Transmission electron microscopy
TRPS	Tunable resistive pulse sensing
UC	Ultracentrifugation
ZP	Zeta potential

1. INTRODUCTION

With growing interest in nanotechnology, various nano-based products and applications have been developed. The use of these nanotech-based materials for medical purposes termed as nanomedicine. Basically, nanomedicine refers to the use of nanoparticles (either of biological or non-biological origin) for the diagnosis, monitoring, prevention and treatment of diseases. Nanoparticles (NPs) have unique characteristics such as size, surface properties, shape, stability, and composition that make them relevant to physiological interactions. The unique physicochemical properties of NPs allow for a wide range of applications in modern medicine and other fields. A few examples of NP applications in biology and modern medicine are fluorescent biological labels, drugs and gene delivery, protein detections and cancer therapy. NPs are useful and have potential applications, but their entry into a physiological environment can present adverse effects, especially in terms of toxicity. For instance, liposome-based drugs showed inherent health problems such as rapid dissolution in the biological environment, poor storage, and inability to maintain stability. This and other unmentioned undesired effects of NPs on biomedical or nanomedicine applications, particularly to human health, lead us to seek alternative solutions for utilizing NPs in a safe manner. Extracellular vesicles (EVs), which are membrane-bound NPs secreted by most cells under physiological and pathological conditions, could offer a potential path to NP safety in the body and will potentially offer us the opportunity to exploit their unlimited potential, for example, for drug delivery system (DDS).

EVs are highly heterogeneous evolutionary conserved entities that play a significant role in transferring functional cargo to nearby or distant cells, thus facilitating intercellular communication. They are known to influence both physiological as well as pathological processes. Novel methods for EV characterization have been developed to measure the size, particle size distribution, surface charge, morphology and concentration of vesicles resuspended in a certain medium.

Naturally, EVs exist as colloidal suspensions when resuspended in media carry a net negative charge due to the presence of glycosylated proteins, lipids, among others on their surfaces. This surface charge of EVs can be estimated by measuring their electrophoretic mobility, which is the basis for calculating their (zeta potential) ZP value. ZP is one parameter used to characterize EVs. ZP can be used as a tool to predict the colloidal stability of NPs or EVs in suspension. ZP allows to assess the initial contact between the NP and the cell membrane. Following this interaction, the uptake rate is determined by the particle size. As a result, ZP and particle size may influence physiological effects. To put it another way, controlling the size and ZP is crucial to the efficient use of NPs for DDS purposes. The colloidal stability of EVs is one of the crucial parameters for understanding their fates under different conditions while maintaining physicochemical properties and cargoes. Several methods can be used to

determine the stability of EVs, including dynamic light scattering (DLS) and transmission electron microscopy (TEM). There is, however, a lack of a standardized methodological approach to EVs, specifically their colloidal stability. There are few studies conducted on the effects of different factors, such as the nature of resuspending media on the ZP of EVs despite their critical role in colloidal stability and pharmacokinetics. Detection and quantification of EVs are among the challenges in EV research. For example, conventional nanoparticle tracking analysis (NTA) is used to determine the particle size and concentration of EVs within a sample. As a result of the wide variation in sizes of EVs, conventional NTA is unable to distinguish EVs from the non-EV total population. This will further complicate already complex and heterogeneous EV entities.

In this thesis, we studied the colloidal stability of EVs under different conditions and how their interaction with fluorescent probe impacts their physicochemical properties. As model systems, we used EVs derived from cultured human JAr cells and biological sources. We determined the impact of different factors such as buffer content, detergent, ionic strength, and pH on the ZP (surface charge) of EVs secreted by the cultured human choriocarcinoma (JAr) cells. This was followed by a comparison of the ZP of EVs isolated from healthy controls and psoriasis patients (i.e. PsA – psoriatic arthritis and PsV – psoriasis vulgaris). Using fluorescence Nanoparticle tracking analysis NTA (FL-NTA), we examined the effect of CellMask® Green (CMG) labelling on the physical characteristics of JAr EVs purified in size-exclusion chromatography (SEC) and in combination with tangential flow filtration (TFF) as well as biological-derived EVs (e.g. BFF – Bovine follicular fluid and seminal plasma).

2. LITERATURE REVIEW

2.1. Nanomedicine and nanoparticles: Definition and terminology

Over the last decades, nanotechnology has been used in our daily lives. This technology has been integrated in multiple fields of study. A growing interest in nanotechnology led to the realization of a number of nano-based applications and products (Webster, 2006; Rengan *et al.*, 2015; Zdrojewicz *et al.*, 2015). The use of these nanotech-based materials for medical purposes is termed as nanomedicine. Simply put, nanomedicine can be defined as the use of nanomaterials (i.e. biological and non-biological origins) to diagnosis, monitoring, prevention and treatment of diseases (Tinkle *et al.*, 2014; Turanlı and Everest, 2016). Nanomedicine also utilizes the different properties of NPs alongside nano-based components and biomedical devices for the purposes listed above (Riehemann *et al.*, 2009).

NPs are materials generally having dimensions in the range of nanoscale, e.g., under 100 nm (Murthy, 2007). In recent years, these materials showed their potential in clinical applications. Thanks to their unique physicochemical properties, NPs can have impact on their physiological interactions, ranging from molecular to systemic level (Lin *et al.*, 2014). NPs do also have limitations, such as exhibiting cytotoxic effects in living organisms, when they are used in clinical settings (Castiglioni, 2014; Takamiya *et al.*, 2016). However, the importance of NPs outweigh their disadvantages and make them an ideal players in modern medicine (Murthy, 2007; Verma and Stellacci, 2010).

Currently, there is no universally accepted definition for either nanomaterials (NMs) or NPs in the scientific community (e.g. in academy and industry). But some efforts have been made to reach a consensual definition due to NPs own novel physicochemical properties that differ significantly from the bulk materials, mainly in terms of their small size. Based on European commission (EC) recommendation, NPs defined as materials that are natural, incidental or fabricated containing particles either in unbound or aggregated state, where one or more dimensions are in the size range of 1–100 nm for half-portion (fifty percent) of the particles (Commission Recommendation, 2011; Bleeker *et al.*, 2013). The EC recommendation for NM definition used mainly as a reference to determine if certain materials considered as NM or not, without classifying them as a hazardous or safe. Other regulatory agencies, such as Food and Drug Administration, recommends the need for further evaluations, safety and effectiveness of nano-based products for betterment of public health (FDA, 2014).

To better understand the distinctive nature of NPs, it is worth to define them basis of their dimensional aspects. NPs can also be considered as zero-dimensional nanomaterials. This definition is linked with NPs having all of their dimensions in the nanoscale range, in contrast to one-dimensional NMs, which

have one dimension above the nanoscale. For instance, this can be seen in nanowires and nanotubes. On a similar note, two-dimensional NMs possess two dimensions higher than the nanoscale and this is also exhibited in self-assembled monolayer films (Murthy, 2007; Soares *et al.*, 2018). Additionally, NPs are composed of three layers: surface layer, shell layer and the core (i.e. the particle itself). Most surface modification and functionalization is introduced at the surface layer using a wide range of small molecules, surfactants and ions. The shell layer and core have distinct content material-wise and the core as the name stands it is the center of the NP (Khan *et.al*., 2019). The size, dimension, different layers and other unexplored properties of NPs make them an interesting nanoscale based entities for many scientific designs and developments.

2.2. Physicochemical characteristics of NPs

NPs possess unique physicochemical properties compared to bulk materials. NPs have unique characteristics such as size, surface properties, shape, stability, and composition that make them relevant to physiological interactions (Patri *et al.*, 2006; Cruz *et al.*, 2018; Ray *et al.*, 2021). Understanding physiological interactions of NPs such as with membranes, biological molecules, metabolic pathways, immune system can lead to developing medical applications that entails improved therapeutic efficacy, minimal side effects and treatment options (Farrera and Fadeel, 2015; Zanganeh *et al.*, 2016; Liu *et al.*, 2019; Tian *et al*., 2020). As such, it is crucial to understand how the different physicochemical characteristics of NPs impact their *in vivo* distribution and behaviour. Despite this, there is no list of minimum requirements. NP characteristics such as size, shape, surface area, surface functional groups, charge, zeta potential, composition can be the first step in their characterization (Lin *et al.*, 2014; Guerrini *et al.*, 2022; Younis *et al.*, 2022).

Size: The size of NP affects interactions with the biological system and additional biological NP-related mechanisms, such us cellular uptake via different routes (Anselmo *et al.*, 2015; Palomba *et al.*, 2018). In engineered NPs, for instance, size is an important factor in regulating the circulation and navigation of NPs in the bloodstream, passing through the physiological (drug) barriers and inducing cellular response (Jiang *et al.*, 2008; Maiorano *et al.*, 2010). Additionally, NPs have a relatively large surface area to volume ratio, and this attribute makes them to more easily interact with cell membranes due to the increase in their contacting surfaces (Huang, Cambre and Lee, 2017). Overall, the small sized NPs (30–50 nm) can have toxic effects as it was reported in different cell lines (Kim *et al.*, 2012) and other biological settings (Horváth *et al.*, 2013). Due to the vast differences in the characteristics of different NPs, it is imperative to determine if size and/or shape are directly related to toxicity.

Surface properties of NPs: The surfaces of NPs are important when NPs interact with surrounding species or environments. Nanomedicine considers the surface properties of NPs to be the key players in biological systems (Patri *et al.*, 2006; Walkey *et al.*, 2012; Bantz *et al.*, 2014). NP's surface composition, charge, energy, and species absorbance or adhesion are a few of the common parameters for determining surface properties (Brodbeck *et al.*, 2001; Vertegel, Siegel and Dordick, 2004; Powers *et al.*, 2006). As an example, NP's superficial layers benefit from the surface composition more than bulk materials. In response to surface energy, NPs could undergo dissolution, aggregation, or agglomeration. As for the surface charge, the way the charges on NP's surface are distributed can affect their ability to bind to receptors and pass through physiological barriers. This means that surface charge determines the stability of NPs in dispersion, which is measured by their zeta potential. There is no feasible way to study the full spectrum of surface parameters of NPs, but individual NP systems can be prioritized according to the surface parameters, whether they are synthetic or natural origins.

Molecular compositions of NPs: NPs can be also classified based on their structure types. These NPs can come in many structural forms, such as liposomes, micelles, virosomes, vesicles, carbon nanotubes, etc. Different types of NPs commonly contain different organic polymers, like organic compounds, lipids, proteins, DNA, and RNA, depending on the origin of the NPs (Wang *et al.*, 2012). Recent studies have identified the toxicological issues associated with NP compositions (Radomski *et al.*, 2005). In addition to the above-mentioned physicochemical characteristics of NPs, the biochemical composition of NPs can play a part in determining the toxicity of NPs. For instance, various cell lines treated with NPs of different compositions and sizes induce intracellular responses with variable toxicity (Sohaebuddin *et al.*, 2010; Nguyen *et al.*, 2015). Additionally, maintaining NP stability for longer periods of time is a challenge for NP-based applications. For instance, in nanomedicine, NP stability is affected by many factors, including the nature of suspensions, pH, particle size distribution, enzymatic degradation, NP-NP interactions, and temperature (Wijenayaka *et al.*, 2015; Zhao *et al.*, 2018; Phan and Haes, 2019). In the later chapters, we will explore the effect of the above-mentioned characteristics of NPs on cellular uptake and toxicity.

2.3. NP interactions: NP-NP and NP-biomembrane interface

The interaction between NPs can be affected by attractive and repulsive forces as well as characteristics of suspending medium where particles interact at solid-liquid interfaces. Distance and surface geometry can also determine the nature of interactions between NPs (Walker *et al.*, 2011). In another scenario, when NPs come into contact with a biological membrane or environment, they can cause unwanted effects such as aggregation, coagulation, adsorption and

morphological changes (Nel *et al.*, 2009). Thus, understanding the interactions of NPs with biological membranes plays a crucial role in reducing the risks posed to the human health (Verma and Stellacci, 2010; Shang, Nienhaus and Nienhaus, 2014). These interactions can be seen from two perspectives: Biological and molecular processes in different diseases occur at the nanoscale as a result of misfolded proteins and altered genes. A better understanding of these processes will lead to the development of engineered NPs that will target the necessary specific action in the body (Kim *et al.*, 2012; Lu *et al.*, 2015). Second, when the surface of NP is in contact with the biological medium, biomolecules derived from the biological medium will cover the surface of NP. This nano-biointerface is known as NP corona, and it has extreme importance to understanding how NPs interact with cells to cause biological responses (Lynch and Dawson, 2008; Salatin *et.al.*, 2015). On the surfaces of NP, proteins and nucleic acids are among the targets for biomolecular interactions (Niemeyer, 2001) (Fig. 1). Proteins with multiple binding sites interact with NP surfaces in different ways, such as specific or non-specific adsorption (Firkowska-Boden, Zhang and Jandt, 2018; Gago *et al.*, 2022). For instance, the soft protein layer or corona on the NP surface has low structural stability and provides active sites for NP surface interactions apart from other physicochemical features (Lundqvist *et al*., 2004). It is important to remember that among the physicochemical properties of NPs, the surface properties play a major role in initiating protein-mediated cellular responses when NPs interact with proteins bound to receptors on cell membranes (Liu and Webster, 2007).

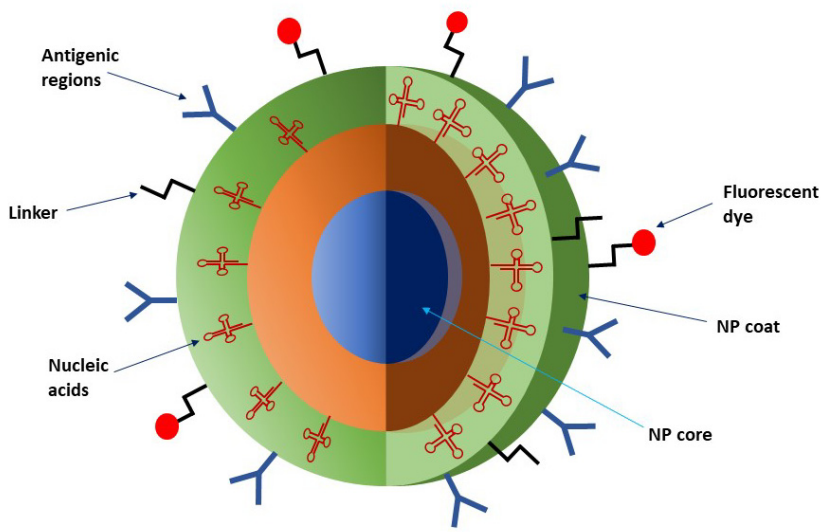

Figure 1. General scheme of a NP. Proteins and nucleic acids can interact with the surfaces of NPs. Surface manipulation of NPs can also be used to produce multifunctional NPs.

The NP-biomembrane interactions can be influenced by the NP's physicochemical properties. Plasma membranes are usually composed of phospholipids and membrane proteins (de Meyer and Smit, 2009; Yue and Zhang, 2012; Porat-Shliom, Weigert and Donaldson, 2013). To maintain cell integrity, cell membranes must remain impermeable to large molecules and NPs. Yet, the adsorption of NPs onto the membrane is still capable of affecting the nature and properties of the membranes, including their fluidity, tension (stiffness), fusion (curvature), and lipid intercalation (insertion) in/on them. The adsorption of NPs also alters the morphology of the cell membrane (Aranda-Espinoza *et al.*, 1996; Li *et al.*, 2012) and leads to different membrane responses. The NP properties, coupled with the nature of the suspension medium, can govern the NP-biointerface, which can result in changes in biological behavior and influence cell fates (Nel *et al.*, 2009; Monopoli *et al.*, 2012).

2.4. Characterization and evaluation methods of NPs

NPs are characterized by their sizes, surface charges, and molecular compositions. This is not the only basis for characterizing NPs since their states (for example, solid form or resuspended in solution) as well as their interactions with biological systems, both *in vitro* and *in vivo*, need also be included. NPs can be characterized using a wide variety of techniques similar to those used to characterize well-established pharmaceuticals (or nanoscale-based small molecules) (Mourdikoudis et al., 2018; Titus *et al.*, 2019; Chinecherem Nkele and I. Ezema, 2021). Due to the unique properties and attributes of NPs, different approaches can be used to categorize and evaluate these properties. Among them are counting methods, which offer the capability of measuring particle size, distribution, and concentration of particles using DLS, and NTA (Filipe *et.al*., 2010; Lai *et al.*, 2015). For the evaluation of NP morphologies, microscopic methods such as scanning electron microscopy (SEM) and TEM are used (Ponce, Mejía-Rosales and José-Yacamán, 2012; Michen *et al.*, 2015). Zeta potential (ZP), which is a parameter that describes the surface charge of particles resuspended in a medium. Based on the principle of electrophoresis, the electrophoretic mobility of particles in dispersion can be used to derive the ZP of NPs (Xu, 2008; Clogston and Patri, 2011). To fully understand the unique properties of NPs, advanced characterization tools are necessary in conjunction with the advent of nanotechnology to characterize the NP's properties effectively. Different methods used for predicting NPs' physicochemical properties are described in the literature (Joudeh and Linke, 2022). Some of these selected methods for evaluating NPs are discussed in the later chapters of this thesis.

2.5. Applications and toxicity of NPs

The unique physicochemical properties of NPs allow for a wide range of applications in modern medicine and other fields. A few examples of NP applications in biology and modern medicine are fluorescent biological labels (Wang *et al.*, 2006), drugs and gene delivery (Prabu, Suriyaprakash and Thirumurugan, 2017; Li *et al.*, 2020; Mitchell *et al.*, 2021), protein detections (You *et al.*, 2007; Nietzold and Lisdat, 2012; Retout *et al.*, 2016), optical biotagging in biological assays (Perevedentseva *et al.*, 2020; Lin *et al.*, 2022), and cancer therapy (Yao *et al.*, 2020; Yang *et al.*, 2022). Of the mentioned applications of NPs, their use in drug delivery is the most prominent. This is mostly due to the wide adoption of polymeric and liposomal drug delivery systems in clinical settings (Murthy, 2007). Liposomes are artificial vesicles with at least one phospholipid bilayer. Due to their small size, hydrophilic and/or hydrophobic nature, and biocompatibility, liposomes are suitable for use as DDS (Gonda *et al.*, 2019). There are currently more than 20 liposome-based formulations in pre-clinical and clinical trials. These liposome formulations are used in the treatment of different types of cancer such as lung (Ahn *et al.*, 2014), metastatic pancreatic (Pelzer *et al.*, 2017), ovarian (Graziani *et al.*, 2017), liver tumor (Lyon *et al.*, 2018), soft-tissue sarcoma (Bonvalot *et al.*, 2019) and other diseases (Adams *et al.*, 2018; Krauss *et al.*, 2019). Some of the current liposome formulations used in clinical trials are described in the literature (Beltrán-Gracia *et al.*, 2019; Taléns-Visconti *et al.*, 2022). Several ligands (drugs) can be encapsulated within liposomes in order to improve their pharmacokinetics and to reduce the clearance of the drugs by immune cells. This results in a long residence and circulation time in the blood. Liposomes are also able to effectively interact with mammalian cell membranes due to their structural similarity. For example, MM-302, a liposomal formulation of doxorubicin conjugate with antibody, specifically targets HER2 overexpressing breast cancer cells (Miller *et al.*, 2016). Several metal NPs were investigated as potential antimicrobial or antiviral agents. Silver NPs were used against bacteria and viruses, for instance, as a result of their antimicrobial and antiviral activities (Galdiero *et al.*, 2011; Rai *et al.*, 2014; Urnukhsaikhan *et al.*, 2021). It has been demonstrated that biodegradable NPs can be efficiently used as vehicles for gene or drug delivery, improving pharmacokinetics and distribution (Zhang and Saltzman, 2013).

NPs are useful and have potential applications, but their entry into a physiological environment can present adverse effects, especially in terms of toxicity. Some of the examples of toxicity of NPs are mentioned below. Liposome-based drugs showed inherent health problems such as rapid dissolution in the biological environment, poor storage, and inability to maintain stability (Khan *et.al*., 2019). The toxicity of zinc oxide NPs on kidney function was assessed (Raisi Dehkourdi *et.al.*, 2017). In this study, zinc oxide NP exposure and its adverse effect on renal function were toxic compared to polyethylene glycol (PEG)-coated NPs. In another example, the nature of the

NP surface determines how NP well interacts with cells and lipid bilayers (Verma and Stellacci, 2010). Alterations to the surface of NP can change their optical, electrical, or physicochemical properties, leading to their cytotoxic reactions by affecting cellular accumulation, distribution, and toxicity (Favi *et al.*, 2015). This and other unmentioned undesired effects of NPs on biomedical or nanomedicine applications, particularly to human health, lead us to seek alternative solutions for utilizing NPs in a safe manner. EVs, which are membrane-bound NPs secreted by most cells under physiological and pathological conditions, could offer a potential path to NP safety in the body and will potentially offer us the opportunity to exploit their unlimited potential, for example, for drug delivery.

2.6. Extracellular vesicles (EVs)

2.6.1. Background and biogenesis

Vesicles were discovered in the 1940s, but the term 'exosomes' was used in EV research for vesicles that were isolated from a conditioned medium of sheep reticulocytes. It was found that the isolated vesicles had transferrin receptors, as well as characteristics common to reticulocyte plasma membrane (PM). The authors suggested that exosome release into the external environment might be a mechanism for the shed of distinct membrane functions (Johnstone *et al.*, 1987). Not only mammalian but also chicken embryonic reticulocytes have been shown to secrete transferrin receptor-carrying exosomes, indicating that exosomes are involved in the clearance of obsolete PMs (Johnstone *et al.*, 1991).

EVs are nanosized membrane-bound vesicles that are secreted by most cell types during physiological and pathological conditions. EVs are highly heterogeneous evolutionary conserved entities (Deatherage and Cookson, 2012) that play a significant role in transferring functional cargo to nearby or distant cells, thus facilitating intercellular communication (Valadi *et al.*, 2007; Men *et al.*, 2019). They are known to influence both physiological (Nolte-‘t Hoen *et al.*, 2009; Zhang *et al.*, 2015) as well as pathological processes (Zhang *et al.*, 2015; Li *et al.*, 2018; Osaki and Okada, 2019). EVs are present in cell culture supernatants and in biological fluids, such as serum, urine, and seminal plasma (Caby *et al.*, 2005; Lässer *et al.*, 2011; Hu *et al.*, 2022).

International Society for Extracellular Vesicles (ISEV) categorized EVs to exosomes, microvesicles and apoptotic bodies, based on their size and mechanisms of biogenesis and secretion (Théry, Ostrowski and Segura, 2009; Yáñez-Mó *et al.*, 2015; Théry *et al.*, 2018; Pegtel and Gould, 2019). EVs may also be differentiated by their biophysical properties, including their density, molecular composition, and functions. Exosomes are the smallest subtype of EVs, with diameters ranging from 30 to 150 nm. Exosome biogenesis begins within endosomal compartments. The exosomes are formed by inward budding of the

PM into early endosomes, whose endosomal membranes invaginate and bud to generate intraluminal vesicles (ILVs) within the organelles' lumen (Morvan *et al*., 2012). The matured late endosomes that carry ILVs are commonly known as multivesicular bodies (MVBs). During their transit to the trans-Golgi network for endosome recycling, MVBs have two fates. These MVBs are either delivered to lysosomes for proteolytic degradation or fuse with the PM and are secreted as exosomes in the extracellular milieu (Williams and Urbé, 2007; Wang *et al*., 2011; Koike and Jahn, 2019)(Fig. 2). The biogenesis and secretion of exosomes (ILVs) are a coordinated process that is driven by an endosomal-sorting complex required for transport (ESCRT) dependent and ESCRT-independent pathways (Trajkovic *et al.*, 2008; Stuffers *et al.*, 2009; Grassart *et al.*, 2014). Microvesicles (MVs) are highly heterogeneous membrane vesicles with diameters ranging from 100 to 1000 nm. MVs originate from outward budding and blebbing of the PM. What caused the shedding of MVs from PM is associated with a multitude of factors. These factors include the reorganization of phospholipids, the protruding of the phosphatidylserine (PS) to the outer surfaces, and shrinkage of the actin-myosin compartment (Muralidharan-Chari *et al.*, 2009). Apoptotic bodies are the largest EVs with a diameter range of 1000–5000 nm and secreted as blebs from apoptotic membrane when a cell undergoes apoptosis (van Niel, D'Angelo and Raposo, 2018). The presence of different machineries and intracellular sorting pathways involved in exosome and MV biogenesis might linked with their heterogeneity nature in terms of size and molecular contents. Thus, ISEV recommends to adopt "EVs" as a collective term to represent exosomes and microvesicles to avoid in its naming or nomenclature of these type of vesicles (Théry *et al.*, 2018).

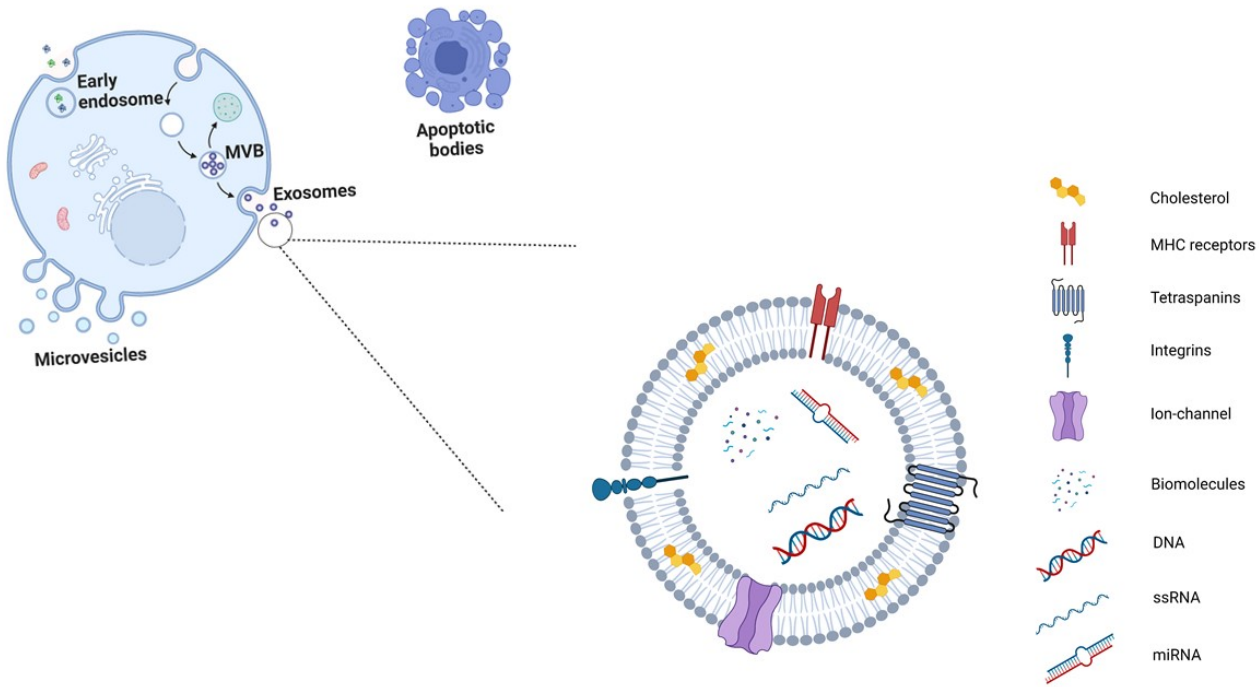

Figure 2. Biogenesis of EVs. Exosomes, microvesicles, and apoptotic bodies are classified according to their size and mechanisms of biogenesis and secretion. Cell-culture conditioned media and biological fluids can be used as sources of EVs. EVs carry functional cargoes that could serve as promising delivery vehicles for medications.

2.6.2. Isolation of EVs

The purpose of a successful isolation method holds both the enrichment of EVs from a given sample (e.g., cell-culture conditioned media to biofluids) and removal of non-EV material, for obtaining isolated samples with high yield and purity with minimal loss of EVs (Théry *et al.*, 2018; Witwer *et al.*, 2021). Various EV isolation approaches employ principles that focus on the general EV characteristics, such as their size, density, charge and proteome. It is worth remembering that isolation of EVs from biological sources is challenging due to the complex nature of body fluids. Each biofluids offers its own complexities ranging from the presence of high protein content (e.g. milk) to low concentration of EVs-derived from urine or lipoproteins that excel EVs number. For example, isolation of EVs from blood is impacted by duration between blood collection and processing, isolation method used, highly viscous nature of blood, co-sedimentation of high-density lipoprotein (HDL) and low-density lipoprotein (LDL) proteins (Yuana *et al.*, 2014; Sun, Saito, and Saito, 2019; Brennan *et al.*, 2020). Some of the currently used EV isolation methods are described below:

I. *Centrifugation-based techniques*: Centrifugation is one of the common and gold standard EV isolation methods (Zarovni *et al.*, 2015). Centrifugation involves the use of centrifugal force to sediment the particle of interest based on the difference in size, shape and density. In other words, centrifugation separates particles, in this case vesicle sub populations, on the basis of different sedimentation coefficients. Because larger and dense particles have high sedimentation coefficient and they tend to sediment in a short time and the opposite is true for smaller and less-dense particles. This can be observed in case of differential centrifugation, where multiple sequential centrifugation steps are used for different purposes (e.g. removal of cells and cell debris to pelleting larger vesicles or apoptotic bodies) with the increment in the centrifugal forces (Livshits *et al.*, 2015).

   Isolation of vesicles using centrifugation is also affected by the initial sample volume, its viscosity (Momen-Heravi *et al.*, 2012), temperature, duration of centrifugation steps as well as the choice of rotor type (e.g., fixed angle or swing-out) (Cvjetkovic *et.al*, 2014). Because of the complex nature of vesicles, the complete separation of vesicles in terms of a known size or density is still ideally unattainable**.**

   It is also worth to remember that the use of high centrifugal force, such as in the range of 100,000 to 200,000 xg, may lead to vesicle fusion as well as co-sedimentation of the vesicle pellets with proteins, and promote EV aggregation, thus influencing TEM and further downstream EV studies (Van Deun *et al.*, 2014; Linares *et al.*, 2015; Yang *et al.*, 2017). Because of the effect of ultracentrifugation (UC) on the purity and downstream assays, it is recommended to merge UC with density gradient centrifugation and/or other techniques. By doing so, EV containing pellet is resuspended and further separated by flotation into preconstructed density gradient made

from either sucrose (K. Li *et al.*, 2018) or iodixanol cushions (Onódi *et al.*, 2018). A note of caution is due here since the above combinations do not guarantee an absolute EV purity, which is resulting from the overlap of common contaminants (e.g. low or high density lipoproteins) with those of EVs (Karimi *et al.*, 2018).

II. *Size-based techniques*: Filtration-based isolation largely dependent on the size and molecular weight cut-off (MWCO) of the nanomembrane being used. Ultrafiltration being one of size-based techniques, it can employ (nano)membrane filters with unlimited/ specific MWCO or size exclusion limit to separate EVs from cell-culture or biofluids. In other words, particles larger than the MWCO of the membrane are retained by the filter, whereas particles smaller than the MWCO are permeated through the filter as waste (Li *et al.*, 2017). Tangential Flow Filtration (TFF) has also similar working principle with that of conventional ultrafiltration. In TFF, EV containing sample solution is tangentially flow over a defined hollow fiber MWCO (~ 100 or 500 kDa) filter to permeate smaller particles (soluble proteins and other contaminants), while retaining concentrated EV sample in the retentate. Unlike conventional ultrafiltration, the EV isolation by TFF prevents clogging and vesicle trapping on the filter unit (Heinemann *et al.*, 2014). However, EV isolation by TFF yields EVs with high protein contamination and there are some concerns on the effect of transmembrane pressure on filtration membrane preserving EV integrity. To avoid this situation, it is recommended to apply a monitored and regulated trans-membrane pressure (shear stress) on the membrane not to cause deformation and lysis of EVs (Zeringer *et al.*, 2015; Li *et al.*, 2019).

    EV isolation by size-exclusion chromatography (SEC) is based on size and the working principle is adopted from the separation of peptides from amino acids. In SEC, the starting sample (e.g. conditioned media/ biofluid) is used as a mobile phase, while the porous gel filtration polymer (e.g. sepharose) used as a stationary phase. In a SEC column, the smaller particles can enter the resin pores of the stationary phase, leads to an increased retention time and delaying elution. Whereas, larger particles (EVs) that cannot enter the pores are excluded and elute before the smaller particles (proteins etc.) (Böing *et al.*, 2014; Gámez-Valero *et al.*, 2016). Though SEC isolation method preserve the vesicle integrity, it fails to efficiently separate EVs from lipoproteins of similar sizes in the case of purifying serum and plasma-derived EVs**.**

III. *Immunoaffinity-based approaches*: Immunoaffinity capture-based approaches can also be used to isolate EVs from cell-culture supernatants or complex biofluids based on the antigen expression on the surface of EVs. Immunoaffinity capture methods rely on using the interaction between specific protein on the EV surface and magnetic beads (resins) coated with antibodies. In other words, once the specific EV surface protein is identified, a specific EV subtype can be captured using receptors immobilized on the magnetic beads. Since affinity-based isolates EVs by selective

receptor-ligand interactions, it results in high specificity and purity for subset of EVs (Sharma *et al.*, 2018). Depend on the intended purpose of isolation, a variety of proteins, such as tetraspanins (CD9, CD63, CD81) and cancer-related markers (EpCAM, ASGR1, Tim4) can be targeted (Conde-Vancells *et al.*, 2008; Tauro *et al.*, 2012; Nakai *et al.*, 2016).

Despite yielding isolates of high purity and specific EV subtypes, immunoaffinity isolation heavily depend on the choice of high-affinity antibodies and running costs. In addition, the biological activity of isolated EVs can be affected by the use of non-neutral pH and non-physiological salt concentrations in the affinity-based methods (Zhang *et al.*, 2018).

IV. *Polymer-based precipitation*: Precipitation is an effective method to concentrate and precipitate viruses and other small particles. The biophysical properties shared by viruses and EVs led to the adoption of precipitation method for the enrichment of EVs. In general, precipitation of EVs is done using hydrophilic polymers, such as PEG into the sample. Upon the addition of PEG, the solubility and hydration of EVs reduced and precipitation occurs (Zeringer *et al.*, 2015). After the completion of precipitation process, EVs can be pelleted by centrifugation at lower speeds. Though PEG-based EV isolation method is rapid and simple to use, the PEG polymer can also precipitate other extracellular proteins and lipoprotein aggregates (Macías *et al.*, 2019). This is why a second isolation method is recommended following PEG treatment of EVs to avoid any interferences of precipitating agents in the biological activity of EVs (Paolini *et al.*, 2016). Commercially available isolation kits that have been developed, such as ExoQuick and Total Exosome Isolation kits that also work based on the precipitation of EVs.

V. *Microfluidics-based isolation*: Microfluidic platforms are one of the emerging technologies for EV isolation from small sample volumes. Microfluidic devices have been developed for immuno-capture using EV targeting antibodies immobilized on a microfluidic chip. Aptamers and oligonucleotides can be used as alternative to antibodies for affinity capturing and provide a reporter signal upon EV surface binding (Zhou *et al.*, 2016; Xu *et al.*, 2020). Microfluidic devices allow the integration of innovative sorting mechanisms such as acoustic trapping, size, electrophoretic and electromagnetic techniques for EV isolation (Davies *et al.*, 2012; Santana *et al.*, 2014; Lee *et al.*, 2015; Liu *et al.*, 2017). The advent of microfluidic-based isolation platforms plays a vital role in bringing diagnostic and therapeutic potentials of EVs to clinical practice.

2.6.3. Characterization of EVs

Selection of EV isolation method is equally as important as characterization of EVs. Firstly, a successful EV isolation is verified after detection of EVs. Secondly, quantifying (size, concentration) and characterizing the molecular composition of EVs provide insights about the physical and content of the isolated EVs. Novel methods for EV characterization have been developed to measure the size, particle size distribution, surface charge, morphology and concentration of vesicles resuspended in a certain medium. These include atomic force microscopy (AFM) (Ju *et al.*, 2020; Yurtsever *et al.*, 2021), NTA (Dissanayake *et al.*, 2021), and resistive pulse sensing (Maas, Broekman and de Vrij, 2017). These analytical characteristics of EVs, which is based on EV's physical characteristics and their molecular cargoes, is explained below.

Nanoparticle tracking analysis: NTA is one of the techniques used to characterize NPs in suspension. NTA measures the size and concentration of particles in suspension. Similarly to DLS, NTA records the Brownian motion of particles in suspension. Particles in suspension are illuminated with a laser light and the scattered light of the particles is captured using a light-sensitive camera (CCD – Charge-coupled device). The software tracked the mean-square displacement of particles per time interval and calculated the size of each particle individually using Stokes-Einstein equation (Fig. 3). Note that NTA do not only determine the size distribution of particles but it can also measure their concentration in the given sample (Dissanayake *et al.*, 2021; Vogel *et al.*, 2021; Auger *et al.*, 2022).

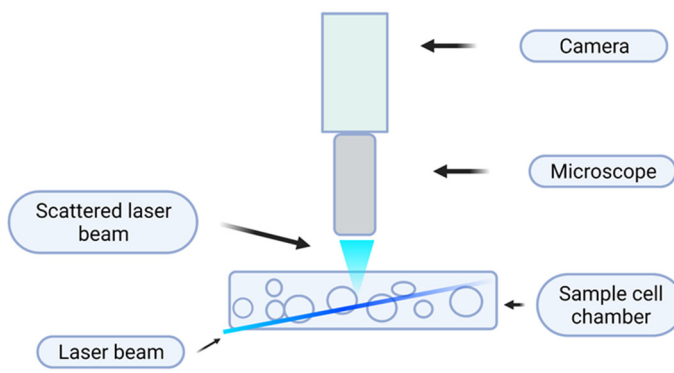

Figure 3. Principle of NTA measurements of NPs. NTA records the Brownian motion of particles in suspension that are illuminated with laser light. NTA video camera captures the scattered light of the particles. Using the Stokes-Einstein equation, the mean-square displacement and size of particles can be calculated.

NTA can determine vesicles in the size range of 10–1000 nm in diameter, which falls within the size of EV subsets (i.e. exosomes and microvesicles). The challenge with NTA is that it fails to distinguish between EVs and other particles of

similar size (Szatanek *et al.*, 2017). This scenario is surfaced when most EV isolation methods are contaminated with protein aggregates and other NPs. This is why the adoption of fluorescence to NTA hopes to address this shortcoming. Using lipophilic dyes or fluorochrome-conjugated antibodies, fluorescence-NTA (FL-NTA) can detect fluorescently-labeled NPs enclosed by lipid membranes or enhanced with EV surface protein markers (Carnell-Morris *et al.*, 2017; Thane *et al.*, 2019).

Flow cytometry: Flow cytometry (FC) is a high-throughput technology, which is capable of analyzing the presence of different markers expressed on the surface of EVs. However, conventional FC has a limitation in detecting smaller particles less than 100 nm owing to its limited sensitivity and resolution. To circumvent this obstacle, EVs are adhered to magnetic carrier particles or latex beads that are coated with antibodies which recognize antigens on EVs (Suárez *et al.*, 2017). In some cases, double labelling of EVs with lipophilic dyes and proteins enhance signal intensity as well as distinguishing EVs from non-EV contaminants (Baj-Krzyworzeka *et al.*, 2006). Next-generation flow cytometers need to allow accurate detection of EVs at single particle level (Tian *et al.*, 2018; Choi *et al.*, 2019; Welsh *et al.*, 2020; Salmond *et al.*, 2021).

Microscopy: Microscopy-based approaches are used to assess the morphology of EVs. Accurate EV imaging involves the deployment of high tech imaging methods with resolution detection limits. Due to prior sample preparation to EV imaging, not all visualization methods are capable of revealing the inherent EV morphology. Electron microscopy, which commonly holds TEM and SEM, is the prominent choice for visualization of EV morphologies. TEM being the standard imaging technique that entails internal information of EVs when beams of electron pass thorough the EV sample, while in SEM scattered electrons are detected to obtain info about the surface topography of EVs (Skoog *et.al.*, 1998). TEM analysis is circumvented by extended sample preparation steps, which can lead to dehydration of EVs, and likely to cause EVs to have a hole in their cup-shaped morphologies (Wu *et al.*, 2015). To overcome EV sample deformations in TEM, cryo-electron microscopy is used to maintain the native spherical shape as EV samples still remain in their native hydrated state (Cizmar and Yuana, 2017). Information about the three-dimensional of EVs can be assessed by using AFM. In AFM, EVs can be either immobilized on surfaces or performed in liquid, which likely maintain their physical characteristics. Super-resolution microscopy can also be used to visualize EVs in three dimensions and localize tetraspanin complexes on the surface of single EVs (McNamara *et al.*, 2022).

Tunable Resistive pulse sensing (TRPS): Using TRPS, particles in the size range of 100 nm to 100 μm and their concentration can be measured in suspension. TRPS equipped with different pore sizes have been used for the measurement of size and concentration of EVs (Salmond and Williams, 2021). In principle, TRPS has two fluid cells connected with electrodes, one carrying suspension and the other half cell contains particle free electrolyte. The cells are connected with a non-conductive nanomembrane. To measure the size and

concentration of resuspended particles, an electric field is applied onto the electrodes. As a result, charged particles migrate to either anode or cathode via the nanopore interface. The migration of charged particles causes a change in the electrical resistance of the buffer. Thus, the size and concentration of charged particles can be calculated from the changes in the resistive pulse of the buffer and the total volume of migrated charged particles (Maas, Broekman and de Vrij, 2017). Using standards with known particle sizes, TRPS can be used to measure particles size, concentration and zeta potential. A detailed information about TRPS and other EV characterization methods have been described in the literature (Zhao *et al.*, 2021).

2.6.4. Opportunities and challenges associated with EVs

Even though EV research is in its infancy stage, there has been a growing interest in EVs. The importance of EVs in intercellular communication, disease progression, and cell proliferation makes them potential therapeutic platforms. Moreover, EVs possess unique properties that make them also a promising alternative to traditional (liposome) nanoparticles. Identification of disease-derived EVs would enable us to control the spread and transmission of some diseases. For instance, it is possible to inhibit the expression of disease-causing EVs by blocking their EV biogenesis pathways. This will result in a reduction in their EV production, secretion, and uptake. Ceramide, for example, is one of the lipids found in ESCRT-independent pathways. Neutral sphingomyelinase 2 (nSMase2) mediates the synthesis of this lipid (Trajkovic *et al.*, 2008). The nSMase2 inhibitor GW4869 was found to reduce ceramide production, resulting in lower EV production (Essandoh *et al.*, 2015). In addition, EVs can be used as DDS with high delivery efficiencies of their cargo to the cytoplasm. This will be a new path to look for EV-based targeted drug delivery for different diseases (Kandimalla *et al.*, 2021).

In spite of these fascinating roles of EVs, there are several challenges associated with exploiting the full potential of these tiny NPs. Two of the main challenges of working with EVs are their isolation and characterization. Pre-analytical variables for EVs are lacking, especially when they are isolated from biological sources. EVs are also likely to co-isolate with non-vesicular particles. In terms of characterization methods, it is difficult to isolate EVs of specific size groups due to the heterogeneous nature of EVs. It is crucial to understand the biological significance of the broad size diversity of EVs in relation to EV heterogeneity. Furthermore, NTA is a reliable quantitative method to measure particle size distributions and concentrations. But it may not offer sufficient resolution to determine NPs with smaller diameters, and it may not discriminate EVs from non-EV populations. A lack of standardization also exists among EV studies within the community. Particularly, the influence of suspension medium on colloidal stability of EVs has not been extensively studied like synthetic NPs, for example, liposomes which can affect EVs as DDS (Sabín *et al.*, 2006; Toimil *et al.*, 2012; Lombardo *et al.*, 2019; Lombardo and Kiselev, 2022).

2.6.5. The need to evaluate the stability of EVs

Whether in an aqueous or biological medium, colloidal suspensions possess a surface charge. In a nutshell, these charges originate from the nature of the particle and the surrounding environment (e.g. the medium). EVs exhibit distinct physicochemical characteristics such as size, charge, and morphology. It is crucial to maintain these EV characteristics for the proper function of EVs in various applications, such as preservation, storage, and surface engineering. Therefore, overcoming the issue of EV aggregation. Naturally, EVs exist as colloidal suspensions when resuspended in media (Hood *et al*., 2014). In-depth knowledge of the *in vivo* interactions of EVs and their fate within the body will expand their potential applications in nanomedicine. Despite this, particle interactions in dispersed systems are complex due to variations in the surface charges of particles. Naturally, nonfunctional EVs possess a net negative charge due to the presence of glycosylated proteins on their surfaces. This surface charge of EVs can be estimated by measuring their electrophoretic mobility (EPM), which is the basis for calculating their ZP value. ZP can be used as a tool to predict the colloidal stability of NPs or EVs in suspension. In this regard, EVs carrying genetic cargo present a multifaceted platform for the advancement of improved DDS. However, there is little information available on how to maintain the stability of isolated EVs before functional analysis or therapeutic use. The stability of EVs relies on the ability to preserve their physicochemical characteristics and cargo contents intact during storage or experiments. As an example, in terms of EV size, NP stability can be viewed as maintaining EV dimensions (size) upon dye labelling, interaction with a biological medium, and during an EV uptake experiment (Mondal *et al.*, 2019; Song *et al.*, 2020). The scope of this thesis aims to understand the colloidal stability of EVs under different physical conditions and how their interaction with fluorescent probes impacts their physicochemical properties.

The EV aggregation can be minimized by applying some barriers in the dispersed systems. This can be achieved via steric or electrostatic stabilizations as well as changing the surface charge distribution of particles, for example, altering the pH, as demonstrated in liposomes (Shao *et al.*, 2017). When collision frequency is lowered by decreasing concentration of particle or duration of storage, the probability of aggregation will also decrease. Additionally, the abundance of relatively high electrostatic repulsion averts the aggregation of charged NPs, such as EVs, and facilitates their interaction with cells (Dan, 2002; Lombardo, 2014). EV stability studies generally focus on preserving EVs, particularly examining the effects of storage temperature and freeze-thaw cycles. These studies have shown inconsistent results between labs (Sokolova *et al.*, 2011; Lee *et al.*, 2016; Maroto *et al.*, 2017; Yuan, Li and Wang, 2021; Görgens *et al.*, 2022). A number of methods can be used to determine the stability of EVs, including DLS and TEM. There is, however, a lack of a standardized methodological approach to EVs, specifically their colloidal stability. Overall, the colloidal stability of EVs is one of the crucial parameters for under-

standing their fates under different conditions while maintaining physicochemical properties and cargoes. A smooth transition from the benchtop to the therapeutic or diagnostic potentials of EVs can be achieved with rigorous standardization practice in isolation and characterization of EVs.

2.6.6. Models used to study EV's colloidal stability and their interaction with fluorescent probes

When EVs are resuspended in an aqueous physiological buffer or biological medium can be considered as a colloid (Hood *et al*., 2014). As NPs, nonfunctional EVs have a net negative surface charge due to the presence of membrane and glycosylated proteins on their surfaces. Their surface charge or ZP can be determined from electrophoretic mobility (EPM) measurements of NPs in a suspension. The colloidal dispersions of EVs carry charged particles like liposomes or other suspensions. ZP determines particle-particle and particle-medium interactions in dispersed systems and predicts their colloidal stability (Hunter, 1981). These interactions in either colloidal or biological systems are highly complex and to gain understanding of them requires knowledge about the surface landscape of NPs. ZP is one of the most important physical parameters for studying this collective property and behaviour of NPs in different systems. ZP measurements can provide relevant information about NP fate and toxicity in suspensions and biological systems. Studies have shown a correlation between ZP and NP behaviour in suspensions (Berg *et al.*, 2009; Jiang, Oberdörster and Biswas, 2009), cellular uptake (Jeon *et al.*, 2018), and potential toxicity (Patil *et al.*, 2007; Schwegmann, Feitz and Frimmel, 2010; Bhattacharjee *et al.*, 2013), indicating the effects of surface charge on biological processes.

To fully understand the ZP concept, one needs to be familiar with the nature of surface charge on and surrounding particles, as well as the selection of the theoretical electrokinetic models applied to describe charge distribution bound to the particle and in the surrounding medium (O'Brien and White, 1978; Delgado *et al.*, 2007). The surface charge of a particle is defined by measuring the ZP, which is the electrokinetic potential at the slipping plane of a colloidal particle moving under the influence of an external electric field (Hunter, 1981; Delgado *et al.*, 2007). When a charged particle suspended in a liquid media, ions, and molecules of opposite charge are strongly adhered around the particle surface; this is referred to as the stern layer (Fig. 4). Beyond the stern layer, a second diffuse outer layer with loosely bound ions develops. These two layers collectively form the electrical double layer (EDL). The edge of EDL is termed as the slippery plane. Due to Brownian diffusion or applied force, ions in the diffuse layer move with the NP, whereas ions beyond the slipping plane remain within the bulk dispersant. Thus, it is at this hypothetical slipping plane boundary where the ZP is measured.

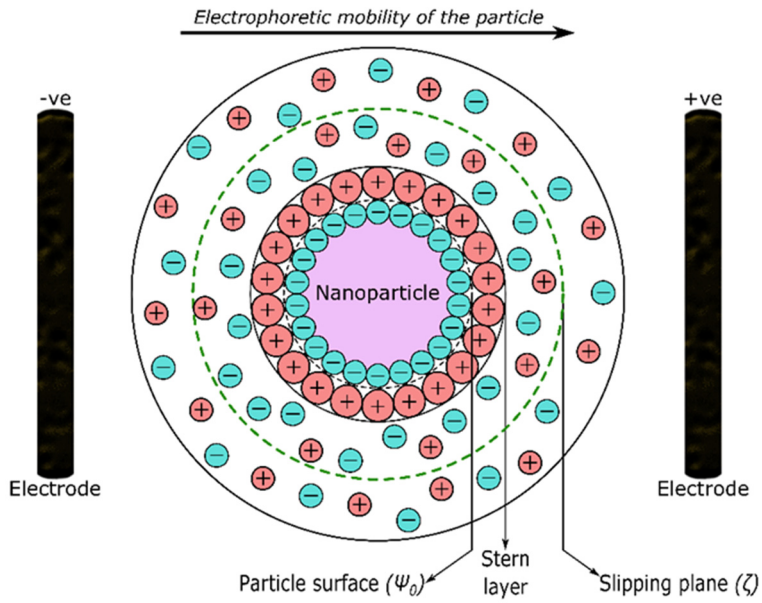

Figure 4. A scheme showing the EDL on an anionic particle, such as the EVs. On the top of particle, a strongly adherent layer of cationic ions (Stern layer) develops, while beyond it, a diffuse layer consisting of ions of both charges is formed. During electrophoresis the particle moves toward the positive electrode with the slipping plane as shown here.

ZP is calculated from EPM under the influence of an electric field. This phenomenon is known as electrophoresis. In this situation, the electrophoretic velocity of a particle is proportional to the applied electric field. As stated in von Smoluchowski equation, the electrophoretic mobility of charged particles (μ_e) defined on the basis of the physical properties of dispersion and ZP (Kaszuba *et al.*, 2010). However, depend on the nature and physical properties of the particle under study, different electrokinetic theories can applied to define the respective μ_e (Delgado *et al.*, 2007). Both EVs and plasma membrane carry a negative surface charge when suspended in a physiological medium. But manipulations on the surface charge of EVs and the nature of the surrounding medium can affect the net effective ZP of vesicles. The magnitude of ZP can fluctuate due to several factors, such as interactions at particle-medium interfaces, pH, and concentrations of ionic species in the medium. There are few studies conducted on the effects of these factors on the ZP of EVs despite their critical role in colloidal stability and pharmacokinetics. Hence, EVs derived from cell culture and biological sources can be used as models to investigate the colloidal stability of EVs under different physical conditions and how their interactions with fluorescent probes affect their physiochemical characteristics.

2.7. Summary of literature review

The unique physicochemical properties of NPs make them potentially essential components of modern medicine. As a result of their small nanoscale size range, they can be applied to a wide range of clinical applications with some adverse side effects. NP size is an important aspect of cellular uptake by different routes and interactions with biological systems. A larger surface-to-volume ratio has been observed in NPs, which will facilitate their interaction with cell membranes. Aside from their size, NPs also possess surface characteristics that enable them to interact with their surrounding via charges. The charge distribution on the NP surface can influence their ability to bind to membrane receptors and trigger cellular responses. NP interactions are determined by electrostatic interactions between different forces and the contents of the suspending medium. A change in the surrounding environment can cause these interactions to behave differently. It has been demonstrated that NP interaction with biological systems results in the formation of functional corona coats that are capable of triggering biological responses. This will further suggest that the surface of NPs is one of the most relevant physicochemical properties of NPs which requires further study.

Due to their unique physicochemical characteristics, NPs have a wide range of applications across a wide range of fields. The application of these NPs in biology and modern medicine is extensive, ranging from fluorescent biological labelling to DDS. In spite of their many useful applications, synthetic NPs have their own limitations. It has been found that liposome-based drugs are unstable in biological environments, zinc oxide NPs are capable of causing renal failure, and some modifications to NP surfaces may result in adverse cytotoxic effects. Considering these adverse features of synthetic NPs, we may need to search for alternative solutions to fully utilize NPs in a safe and effective manner.

EVs are membrane-bound NPs secreted by most cell types under physiological and pathological conditions. As well as serving as drug delivery vehicles, EVs play an important role in intercellular communication. Because of the complex mechanism of biogenesis and secretion pathways, these tiny particles are heterogeneous in terms of their size, cargo, and surface proteins. The challenge in the field is to isolate and characterize these EVs, given their nature and the lack of effective isolation and characterization methods. NTA, for example, is a popular quantitative method to measure the particle size and concentration of EVs in a sample. Additionally, conventional NTA fails to exclude EVs from non-EV total populations. Last but not least, there is no standardization for the measurement of surface charge (i.e. ZP) in different buffer conditions in order to maintain the colloidal stability of EVs for a variety of purposes. The development of stable and multifunctional EVs depends on understanding these interactions between EVs in different systems and maintaining their physicochemical characteristics. It is evident that there are factors that facilitate the aggregation of EVs where their function will be adversely impacted. The

stability of EVs, however, can be assessed through rigor standardized ZP measurements including several factors that contribute to long-term stability. Therefore, various regulatory agencies and researchers in the EV field need to be aware of the importance of maintaining colloidal stability in different conditions for the safe use of these NPs for effective DDS, diagnosis, and treatment of diseases.

3. AIMS OF THE STUDY

The general aim of this doctoral project was to determine the colloidal stability of EVs under different physical conditions and study the applicability of FL-NTA for detection of membraneous EVs.

The specific aims of the study include:

Aim 1: To determine the impact of different factors such as phosphate buffer content, detergent, ionic strength, and pH on the apparent zeta potential (surface charge) of EVs secreted by the cultured human choriocarcinoma (JAr) cells.

Aim 2: To characterize and compare the physical characteristics of EVs derived from blood-serum of psoriatic arthritis (PsA) and psoriasis vulgaris (PsV) patients with healthy controls.

Aim 3: To study the effect of membrane dye (CMG) labelling on the size, concentration, and zeta potential (ZP) of JAr EVs isolated using different methods, as well as EVs derived from biological fluids (e.g. bovine follicular fluid and seminal plasma).

4. MATERIALS AND METHODS

A detailed description of the materials and methods used in this thesis can be found in the original publications (I–III). This section summarizes the methods used in the studies.

The Ethics Review Committee on Human Research at the University of Tartu approved the protocol for collecting blood serum samples and conducting the research (protocol code 265/T-21).

4.1. Overall experimental plan

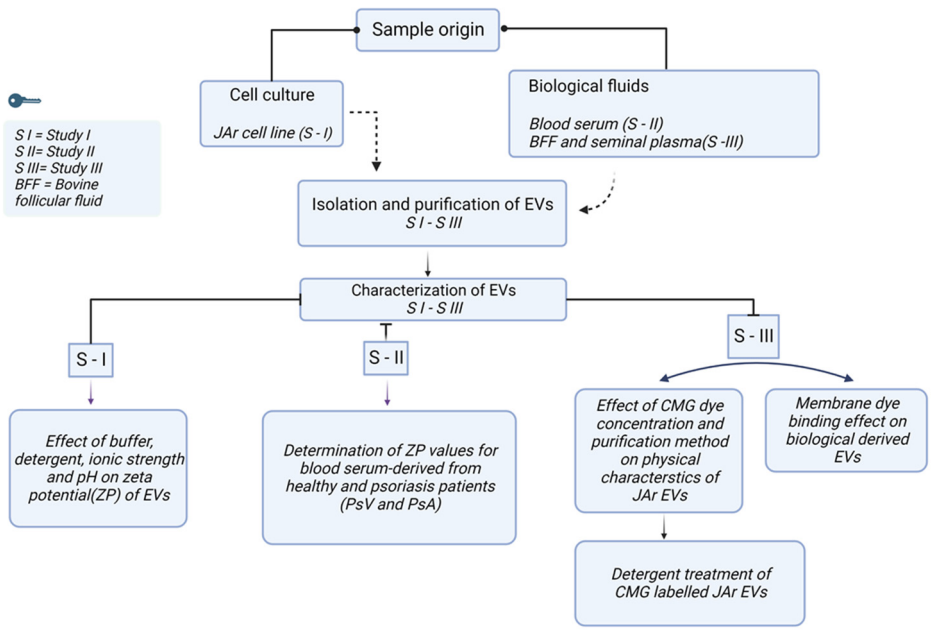

Figure 5. Overall study design.

4.2. Cell culture

4.2.1. JAr cell culture (Study I and III)

A human first-trimester choriocarcinoma cell line (JAr) was obtained from the American Type Culture Collection (ATCC, HTB-144, Teddington, UK.). Cells were grown in RPMI 1640 medium (Gibco, Scotland) containing 1% penicillin/ streptomycin, 1% L-glutamine, and 10% FBS at 37°C under a humid 5% CO_2 level. Once cells reached 80% confluence, the conditioned medium was discarded. Later, the cells were washed only with an unsupplemented RPMI 1640

medium to remove any traces of fetal bovine serum (FBS) remaining from the culture. This was followed by replacing the unsupplemented medium with a fresh RPMI 1640 medium containing 1% penicillin/streptomycin, 1% L-glutamine, and 10% EV-depleted FBS. Cells were cultured for 24 hours under the same conditions as described above. The conditioned medium was collected after incubation to isolate EVs.

4.2.2. Ultrafiltration-based EV depletion from FBS (Study I and III)

An earlier methodology was used to deplete EVs in FBS (Kornilov *et al.*, 2018). Using an Amicon Ultra-15 centrifugal unit (100 kDa, MerkMillipore, Darmstadt, Germany), FBS was ultrafiltered at 5000 xg for 30 minutes. Following centrifugation, the particle concentration of the collected filtrate was determined by NTA. In this way, vesicles from FBS will be eliminated when cells are cultured in an EV-depleted medium.

4.3. Isolation of EVs

4.3.1. Collection and handling of JAr conditioned media from cell culture model (Study I and III)

In studies I and III, EVs were obtained from conditioned media of cells grown for 24 hours in an EV-depleted medium. In the pre-cleaning EV isolation steps, the conditioned medium was removed from the cells and transferred to sterile 50 mL Falcon conical tubes for successive centrifugation at 4°C . The medium underwent sequential centrifugation at 400 xg, 4000 xg, and 10000 xg to remove cells, cell debris, and apoptotic bodies, respectively. The supernatant obtained from the last step of centrifugation was further concentrated to 500 µL using Amicon Ultra-15 centrifugal unit (10 kDa, MerkMillipore, Darmstadt, Germany). The concentrated medium was then used to isolate EVs using benchtop SEC columns as presented in a previous work carried out by the Fazeli's group (Es-Haghi *et al.*, 2019).

4.3.2. Collection and processing of biological fluids (Study II and III)

The EVs used in studies II and III were obtained from biological fluids. Twelve patients with psoriasis vulgaris (PsV) or psoriatic arthritis (PsA), as well as 12 healthy controls, were the source of EVs for study II. While in study III, BFF and seminal plasma were used as sources of EVs. Blood serum obtained from healthy and patient samples was also centrifuged sequentially in the same ways as the JAr conditioned medium. The second centrifugation step, however, was done at a lower force of 2000 xg to remove cell debris. The supernatant obtained

from the last centrifugation was concentrated to 500 µL before being loaded onto qEVoriginal/70nm SEC columns (ICO-70, Izon Science, Oxford, UK). BFF and seminal plasma samples were sequentially centrifuged at comparable centrifugal forces (Hasan *et al.*, 2020).

4.3.3. Size-Exclusion Chromatography (SEC) (Study I–III)

In studies I to III, both manual and pre-packed SEC columns were used to isolate EVs. The sepharose beads were manually packed into empty columns for studies I and III, specifically for conditioned medium, BFF, and seminal plasma samples. The beads were made up of a cross-linked 4% agarose matrix with a diameter of 90 µm (Sepharose 4 Fast Flow, GE HealthCare Bio-Sciences AB, Uppsala, Sweden). For these studies, the column height was 10 cm. After calibrating the column with PBS having a physiological pH range of 7.2 to 7.4, the concentrated medium and/or biological fluid of 500 µL was loaded onto the columns. Vesicle-enriched fractions 6–9 (total volume of 2 mL) were collected from JAr conditioned medium (I). While the EV fractions were eluted at fractions 5–7 and 5–8 for BFF and seminal plasma samples, respectively. The collected fractions were then further concentrated using centrifugal filters with 10 kDa cut-off.

Isolation of EVs from blood serum samples was performed using prepacked SEC columns. Following calibration with PBS, the column was loaded with 500 µL of concentrated blood serum. EV-containing fractions (7–10) were collected and further concentrated as described elsewhere (II).

4.3.4. Tangential flow filtration (TFF) (Study III)

In study III, EVs were isolated using a TFF- Easy filtration system (HansaBio-Med, Tallinn, Estonia) after the conditioned medium was sequentially centrifuged according to TFF supplier. Briefly, TFF filtering units were primarily washed with Milli-Q water using syringes prior to loading conditioned medium. By gently applying forces to the syringes, sterile hollow-fiber polysulfone membranes were used to filter and concentrate the conditioned medium. Polysulfone membranes with pore sizes of 20 nm were used to remove free biomolecules from the conditioned medium. The concentrated conditioned medium was retained in the membrane and transferred to sterile tubes. The concentrated conditioned medium was further concentrated to 500 µL before being introduced to SEC column (Es-Haghi *et al.*, 2019).

4.4. EV characterization

4.4.1. Conventional and fluorescence NTA (Study I–III)

The particle size and concentration of JAr conditioned medium and blood serum-derived EVs were measured using an NTA instrument (Particle Metrix GmbH, Ammersee, Bavaria, Germany). NTA's laser and microscope were auto-aligned using 100 nm polystyrene (PS) standard beads (Applied Microspheres B.V., Netherlands). Precision and trueness of auto-alignment evaluations were done on daily basis before sample measurement. NTA's sample holder was washed with Milli-Q water and PBS following alignment and daily performance of the instrument. Size and concentration of the above-mentioned samples were measured in scatter mode at the following settings: sensitivity 85, shutter 70, and 30 frames per second.

FL-NTA was used to determine the size and concentration of samples used in studies I and III. FL-NTA was conducted using the same procedure as conventional NTA, with a few changes. FL-NTA's laser and microscope were auto-aligned with 100 nm PS and fluorescent Yellow-Green PS standard beads (Applied Microspheres B.V., The Netherlands). The size and concentration of the above-mentioned samples were evaluated in scatter mode at the following settings: sensitivity 72, shutter 100, and 30 frames per second. A sensitivity value of 90 was used to measure the particle size and concentration of fluorescently labeled EV samples. The EV samples used in studies I–III were measured three times at 11 focal planes (positions) in room temperature (RT).

4.4.2. Electron microscopy

4.4.2.1. SEM (Study I)

SEM imaging was done for EVs isolated from a JAr conditioned medium. An aliquot of JAr EV was placed on aluminium foil for overnight drying. A gold sputter coating was applied to the EV sample following overnight drying. A Hitachi S-4300 SEM microscope was then used to image the coated sample.

4.4.2.2. TEM (Study I and III)

TEM is used to determine the morphology of EVs based on the previously described methodology (Théry *et al.*, 2006; Es-Haghi *et al.*, 2019). In brief, a concentrated JAr EV sample was placed on copper-mesh grids and fixed with 1% glutaraldehyde and 2% paraformaldehyde. Following this, EVs were contrasted in uranyl oxalate (Polysciences, Warrington, USA) and oxalic acid (Sigma-Aldrich, Schnelldorf, Germany). EVs were then embedded in a mixture of methylcellulose (Sigma-Aldrich, Schnelldorf, Germany) and uranyl acetate (Polysciences, Warrington, USA). Imaging of the EV sample was done using JEM 1400 TEM (JEOL Ltd. Tokyo, Japan) and Morada TEM CCD camera (Olympus, Germany) at 80 kV.

4.4.3. Western Blot (Study I)

Western Blot analysis was used to confirm the presence of protein markers in EVs. ISEV guidelines were followed in examining the presence of EV tetraspanins (CD9, CD63, and CD81) and cytosolic proteins (HSP70) in the JAr cell lysate and EVs (Théry *et al.*, 2018). Concentrated JAr EV proteins were precipitated in methanol and chloroform. The precipitated EV protein was then resuspended in 0.5 % SDS. A Bradford assay was used to determine the protein concentration of EV protein precipitated in SDS and JAr cells lysed in lysis buffer. Fifty and 30 µg protein was used in either reducing (HSP70) or non-reducing (CD63, CD9, and CD81) Laemmli buffer. Following this, the proteins were resolved on 12% SDS-PAGE and then transferred to PVDF membranes. The membrane was incubated with primary antibodies overnight at 4°C in 5% non-fat milk PBST buffer. Immunoblotting is performed using the following primary antibodies: anti-CD63 (BD Biosciences), anti-CD9 (Santa Cruz), anti-HSP70 (Novus Biologicals), and anti-CD81 (BD Biosciences). Then, the membrane was probed with horseradish peroxidase conjugated goat anti-mouse secondary antibody (Thermo Fisher Scientific) for an hour at RT. We also incubated the membrane with TSG101 and Alix, but these proteins were not enriched in JAr EVs. After each incubation, the membrane was washed with PBST buffer. Protein bands were visualized with a detection reagent (ECL Select™, GE Healthcare) and an ECL imager (ImageQuant™, GE Healthcare).

4.5. ZP (surface charge) measurements of EVs (Study I–III)

ZetaView NTA instrument was used to measure the surface charge (ZP) of EVs isolated from cell culture medium and biological fluids. The ZP measurement was validated using a negatively charged PS latex with a nominal diameter of 200 nm (Applied Microspheres B.V., Netherlands). Following the ZP validation measurements, NTA's sample holder was washed with Milli-Q water and a buffer of interest (0.01 to 10 mM phosphate ion concentration). The ZP of the above-mentioned samples was measured in scatter mode at the following settings: sensitivity 85, shutter 100, and 30 frames per second. Whereas, the ZP of fluorescently labeled EVs was determined at a sensitivity value of 90. The ZP of neat and fluorescently labelled NPs was measured three times at 2 stationary layers. The pH of EV suspensions was adjusted to 6.9–7.2 by adding 0.1 M HCl/NaOH.

4.6. Labelling of EVs with membrane lipophilic dye (Study III)

Various fluorescent dyes are used to label EV membranes. In study III, EVs isolated from biological fluids and cell cultures were stained with 0.1 mg/mL of CellMask™ Green (Thermo Fisher Scientific). As a next step, EVs isolated from JAr conditioned medium were stained with different concentrations of CMG plasma membrane dye. Following that, EV's lipid membrane disruption was confirmed using 0.5 % nonionic NP-40 detergent. The neat and CMG-labeled vesicles isolated from JAr conditioned medium were subjected to NP-40 detergent treatment at a dye concentration of 0.1 mg/mL (see Fig. 16).

4.7. Statistical analysis

In study I, the statistical analyses were performed using R (version 3.6.1, R foundation for statistical computing, Vienna, Austria). As we collected multiple measurements from each of three biological replicates (each carried out on a separate day), the effects of different conditions were assessed using nested one-way or two-way ANOVAs with a 'replicate' error term to account for the inter-dependence of measurements collected from the same biological replicates. For specific intergroup comparisons, Tukey multiple comparison tests were used.

As part of study II, the particle size and ZP of EV samples were normalized to account for differences in nanoparticle concentration by dividing the counts per respective bin (i.e. the total particle concentration in the respective size/ZP bin was divided by the total particle concentration). This results in a value representing the proportion of total counts per bin. The NP size and ZP data were binned at 20 nm and 5 mV, respectively. By using the qt() function in R, the 95% confidence intervals were derived from a t-distribution ($df = N - 1$) for size and ZP values of the studied groups. The density function in R was used to derive density curves for particle size distribution and ZP profiles for healthy controls and patient groups.

In study III, the statistical analyses were performed using Graphpad prism v8.4.2. Two-tailed Student unpaired/paired *t*-test was used for comparing the concentration and ZP of fluorescently labelled and total particles. The effects of different conditions were assessed using one-way or two-way ANOVAs. For specific intergroup comparisons, Tukey multiple comparison tests were used. Data are shown as the mean ± SD (standard deviation) of three independent experiments (biological replicates), and a *p*-value of < 0.05 was considered to be statistically significant marked with an asterisk (*) symbol.

5. RESULTS

5.1. Study I : Factors affecting zeta potential (ZP) measurements of EVs secreted by cultured human choriocarcinoma (JAr) cells

5.1.1. Experimental design for study I

As part of study I, four experiments were conducted. In this study, the effect of phosphate buffer concentrations (1, 0.1, and 0.01 mM phosphate ions dilutions) without and with detergent (0.03 % Tween -20), or in the presence of chloride salts (10 mM of NaCl, KCl, $CaCl_2$, and $AlCl_3$) and different pH values (4, 7, and 10) on ZP of EVs was determined (Fig. 6).

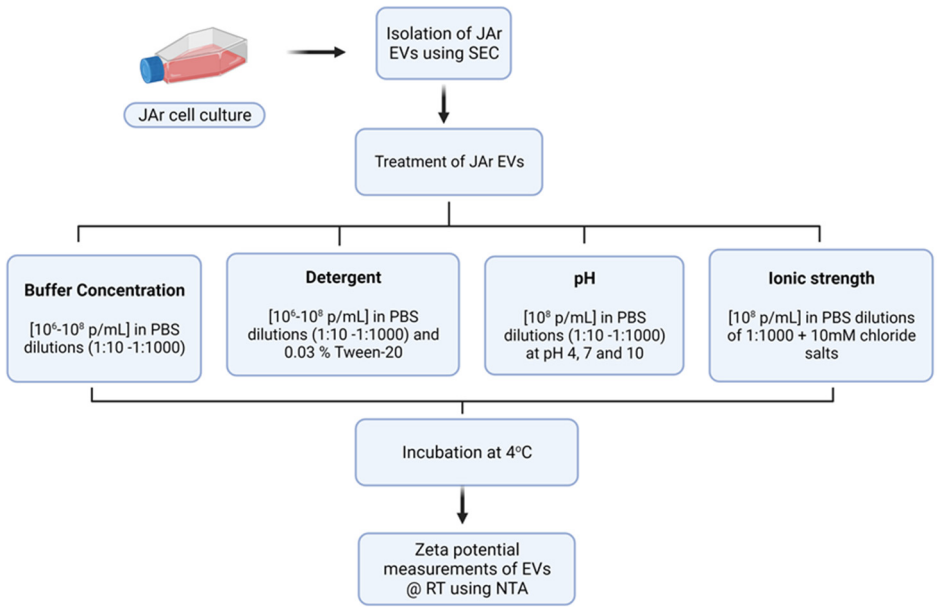

Figure 6. Experimental design for study I.

5.1.2. Biochemical and physical characterization of JAr EVs

Electron microscopy-based approaches (SEM and TEM) were used to determine the morphology and size of JAr EVs. As shown in figure 7, SEM and TEM analysis confirmed the typical cup-shaped and spherical morphologies of EVs. The mean diameter of the EVs (~120 nm) determined using SEM and TEM corroborated well with the NTA particle size measurements (Fig. 8).

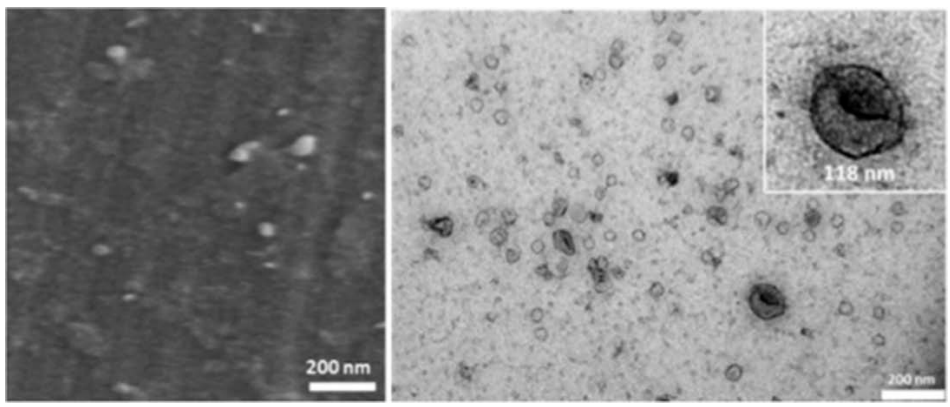

Figure 7. Physical characterization of JAr EVs using electron microscopy. The morphology of JAr EVs was confirmed using SEM (left side) and TEM (right side). TEM analysis showed that the purified JAr EVs have cup-shaped structural features (right side: inset). Scale bar: 200 nm (I).

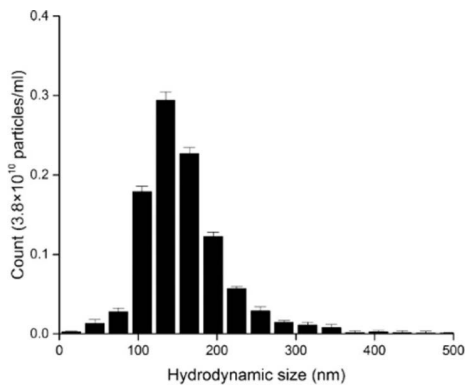

Figure 8. Characterization of JAr EVs using NTA. The particle size distribution and concentration of JAr EVs determined by ZetaView® NTA. Concentration of EVs are shown as particles/mL (mean ± SD , *n* = 3). The (polydispersity Index) PDI value for JAr EVs found to be 1.11 (I).

Western blot analysis showed that the expression of EV surface markers (CD9, CD63, and CD81) along with cytosolic protein (HSP70) in JAr EVs (Fig. 9).

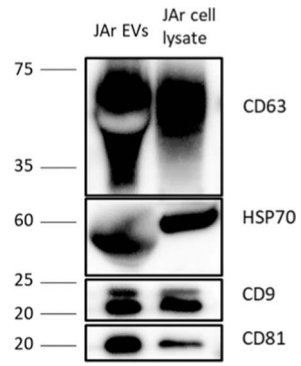

Figure 9. Biochemical characterization of JAr EVs using western blotting. Western blot analysis detected EV tetraspanin protein markers in JAr EVs (such as CD9, CD63, and CD81) and corresponding cell lysates (as a control). These samples also contained a cytosolic protein, HSP70 (I). Control bands for the ladder were cut off.

With the confirmation of the presence of EVs and enriched surface protein markers in the JAr conditioned medium, we investigated the impact of factors such as phosphate buffer content, detergent, ionic strength, and pH on the surface charge (zeta potential) of JAr EVs. ZP measurements were done using ZetaView® NTA.

5.1.3. Effect of phosphate buffer content on the colloidal stability of EVs

To assess the effect of buffer content on the ZP of EVs, phosphate-buffered saline (PBS) was diluted to different phosphate ionic concentrations (0.01, 0.1, and 1 mM) in particle-free water (pH 6.9). Regardless of the concentration of EVs, there was a significant shift in the measured ZP of EVs towards less negative values at the higher molar concentration of PBS ($p < 0.001$; Fig. 10). There was no significant overall effect of EV concentration ($p = 0.434$) on the respective diluted PBS concentrations. Further statistical tests showed that for each concentration of EV, ZP was significantly less negative at 1 mM than 0.01 mM phosphate ion concentration (all $p < 0.01$).

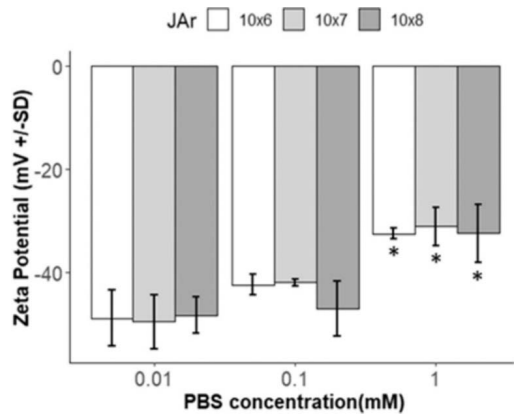

Figure 10. Buffer effect on ZP of EVs. ZP of JAr EVs (mean ± SD, $n = 9$) depicted as a function of concentrations of EVs (10^6 particles/mL (□), 10^7 particles/mL (light gray bins), and 10^8 particles/mL (dark gray bins)) and diluted PBS. EVs suspended in various molar ratios (0.01, 0.1, and 1 mM) of phosphate ions in MQ at pH 6.9. The data measuring points at 1 mM were significantly different ($p < 0.05$) than 0.01 mM, and thus were labelled with an asterisk sign (I).

5.1.4. Investigating the effect of detergent on ZP of EVs

Following the addition of Tween-20, the ZP of EVs shifted towards less negative values. This result was generally constant over different JAr EV concentrations and PBS dilutions (Fig. 11). Thus, there was an overall significant additive effect of Tween-20 ($F(2) = 279.64$, $p = 0.004$). Overall, the effect of Tween-20 was significantly dependent on concentration of buffer ($F(2) = 10.2$, $p = 0.027$) but independent on EV concentration ($F(2) = 0.442$, $p = 0.67$). Post hoc Tukey tests revealed that the effect of Tween to be significant ($p < 0.05$) at all PBS dilutions and EV concentrations except for the measurements at the higher molar concentration (1 mM) of phosphate ion and 10^8 particles/mL ($p = 0.4$) EV concentration.

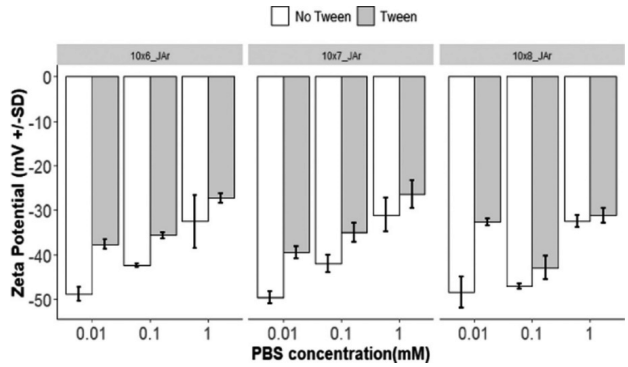

Figure 11. Detergent effect on ZP of EVs. Compiled ZP measurements of JAr EVs under different buffer conditions (mean ± SD, n = 9). EVs resuspended in various molar ratios of phosphate ions (0.01, 0.1, and 1 mM), either without (white bins) or with (▩) Tween-20 (0.03%), at pH 6.9 under three different EV concentrations (10^6 particles/mL, 10^7 particles/mL, and 10^8 particles/mL) (I).

5.1.5. Assessment of the effect of ionic valency and ionic strength on the ZP of EVs

To understand the effect of ions and their ionic strengths on the ZP of JAr EVs, chloride salts were used in this experiment. The measured ZP of EVs became less negative in the presence of the trivalent Al^{3+} (mean change of -18 mV, $p < 0.001$) and divalent Ca^{2+} ions (mean change of -24 mV, $p < 0.001$), but unaffected by monovalent ions Na^{+} and K^{+} (Fig. 12, left side). Furthermore, an increase in ionic strength led to a decrease in ZP values of EV (Fig. 12, right side).

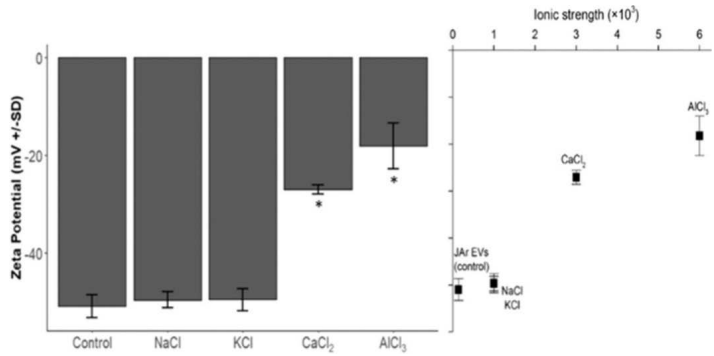

Figure 12. ZP measurements of JAr EVs in chloride salts. EVs were resuspended in 0.01 mM phosphate buffer (pH 6.9) containing 10 mM of different chloride salts (NaCl, KCl, $CaCl_2$, and $AlCl_3$) at varying ionic strengths. The cationic effects on the ZP values of JAr EVs was significantly different ($p < 0.05$) compared to the control, and hence labelled with an asterisk sign (I).

5.1.6. Evaluating the effect of pH on ZP of EVs

To assess the effect of pH on the ZP of EVs, PBS was diluted at different concentrations of phosphate ions (0.01, 0.1, and 1 mM) and at varying pH levels (pH 4, 7, and 10). At low pH, a decrement in ZP value of EVs was observed (Fig. 13). At pH 4 and 10, the change in ZP values of EVs in response to buffer content was more variable than at pH 7. ZP of EV shifted to more negative value as pH increased ($p = 0.007$). In general, more negative ZP was observed at pH 10 than pH 4 at all PBS concentrations (all $p < 0.01$). In terms of PBS dilutions, EVs resuspended in low molar ratios of 1x PBS showed higher ZP values. Post hoc Tukey test revealed that the phosphate buffer content had no effect of pH ($p = 0.304$).

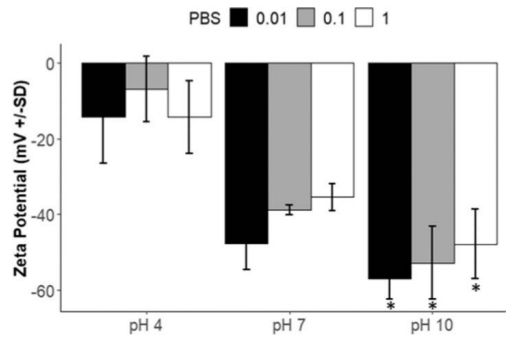

Figure 13. ZP measurements of JAr EVs at different pH and PBS dilutions. Variation of ZP of EVs (10^8 particles/mL) as a function of pH (pH 4, 7, and 10) resuspended in different molar concentrations of phosphate ions (0.01 mM (■), 0.1 mM (gray bins), and 1 mM (white bins)). The data measuring points at 1 mM were significantly different ($p < 0.05$) than 0.01 mM, and thus were labelled with an asterisk sign (mean ± SD, $n = 9$) (I).

Key findings of study I:

- ⇨ In general, the ZP of EVs was influenced by the phosphate buffer concentration used in the experiment. ZP of EVs tends to shift towards less negative values as the molar concentration of phosphate ions increases in the buffer.
- ⇨ Upon the addition of detergent, the magnitude of ZP of EVs shifted towards less negative values. It should be noted, however, that the overall effect of Tween-20 was significantly dependent on the concentration of phosphate buffer, but not on the concentration of EV.
- ⇨ ZP of EVs became less negative in the presence of the trivalent Al^{3+} and divalent Ca^{2+} ions, but remains unaffected by monovalent ions Na^{+} and K^{+}.

In addition, an increase in ionic strength led to a decrease in ZP values of EV.

⇨ ZP of EV shifted to a more negative value as pH increased. At acidic and alkaline pH, the change in ZP values of EVs in response to buffer content was more variable than at neutral pH.

5.2. Study II: Evaluation of the physical characteristics of EVs isolated from blood serum of psoriasis patients

Having confirmed the presence of EVs in the JAr conditioned medium and identifying numerous factors that influence their surface charge (zeta potential), we investigated the effect of ZP on blood serum-derived EVs from healthy controls and psoriatic arthritis patients.

5.2.1. Experimental design for study II

In this study, we compared the physical characteristics of EVs derived from blood-serum of psoriatic arthritis (PsA) and psoriasis vulgaris (PsV) patients with healthy controls. SEC-Purified EVs (10^8 particles/mL) were resuspended in 1 mM phosphate buffer (pH 7.4). Following that, the size and ZP of blood serum-derived EVs (controls and patients) were determined using the NTA instrument (Fig. 14).

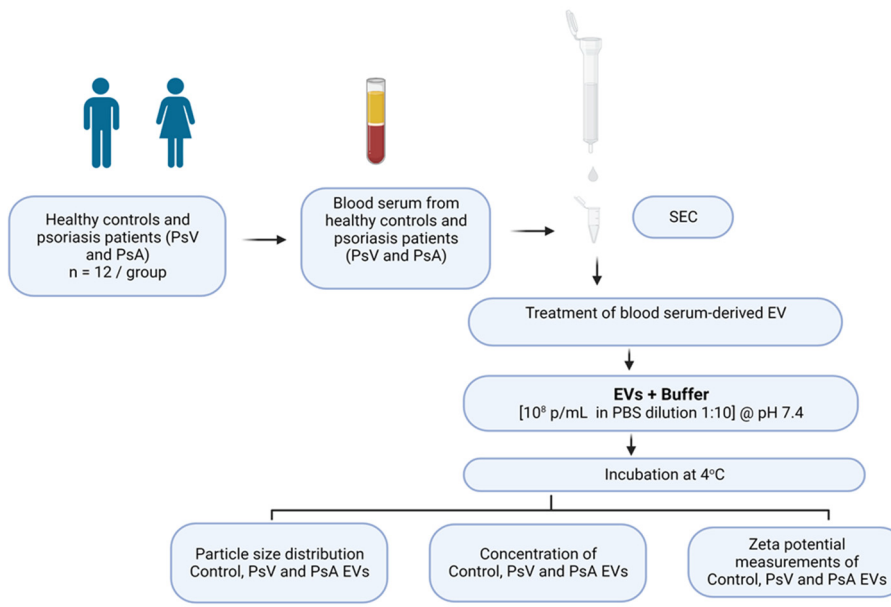

Figure 14. Experimental design for study II.

5.2.2. Physical characterization of serum-derived EVs

TEM was used to determine the morphology and size of serum-derived EVs. The mean diameter of the EVs determined using TEM is in agreement with the NTA particle size measurements. Moreover, Western blot analysis revealed that purified samples were enriched in EV surface protein markers. These results were reported by (Lättekivi *et al.*, 2022) and we acknowledge for his work.

To compare the ZP of EVs between healthy and psoriatic patient groups, EVs were resuspended in 1mM phosphate ion concentration. The results of the NTA size measurements showed that particle concentrations varied among the studied groups, particularly for two of the control samples (Fig. 15A). The particle size distributions of EVs were not significantly different between the healthy controls and patient samples (Fig. 15B). It has been noted that the mean diameter of the EVs falls within the range of 80–120 nm, with some samples having a wider size distribution (Fig. 15C). From the data in figure 15D-E, it is apparent that no significant differences in ZP values of EV observed between healthy controls and patient groups (PsA and PsV).

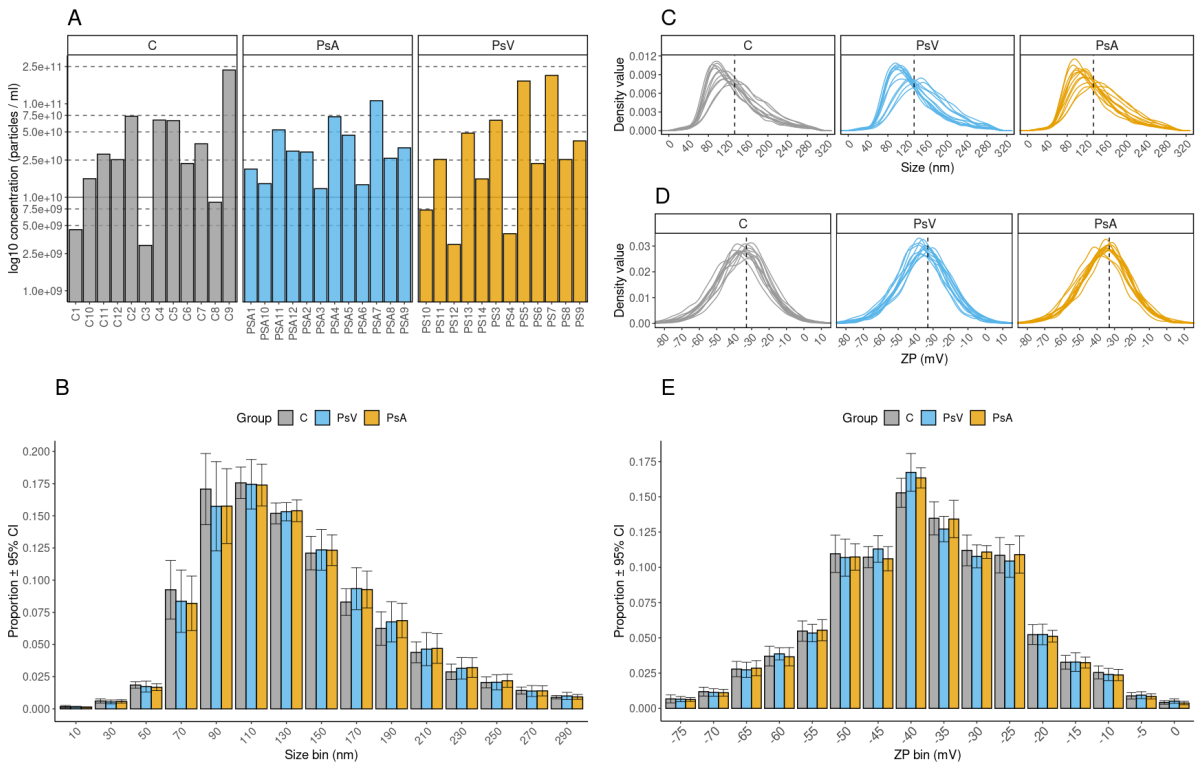

Figure 15. Characterization of blood-serum derived EVs using NTA. (**A**) The total particle concentration for EVs isolated from blood-serum of healthy and psoriasis patients (PsA and PsV). Particle concentration is expressed as a function of the log scale in particles/mL. (**B**) The particle size distribution of EV samples is shown in terms of normalized concentration (i.e. the total particle concentration in the respective size bin was divided by the total particle concentration). (**C–D**) The particle size distribution and ZP profile for healthy controls and patient groups are expressed as density curves. The mean particle size (nm) and ZP for respective groups was marked with dashed vertical lines. (**E**) The ZP of EV samples is shown in terms of normalized concentration per millivolt (i.e. the total particle concentration in the respective ZP bin was divided by the total particle concentration). Error bars illustrate 95% confidence intervals (II).

Key findings of study II:

- ⇨ Variation in particle concentrations were observed among the studied groups.
- ⇨ The particle size distributions of EVs were not significantly different between the healthy controls and patient samples (PsA and PsV).
- ⇨ The mean diameter of the EVs falls within the range of 80–120 nm.
- ⇨ No significant differences in ZP values of EV were observed between healthy controls and patient groups.

In study I and II, we evaluated the physical characteristics of EVs isolated from blood serum and conditioned medium, focused primarily on their surface charge (ZP). Following that, we opted to investigate how EV interactions with fluorescent probes, such as membrane dye affect their physiochemical characteristics. Such investigations are paving the way to the application of FL-NTA rather than conventional NTA for differentiating between membranous EVs and non-EV particles.

5.3. Study III: Evaluating lipophilic membrane dye labelling of EVs using fluorescence nanoparticle tracking analysis

5.3.1. Experimental design for study III

As part of study III, four experiments were conducted. In this study, the effect of CMG labelling was investigated on the size, concentration, and ZP of JAr EVs isolated using different methods, as well as EVs derived from biological fluids (e.g. bovine follicular fluid and seminal plasma) as presented in section 4.3.1 and 4.3.2. and published in the paper (Midekessa *et al.*, 2021) (Fig. 16). CellMask™ Green (CMG) is a lipophilic dye used for labelling EV membranes.

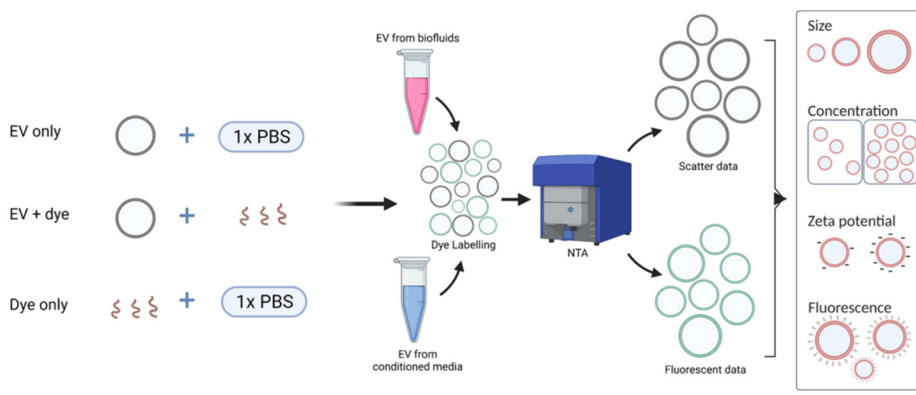

Figure 16. Experimental design for study III.

5.3.2. Effect of CMG concentration on nanoparticles originating from cultured human JAr cells

The first set of analyses examined the impact of CMG dye concentration on the size, concentration, and ZP values of NPs originated from JAr cells. Heterogeneous size distribution was observed for JAr EVs. Based on measurements in fluorescent (*fl*-NPs) and scatter modes (total *t*-NPs) of NTA, the size distribution of EV-only controls and EVs labelled with CMG showed size range values of ~30–400 nm (Fig. 17). With successive increases in the concentration of CMG dye, the particle size distribution for *fl*-NPs originated from JAr cells moved further to smaller particles in the range of ~40/60 to 260/280 nm. Neither scatter nor fluorescent modes of NTA detected CMG nanoparticles in the CMG-only control.

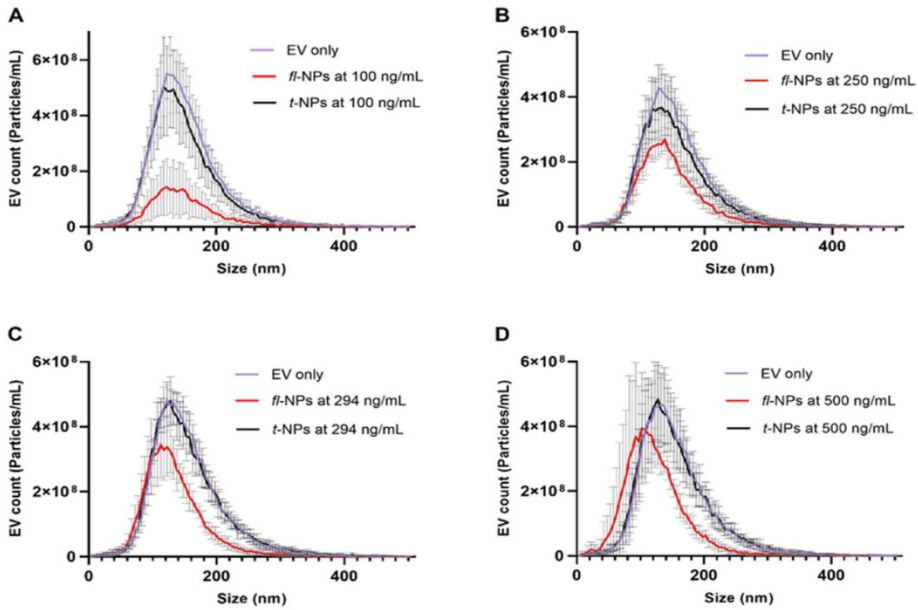

Figure 17. CMG labelling of EVs. (**A-D**) Particle size distribution in scatter and fluorescent mode (504 nm) for EVs isolated from JAr conditioned medium. The size of *fl*-NPs originated from JAr cells was measured at a minimum brightness threshold value of 25 with varying CMG concentrations of 100, 250, 294 and 500 ng/mL respectively. Both EV only (control) and fluorescently labelled NPs of JAr EVs were resuspended in PBS (mean ± SD, $n = 9$) (III).

The results, as shown in figure 18A, indicate that the mean particle size of *fl*-NPs of JAr EVs shifted significantly toward smaller particle sizes with subsequent increases in CMG dye concentration ($p \leq 0.05$), except for the lowest dye concentration ($p = 0.354$). A variation in the concentration of CMG dye also resulted in a difference in the concentration of *fl*-NPs. It was found that JAr

EVs labelled with higher concentrations of dye produced a greater number of fluorescently labeled particles compared to EVs labeled with the lowest dye concentration (Fig. 18B). In addition, there were differences in the ratios of fluorescently labelled NPs in the different EV size bins. To determine if CMG-positive fluorescent NPs vary with different size bins, the size bins were categorized into four groups (40–150, 151–250, 251–350, and 351–450 nm). A consistent CMG labeling ratio was observed for the first three size groups (40–150 nm: 26.17 ± 9.34 %, 151–250 nm: 27.17 ± 9.03 %, 251–350 nm: 29.47 ± 10.17%) at the lowest dye concentration, while the larger size bin (351–450 nm: 41.68 ± 25.46 %) showed higher levels of CMG-positive fluorescent NPs. Different size bins of JAr EVs were found to have different ratios of CMG-positive fluorescent NPs with increasing dye concentrations. In particular, CMG-positive NPs showed high fluorescence at the lowest size bins with CMG concentrations of 250 and 294 ng/mL. For the above-mentioned dye concentrations, a decrease in fluorescent NP concentration was observed with increasing size bins (40–150 nm: 77.32 ± 24.72 %, 151–250 nm: 60.11 ± 13.38 %, 251–350 nm: 45.12 ± 14.10 %, and 351–450 nm: 41.60 ± 13.81 %). For different EV size bins at a concentration of 294 ng/mL, the CMG labelling percentages are as follows: 40–150 nm: 79.40 ± 15.42 % , 151–250 nm: 42.30 ± 5.07 %, 251–350 nm: 27.57 ± 7.22 %, and 351–450 nm : 26.08 ± 9.37 %. This is also true for the highest dye concentration, where the NPs in the smallest size bins were labelled higher than those in the other size bins (40–150 nm: 100.36 ± 21.97 % , 151–250 nm: 41.71 ± 12.01 %, 251– 350 nm: 19.16 ± 5.77 %, and 351–450 nm: 34.56 ± 10.91 %). It is therefore important to interpret these results cautiously, particularly at the highest dye concentrations. Further analysis showed significant differences in the concentrations of total (*t*) and *fl*-NPs of JAr EVs labelled with different concentrations of CMG ($p \leq 0.05$).

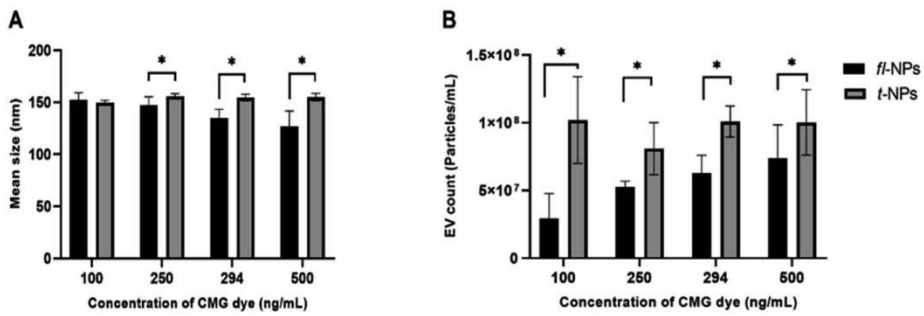

Figure 18. Effect of CMG concentration on particle size and concentration of NPs in CMG-labelled EVs. Comparison of (**A**) mean particle sizes and (**B**) concentration of CMG-positive *fl*-NPs in comparison to total NPs measured after labelling with varying concentrations (100, 250, 294 and 500 ng/mL) of CMG dye. A significant difference in mean particle size and concentration observed between *fl*-NPs and *t*-NPs at all concentrations of CMG, with the exception of the lowest concentration of CMG. Thus, were labelled with an asterisk sign (mean ± SD, $n = 9$) (III).

Fluorescence-NTA was also used to determine the effect of CMG concentration on the ZP of fluorescent and total NPs of CMG labelled EVs. There was a significant shift toward more ($p \leq 0.05$) and less ($p \leq 0.05$) negative ZP values for *fl*-NPs of JAr EVs with the lowest and highest dye concentrations (Fig. 19).

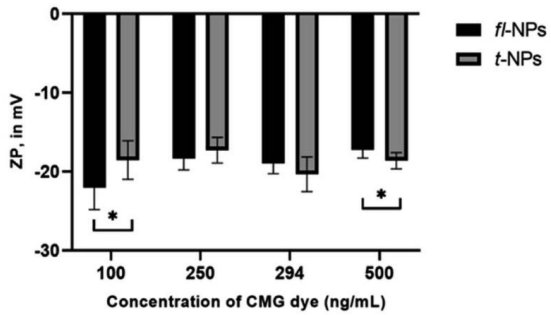

Figure 19. Effect of CMG concentration on ZP measurements of NPs in CMG-labelled EVs. Comparison of ZP of CMG-positive *fl*-NPs in comparison to total NPs measured after labelling with varying concentrations (100, 250, 294 and 500 ng/mL) of CMG dye. A significant difference in zeta potential values between *fl*-NPs and *t*-NPs at lowest and highest dye concentrations. Thus, were labelled with an asterisk sign (mean ± SD, $n = 9$) (III).

5.3.3. Effect of purification method on physical characteristics of fluorescently labelled NPs of JAr EVs

To assess the effect of EV purification method on the physical characteristics of *fl*-NPs of CMG-labelled EVs, JAr EVs were purified with only SEC and in conjunction with TFF. A high particle size distribution (~60 to 280 nm) was observed for the *fl*-NPs of JAr EVs purified in TFF and SEC. The results, as shown in figure 20B, indicate that the mean particle size of *fl*-NPs of JAr EVs purified in TFF and SEC shifted toward larger particle sizes than SEC alone. Further analysis showed that the mean particle size of *fl* and *t*-NPs of JAr EVs purified in TFF and SEC significantly shifted towards larger particles ($p \leq 0.05$). Contrary to this, the mean particle size of *fl* and *t*-NPs of JAr EVs purified in SEC alone remain unchanged ($p = 0.354$).

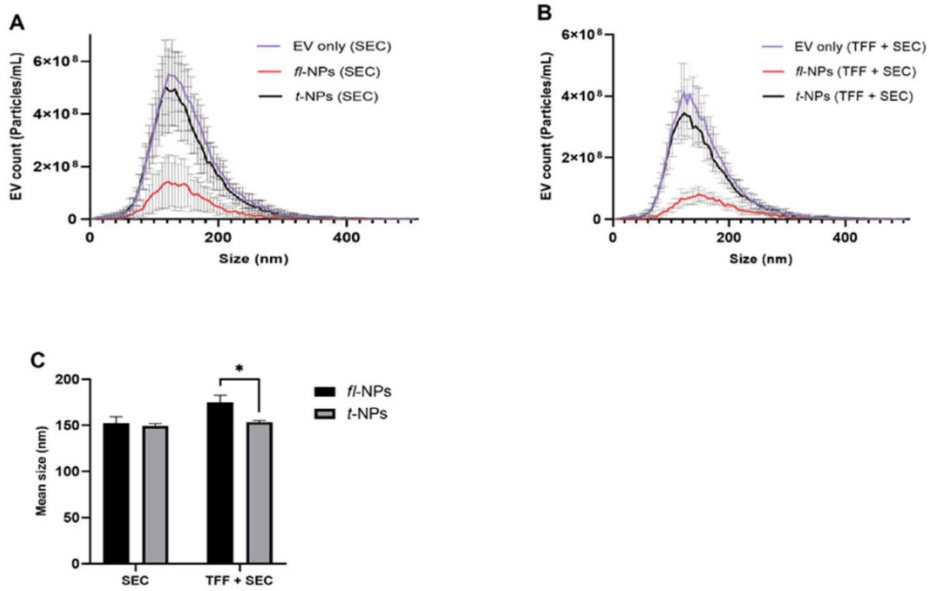

Figure 20. Particle size distribution and mean particle sizes for NPs of CMG-labelled JAr EVs. (**A,B**) Particle size distribution and (**C**) mean particle sizes in scatter and fluorescent mode (504 nm) for EVs isolated from JAr conditioned medium using SEC and TFF plus SEC. The size of *fl*-NPs originated from JAr cells was measured at a minimum brightness threshold value of 25 at CMG concentration of 100 ng/mL. A significant difference in mean particle size observed between *fl*-NPs and *t*-NPs of JAr EVs purified in TFF and SEC. Thus, were labelled with an asterisk sign (mean ± SD, $n = 9$) (III).

There was a difference in concentration for *fl* and *t*-NPs of CMG-labelled EVs purified in SEC, and TFF plus SEC (Fig. 21A). Labelling of EV samples with CMG showed that *fl*-NPs of JAr EV purified in SEC had a relatively high percentage of CMG positive NPs (~29%) than JAr EVs purified in TFF plus SEC (~26%). In addition, there were also differences in the ratios of fluorescently labeled NPs in the different EV size bins purified in SEC only and in combination with TFF (Table 1). Like mentioned above, the size bins for CMG positive fluorescent NPs were categorized into four groups (40–150, 151–250, 251–350, and 351–450 nm). A consistent CMG labeling ratio was observed for the SEC purified EVs in first three size groups at the dye concentration of 100 ng/mL, while the larger size bin showed higher levels of CMG-positive fluorescent NPs. JAr EVs purified in TFF + SEC, however, were found to have increasing ratios of CMG-positive fluorescent NPs with increasing size bins.

Table 1. **Labelling percentage for CMG positive JAr EVs purified in SEC and TFF plus SEC**

Vesicle size ranges (nm)	SEC purified *fl*-NPs (%)	TFF + SEC purified *fl*-NPs (%)
40–150	26.17 ± 9.34	19.23 ± 4.06
151–250	27.17 ± 9.03	34.52 ± 6.57
251–350	29.47 ± 10.17	48.15 ± 16.27
351–450	41.68 ± 25.46	69.58 ± 34.46

FL-NTA was also used to determine the effect of purification method on the ZP of *fl* and total NPs of CMG labelled EVs. Based on ZP measurements (Fig. 21B), ZP of *fl*-NPs of JAr EVs purified with SEC significantly shifted further to more negative values than respective Control (*t*-NPs). T-tests found no significant differences in ZP values for *fl* and *t*-NPs of JAr EVs isolated in conjunction with TFF and SEC ($p = 0.183$). Furthermore, the ZP of *t*-NPs of JAr EVs isolated in TFF and SEC significantly shifted towards a more negative value than the ZP of *t*-NPs of JAr EVs purified in SEC alone ($p \leq 0.05$). Generally, the ZP of EVs only (control) and *fl*-NPs of JAr EVs (purified both in SEC and TFF + SEC) was not affected by the chosen method of EV purification.

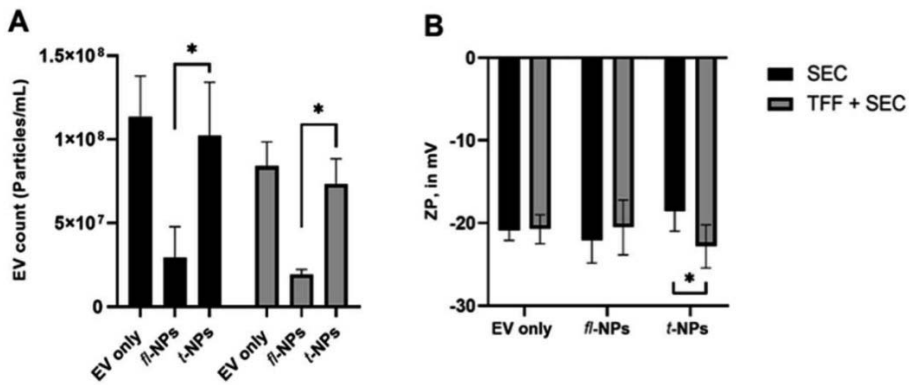

Figure 21. Concentration and ZP for NPs of CMG-labelled JAr EVs isolated in SEC and in conjunction with TFF. Comparison of (**A**) concentration and (**B**) zeta potential of CMG-positive *fl*-NPs in comparison to total NPs measured after labelling with a CMG dye concentration of 100 ng/mL. A significant difference in concentration observed between *fl*-NPs and *t*-NPs NPs of CMG-labelled JAr EVs isolated in SEC and in conjunction with TFF. The ZP of *t*-NPs of JAr EVs isolated in TFF and SEC significantly different from the ZP of *t*-NPs of JAr EVs purified in SEC alone. Thus, were labelled with an asterisk sign (mean ± SD, $n = 9$) (III).

5.3.4. Influence of EV source on fluorescent EV proportions (labelling percentage)

The purpose of experiment 3 was to determine whether the EV source would affect the particle size, concentration, and surface charge of CMG-labelled EVs. This study used SEC purification to isolate EVs from bovine follicular fluid (BFF) and seminal plasma. As expected, biologically-derived EVs also displayed heterogeneity in particle size (Hasan *et al.*, 2020). A high particle size distribution (~33 to 380 nm) was observed for the *fl*-NPs of BFF EVs. While *fl*-NPs of seminal plasma-derived EVs have sizes ranging from ~50 to 380 nm. The results, as shown in figure 22A, indicate that the mean particle size of *fl*-NPs of BFF EVs shifted toward smaller particle sizes than seminal plasma-derived EVs. Figure 22 C-D illustrates the variation in concentration between *fl*-NPs of BFF and seminal plasma-derived EVs. Labelling of EV samples with CMG showed that *fl*-NPs of BFF EV had a significantly higher percent of CMG positive NPs (~60%) than seminal plasma-derived EVs (~18%). There were also differences in the ratios of fluorescently labelled NPs in the different EV size bins for EVs derived from biological fluids (Table 2). In the smallest size bins of BFF EVs, the CMG labelling percentage was higher than in the remaining three size bins, which had almost uniform CMG labelling percentages at the dye concentration of 100 ng/mL. With increasing size bins, an increase in fluorescent NP ratio was observed for CMG-positive fluorescent seminal plasma-derived EVs.

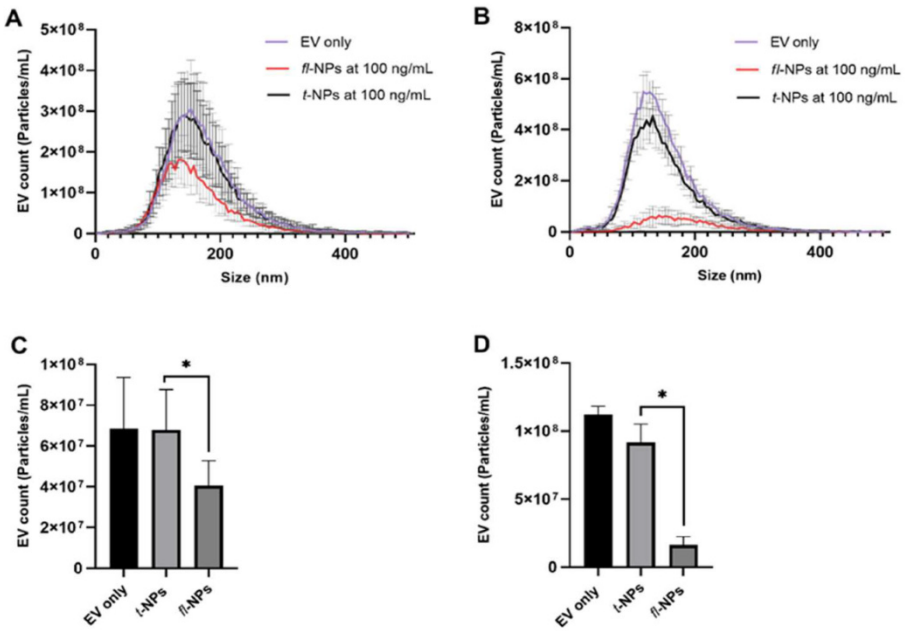

Figure 22. Particle size distribution and concentration for NPs of CMG-labelled biologically-derived EVs. (**A,B**) Particle size distribution and (**C,D**) mean particle sizes in scatter and fluorescent mode (504 nm) for EVs isolated from BFF and seminal plasma, respectively. The size of *fl*-NPs originated from BFF and seminal plasma was measured at a minimum brightness threshold value of 25 at CMG concentration of 100 ng/mL. A significant difference in concentration observed between *fl*-NPs and the corresponding *t*-NPs of BFF and seminal plasma EVs. Thus, were labelled with an asterisk sign (mean ± SD, $n = 9$) (III).

Table 2. **Labelling percentage for CMG positive BFF and seminal vesicles EVs purified in SEC**

Vesicle size ranges (nm)	*fl*-NPs of BFF EV (%)	*fl*-NPs of seminal vesicles (%)
40–150	76.16 ± 19.70	11.07 ± 5.93
151–250	51.81 ± 7.98	25.86 ± 9.27
251–350	47.76 ± 19.18	38.47 ± 13.83
351–450	49.31 ± 21.23	62.90 ± 29.30

Regarding ZP measurements, the ZP of *fl*-NPs of BFF EVs significantly shifted further to a less negative value than respective *t*-NPs ($p \leq 0.05$). T-tests found no significant differences ($p = 0.353$) in ZP values for *fl* and *t*-NPs of seminal plasma (Fig. 23).

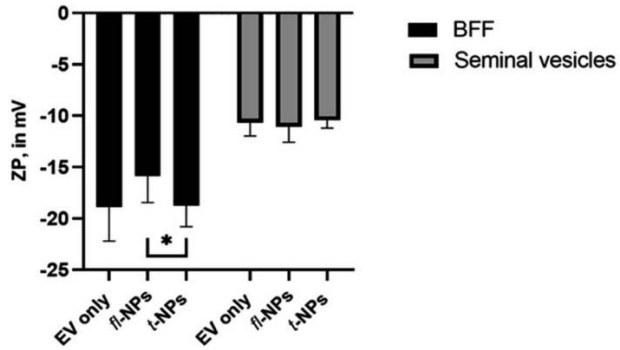

Figure 23. Zeta potential for NPs of CMG-labelled BFF and seminal plasma EVs. Comparison of ZP of CMG-positive *fl*-NPs in comparison to total NPs measured after labelling with a CMG dye concentration of 100 ng/mL. The ZP of *t*-NPs of BFF EVs was significantly different from the corresponding of *t*-NPs of BFF EVs. Thus, were labelled with an asterisk sign (mean ± SD, $n = 9$) (III).

5.3.5. Investigation of the effect of detergent on the EV lipid bilayer in the context of fluorescent EVs

To ensure that the true fluorescent labelling of EV membrane was done during FL-NTA experiment, EVs were treated with NP-40 detergent (0.5%) to lyse the membrane. NP-40 detergent treatment demonstrated that the concentration of *t*-NPS in JAr EVs decreased from ~$1x10^8$ particles/mL before treatment to ~$2.3x10^7$ particles/mL following detergent treatment. As a result, particle concentration decreased by approximately 74–76% (Fig. 24). In the case of fluorescent NPs, the reduction in particle concentration was prominent that the concentrations of *fl*-NPs after NP-40 treatment fell below the detection limit of FL-NTA.

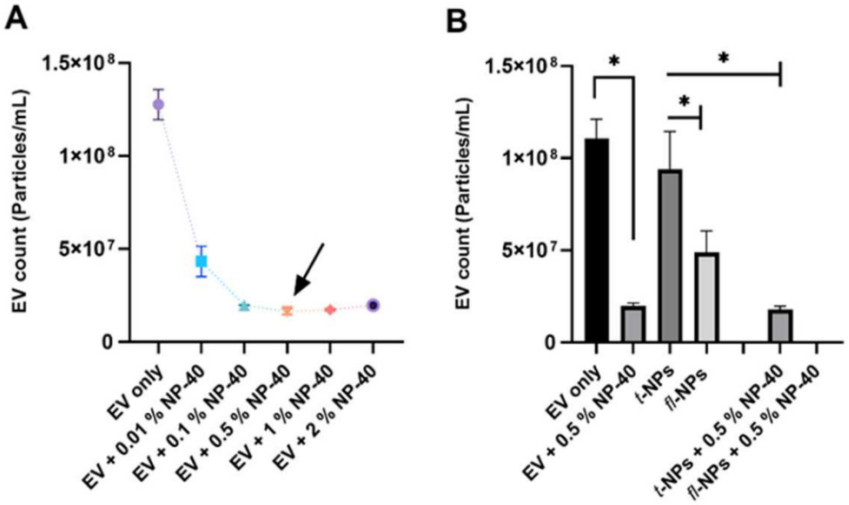

Figure 24. NP-40 treatment of EVs derived from cultured human JAr cells. (**A**) Different concentrations (0.01%, 0.1%, 0.5%, 1%, and 2%) of NP-40 were applied to EVs. NP-40 concentrations of 0.5% disrupted the membrane of EVs and resulted in saturation. (**B**) JAr EVs were treated with NP-40 detergent at a concentration of 0.5 % with or without the presence of CMG dye. The particle concentration of *t*-NPS in JAr EVs decreased significantly after 0.5 % NP-40 detergent treatment. Thus, were labelled with an asterisk sign (mean ± SD, $n = 9$) (III).

Key findings of study III:

- ⇨ With successive increases in the concentration of CMG dye, the particle size distribution and mean particle size for *fl*-NPs of CMG-labelled JAr EVs moved further to smaller particles size ranges.
- ⇨ JAr EVs labelled with higher concentrations of dye produced a greater number of fluorescently labelled particles compared to EVs labelled with the lowest dye concentration. Additionally, there was a significant shift toward more and less negative ZP values for *fl*-NPs of JAr EVs with the lowest and highest dye concentrations.
- ⇨ The mean particle size of *fl* and *t*-NPs of JAr EVs purified in TFF and SEC significantly shifted towards larger particles. Contrary to this, the mean particle size of *fl* and *t*-NPs of JAr EVs purified in SEC alone remain unchanged.
- ⇨ Labelling of EV samples with CMG showed that *fl*-NPs of JAr EV purified in SEC had a significantly higher percentage of CMG positive NPs (~29%) than JAr EVs purified in TFF plus SEC (~26%). In another scenario, labelling of EV samples with CMG showed that *fl*-NPs of BFF EV had a significantly higher percent of CMG positive NPs (~60%) than seminal plasma-derived EVs (~18%).

6. DISCUSSION

Broadly, this thesis aimed to determine the colloidal stability of EVs under different physical conditions and with the introduction of lipophilic membrane dye, FL-NTA technology can be used to distinguish EVs from non-EV populations and to check the purity of EVs. In order to accomplish this, EVs derived from cultured human JAr cells and biological sources were used as model systems. We showed that ZP of JAr EVs was affected by the nature of the phosphate buffer with or without the presence of detergent as well as the ionic strength and pH of the buffer due to the compression of EDL surrounding the surface of EVs. Thus, lowering the repulsive forces between EVs resuspended in the respective buffers and favour aggregation of EVs. Furthermore, we compared the ZP of EVs isolated from healthy controls and psoriasis patients (e.g. PsA and PsV). Following that, a range of pre and post-acquisition parameters have been optimized for the FL-NTA instrument. We investigated the detection of fluorescent NPs at different sensitivity levels, shutter values, and minimum brightness levels using CMG labelled JAr and HCT CD63 positive EV standards. Optimizing the above-mentioned parameters of FL-NTA is crucial for the proper detection and analysis of fluorescent NPs. It is important to ensure that the camera is set at an optimal level i.e. sensitive enough to detect the smallest NPs in a given sample. Changing the minimum brightness threshold level of the camera, for instance, affects the detection of fluorescent NPs in two ways: only large and bright NPs will be detected if the minimum brightness threshold is raised. Thus, hiding the smaller NPs from the total fluorescent NPs count. Alternatively, decreasing the minimum brightness threshold level can increase the detection of background noise, which can be registered as fluorescent NPs. For this reason, it is recommended that both pre and post-acquisition parameters of FL-NTA be refined for detection and reliable data of fluorescent NPs in heterogeneous EV samples. After optimizing a range of parameters of FL-NTA, we examined the effect of CMG labelling on the physical characteristics of JAr EVs purified in SEC and in combination with TFF as well as biological-derived EVs (e.g. BFF and seminal plasma). According to the results of FL-NTA, this technology has the potential to distinguish EVs from non-EV populations as well as check the purity of EVs derived from cell culture conditioned media and biological fluids. By excluding the signal from unlabelled NPs, FL-NTA detects fluorescently-labelled NPs enclosed by lipid membranes in a given sample (Oesterreicher *et al.*, 2020). It is clear from these studies that it is important to characterize EVs under standardized ZP measurements and to exploit the potential of FL-NTA for the detection of membrane dye-labelled EVs under different conditions.

The assessment of ZP measurements of either unlabelled or labelled EVs with controlled variables (the pH, ions in the buffer, other additives and applying the right electrokinetic model) may serve as a direct characterization tool for EVs. Naturally, these EVs possess a net negative charge due to the presence

of glycosylated and membrane proteins on their surfaces. Thus, EVs carrying genetic cargo provide a versatile platform for the advance of improved DDS with surface modifications. Further studies are required to determine the colloidal stability of EVs and to determine whether different methods of EV surface engineering maintain their functional cargoes. In order to achieve this, ZP can be applied in a controlled environment to determine the nature of engineered EV surfaces and their distribution of charges. In relation to the results of the FL-NTA. Using lipophilic dye, FL-NTA demonstrated its ability to distinguish EVs from non-EV populations and could be used as a purity indicator for EV samples while maintaining the physicochemical characteristics of CMG positive EVs. All of these findings demonstrate that various factors can affect the stability of isolated EVs before they can be used for functional analysis and therapeutic purposes. Moreover, it is important to use fluorescence in conjunction with conventional NTA for the proper detection and analysis of fluorescently labelled EVs.

6.1. Isolated and characterized JAr EVs confirm the physical and biochemical properties EVs

In recent years, there has been increasing interest in EVs use as DDS due to their multifunctional roles in cellular communication and potential use as therapeutics. There are, however, several challenges related to EV research, including their heterogeneity (Li *et al.*, 2019), which complicates their isolation and characterization. By using SEC, we were able to isolate EVs from cultured human JAr cells. Physical characterization of JAr EVs using NTA and electron microscopy (SEM and TEM) confirmed heterogeneous particle size distributions. The SEM and TEM measurements correlated well with the NTA particle size measurements, although the latter provided a broader distribution of particle sizes (~30–300 nm) for isolated EVs. Western blot analysis confirmed that EV surface marker proteins were present in purified JAr EVs and corresponding cell lysates, suggesting successful SEC isolation of EVs. A negative surface charge of EVs was attributed to these surface marker proteins. Additionally, the negative surface charge of EVs can be determined via ZP measurements. Different studies have demonstrated significant differences in ZP for EVs derived from cultured human cells (Baran *et al.*, 2010; Akagi *et al.*, 2015; Kesimer and Gupta, 2015; Nishino *et al.*, 2020). Therefore, further research should be conducted to examine and compare ZP data obtained under different conditions. In the following section of this thesis, the factors influencing the ZP of EVs isolated from cultured human JAr cells are discussed in greater detail.

6.2. The nature of the medium, pH and ionic strength influence the ZP value of EVs

Surface charges of NPs can be affected by the nature of the dispersion system. With increasing phosphate ion concentrations, the ZP of EVs shifted towards less negative values. One of the reasons for this effect was the presence of more counterions in 1mM than the other two more diluted concentrations of phosphate buffer. The presence of counterions is also related to higher conductivity observed at higher concentrations of phosphate buffer (i.e. 1 mM [1070−1151 μS/cm] >> 0.01 mM [26−46 μS/cm]). Availability of high conductivity at 1 mM phosphate buffer concentrations can affect the distribution of the ions surrounding the EV surface. In particular, the thickness of an electrical double layer (EDL) surrounding the NP is inversely proportional to the ionic strength of the medium. This results in the compression of the EDL. The presence of higher conductivity of the medium may also influence the stability of EDL by reducing electrostatic repulsive forces between EVs, thereby affecting the colloidal stability of EVs and resulting in a less negative ZP value. This study supports evidence from previous observations (Sokolova *et al.*, 2011; Kesimer and Gupta, 2015; Soares Martins *et al.*, 2018). This finding is contrary to previous studies which have suggested that low molar concentrations of phosphate buffer resulted in less negative ZP value for normal trabecular meshwork-derived EVs (Beit-Yannai, Tabak and Stamer, 2018; Tabak, Schreiber-Avissar and Beit-Yannai, 2021). A number of factors could have contributed to these differences, including the phosphate buffer concentrations tested, the nature of EV studied, the concentration of EV, etc. The effect of NP concentration on the ZP was observed for EVs resuspended in 0.1 mM phosphate ion concentration.

The ZP shifted towards less negative values for EV concentrations of 10^6 and 10^7/mL compared to 10^8/mL resuspended in the same phosphate buffer content. A shift in ZP with lower concentrations may be a consequence of too diluted samples and the high electrophoretic mobility of EVs in the resuspended medium. For this reason, NPs should be used at an optimal concentration that can provide a sufficient signal-to-noise ratio. Overall, it is not possible to establish a correlation between ZP and EV concentration.

Our study also used Tween-20 to determine the degree of adsorption onto EV surfaces. The addition of detergent to the phosphate buffer contents resulted in less negative ZP values of EVs. Generally, ZP values of EVs resuspended in a buffer containing 0.03 % Tween-20 were less negative. Tween-20, however, had no effect on ZP for all combinations of EV and phosphate buffer. For instance, Tween-20 had no effect on ZP measurements for the highest EV concentration that was resuspended in 1mM phosphate buffer. These surfactants influence the stability of colloidal systems by controlling the surface charge of particles as well as the hydrophobic-hydrophilic balance on their surfaces (György *et al.*, 2011). The presence of Tween-20 in the phosphate buffer may have led to Tween-20 molecules adsorbing to the EV surface and lessened

colloidal stability. Further reductions in the ZP of EVs may have been attributed due to the surfactant's ability to adsorb onto EV surfaces near the critical micellar concentration (CMC). Overall, the ZP of EVs seems to have stabilized due to the non-ionic interactions of Tween-20 resuspended in low molar concentrations of phosphate buffer hampering with the steric repulsion between EVs. Previous studies have not examined the effect of detergents on ZP or its relationship to the colloidal stability of EVs. However, some studies reported the use of detergents for vesicle solubilization (Osteikoetxea *et al.*, 2015; Yakovlev *et al.*, 2020).

Ionic strength and pH are crucial factors that can influence the fate of EVs in cellular physiology (Moore *et al.*, 2015; Liu *et al*., 2017). The current study found that the ZP of JAr EVs became less negative in the presence of trivalent and divalent ions compared to monovalent ions. It has been noted above that decreases in ZP in polyvalent ions are caused by shrinkage of the EDL around EV surfaces as a result of increased binding by higher valency ions. The compression of the EDL layer surrounding EV surfaces further promotes NP aggregation due to frequent NP-NP collisions, leading to aggregation, as indicated by a reduction in ZP. Interestingly, monovalent ions did not affect the ZP of EVs. This finding broadly supports the work of other studies in this area linking the effect of different EV formulation conditions (e.g. buffer type, ionic strength, and pH) on the stability of frozen and thawed EVs (Trenkenschuh *et al.*, 2022).

The present study also investigated the effect of pH on the ZP of JAr EVs. A decrease in pH (acidic pH) resulted in a decrease in ZP of EVs, and vice versa. The surface charge of EVs is naturally negative. However, the addition of EVs to an acidic environment probably neutralized some of the EV negative surface charges, leading to weaker electrostatic repulsive forces. This further fosters the aggregation of EV particles, as indicated by a lesser ZP value. On the contrary, the addition of EVs into a basic (OH-) environment exposed more negative sites on the EV surfaces due to the deprotonation of surface groups. This situation favors electrostatic repulsions between EV particles and increases their colloidal stability. This study supports evidence from previous observation with different perspective. (Cheng *et al.*, 2019) reported that EVs stored at pH 4 yielded lower particle numbers than at pH 10, thus indicating an acidic environment has a destructive effect on EVs. Based on these findings, physicochemical characterization may be prioritized for the successful translation of EVs into nanomedical formulations or therapeutic applications.

6.3. ZP of blood-serum derived EVs did not differ between healthy and psoriasis patients

As part of this study, we also assessed the physical characteristics of blood serum EVs derived from healthy and psoriasis patients (PsV and PsA). There is evidence that the size distribution or ZP of EVs is strongly related to their

cellular origin (Sokolova *et al.*, 2011; Kato *et al.*, 2013; Freeman *et al.*, 2018). In this study, however, the difference in particle size distributions and ZP of EVs was not observed between the healthy controls and patient samples (PsA and PsV). EVs' colloidal stability can be indirectly affected by the long-term storage effect of samples on their surface properties, which could affect their biological surface properties and cargo proteins (Maroto *et al.*, 2017; Santucci *et al.*, 2019; Görgens *et al.*, 2022). This can be one of the reasons why we did not detect a difference in ZP between the studied groups. In future investigations, it is possible to use different and sensitive technologies that can distinguish the physical characteristics of EVs derived from healthy and diseased samples. In the following section of this thesis, the factors influencing the CMG labelling on human JAr cells and biological source-derived EVs are discussed.

6.4. CMG labelling alters the size of fluorescent nanoparticles

Concentration of membrane dye, the choice of purification method, detergent treatment and source of EVs effect the optimum labelling of EVs using FL-NTA. We have used CellMask™ Green (CMG) plasma membrane dye to label the membrane of EVs (CellMask™ Green Plasma Membrane Stain). Conventional NTA is limited in its ability to distinguish vesicular particles from non-vesicular particles or particle aggregates within a given sample population. Due to this reason, we labelled EVs with CMG and track their emitted fluorescent light using the fluorescent mode of NTA. With the aid of FL-NTA, we were able to distinguish NPs of EV origin from non-EV origins. We have optimized the FL-NTA camera's sensitivity threshold level to ensure proper analysis and detection of fluorescent nanoparticles (*fl*-NPs)(Gardiner *et al.*, 2013).

Increases in CMG dye concentrations resulted in a decrease in the mean size of the *fl*-NPs. It was found that at the highest concentration of CMG dye, *fl*-NPs were in the size range of ~15 to 40 nm. TEM/SEM imaging of CMG-labelled EVs would provide insight into the size shift of particles at high dye concentrations. In the scatter mode of NTA, these NPs were not detected. This finding is consistent with that of Carnell-Morris *et al.*, 2017 who used CellMask™ Orange to label plasma-derived EVs. Using FL-NTA, the authors also detected *fl*-NPs in a size range of ~ 15 to 30 nm that were not detected when using scatter mode (Carnell-Morris *et al.*, 2017). Shimomura *et al.*, 2021 studied EVs labelled with Mem and PKH dyes, and found that there was no effect on the size of Mem-labelled EVs. We observed a similar result when we used the lowest concentration of CMG for labelling EVs and detecting the corresponding *fl*-NPs. In contrast, Dehghani *et al.*, 2020 reported that successive increases in PKH dye concentrations caused an increase in *fl*-NPs of PKH-labelled EVs. Thus, suggesting formation of PKH aggregates (Pužar Dominkuš *et al.*, 2018, p. 26). A number of factors could have contributed to these differences, including the type and EV concentrations used, the structure and properties of the

dyes, incubation temperature, sensitivity of the NTA camera etc. In addition, there were differences in the ratios of fluorescently labelled NPs in the different EV size bins (i.e. 40–150, 151–250, 251–350, and 351–450 nm). A consistent CMG labelling ratio was observed for the first three size groups at the lowest dye concentration, while the largest size bin showed higher levels of CMG-positive fluorescent NPs. With the lowest concentration of dye, the mean particle size is maintained for cumulative size ranges of fluorescent NPs. However, an increase in dye concentration led to a high labelling of the smallest size bins, specifically vesicles in the exosome size ranges. There is a wide variation in the distribution of lipids in EVs' membrane (Brzozowski *et al.*, 2018; Skotland *et al.*, 2020). For example, exosomes contain a higher order of lipids than microvesicles. The higher percentage of *fl*-NPs observed at the smallest size bins could be attributed to this. It is necessary to conduct further research in order to determine why the smallest size bins of EVs are more fluorescent than the rest of the size bins. Therefore, these results should be interpreted cautiously, particularly at the highest dye concentration.

6.5. EV purification method and source of EVs attribute to variations in the proportion of CMG-positive fluorescent nanoparticles

One of the major concerns in the EV field is the lack of an accurate EV quantification method. By adopting FL-NTA, we can determine the percentage (i.e. concentration of NPs measured at fluorescent mode to that of scattered mode) of EVs in a given heterogeneous population of NPs. Different isolation methods can result in different biophysical properties for the same cell line, further complicating EV quantification (Sharma *et al.*, 2020). Here, we used FL-NTA as a EV purity index tool to compare the efficiency of different EV purification methods. A comparison in EV purification efficiency of JAr cell-derived EVs purified in SEC alone or coupled with TFF revealed that *fl*-NPs of JAr EV purified in SEC had a relatively higher percent of CMG positive NPs (~29%) than JAr EVs purified in TFF plus SEC (~26%). Due to the fact that the TFF + SEC method is developed for large-scale EV productions (Watson *et al.*, 2018), we were expecting to separate and remove protein contaminants and yield a pure EV sample. However, the particle size of *fl*-NPs for JAr EVs purified by TFF + SEC was larger than that of EVs purified by SEC alone. Likewise, JAr EVs purified in TFF + SEC had increasing ratios of CMG-positive fluorescent NPs with increasing size bins (i.e. smallest to largest: 19.2 to 69.6%), as opposed to *fl*-NPs purified in SEC alone with consistent ratios. Moreover, the particle mean size of *fl*-NPs in SEC purified EVs is retained, indicating that the SEC method is capable of preserving both physicochemical and functional properties (Mol *et al.*, 2017). Colloidal stability of EVs can be affected by EV source and purification method (Zonneveld *et al.*, 2014; Nordin *et al.*, 2015). In

this case, *fl*-NPs of JAr EVs purified in SEC showed more negative ZP values than those purified in TFF + SEC, indicating these particles are less prone to aggregation. Overall, the importance of defining the true identity of EVs in FL-NTA will allow us to validate EV tracking studies where a change in particle size is known to affect EV uptake and biodistribution (Pužar Dominkuš *et al.*, 2018; Staufer *et al.*, 2022).

This study also aimed to determine whether the EV source would affect the particle size, concentration, and surface charge of CMG-labelled EVs. The particle size distribution of *fl*-NPs derived from seminal plasma was shifted towards larger particles than that of *fl*-NPs derived from BFF EVs. Interestingly, biologically derived EVs showed a greater variation in labelling ratios than cell culture-derived EVs. For instance, the labelling ratio for CMG positive *fl*-NPs for BFF and seminal plasma derived EVs was ~60 % and ~18 % respectively. There were also differences in the ratios of fluorescently labelled NPs in the different size bins for EVs derived from these biological fluids. The fluorescent NP ratio increased with increasing size bins for fluorescent seminal plasma-derived EVs (smallest to largest: ~11.1% to 62.9%). Conversely, the fluorescent NP ratio for BFF derived EVs was higher in the smallest size bins (~ 76.2%), while it was relatively uniform for the rest (~ 50.1%). Comparison of the findings with those of other studies confirms lipophilic dye labelling of biologically derived EVs provides relatively higher fluorescent particles than that of cell culture-derived EVs (Dlugolecka *et al.*, 2021). However, this is not always the case as the JAr EVs have a greater proportion of *fl*-NPs than the seminal plasma-derived EVs. It has been reported that seminal plasma does not contain lipids, but there are relatively small amounts of lipids in seminal plasma of bovine, boar and human (Komarek *et al.*, 1964; Vignon *et al.*, 1989; Am-in *et al.*, 2011). There is a possibility that this condition influences the binding of CMG to the membranes of seminal plasma-derived EVs. Other factors could have contributed to these differences, including the structure and complex composition of lipid, the permutations of head groups and fatty acid chains, hydrophobicity and head group polarity, etc (van Meer *et al.*, 2008; Ramirez *et al.*, 2018; Skotland *et al.*, 2019). Additionally, these differences may also result in different ZP values for BFF and seminal plasma-derived EVs. Overall, the source of EVs can affect the proportion of CMG-positive *fl*-NPs.

6.6. Detergent treatment of EVs confirms the true EV membrane labelling

The addition of a non-ionic detergent, such as NP-40 to EV suspensions was used to confirm the true fluorescent labelling of JAr EV membranes. A significant loss in particle number was observed when unlabelled and CMG-labelled EVs were treated with 0.5 % of NP-40. Using the fluorescent mode of NTA, detection of complete elimination of NP-40-treated *fl*-NPs indicated that the NPs under study were surrounded by lipid bilayers. Detergents such as these

may cause an imbalance of forces on the surfaces of the NPs, thereby affecting the membrane of the EVs. It is evident from these types of conditions that the particles under study were enclosed with membranes. These results provide information about the true membrane labelling of EVs in a heterogenous population of NPs. However, this conclusion should be viewed with caution if the protocols used for labelling and experimental conditions are not rigorously optimized and standardized. Overall, FL-NTA can be used as a EV purity index tool to compare and check the purity of heterogeneous EV samples.

6.7. Future directions

In this thesis, we have investigated the impact of different factors (nature of the buffer with or without the presence of detergent, the ionic strength and pH of the phosphate buffer) on the ZP of EVs secreted by the cultured human choriocarcinoma (JAr) cells. Additionally, we compared the physical characteristics (e.g. particle size distribution, concentration and ZP) of EVs isolated from healthy controls and psoriasis patients (i.e. PsA and PsV) and systematically evaluated the CMG labelling on EVs isolated from cell-culture and biological sources using FL-NTA. In the first study, the results suggested the need for EV characterization to be conducted with rigor and on the basis of standardized ZP measurement protocols. It is necessary to expand the idea and use of ZP so that it can be widely applied in the EV field. This includes the following:

- Firstly, we need to investigate the appropriate storage buffer conditions in order to maintain the native and stable properties of EVs. In order to accomplish this, we need to test different buffer formulations and additives in order to preserve the fate of EVs following purification and storage.
- Secondly, ZP value can be used for process development and mass production of EVs.
- Thirdly, ZP value of EVs suspended in clinically used drug buffers provide valuable insight into pharmacokinetic studies.

Additionally, it will be necessary to explore highly sensitive and advanced technologies to profile EVs in the blood. It is possible also to establish a sensitive and accurate detection of ZP using the latest particle tracking technologies by introducing antibody or aptamer to EVs. This way a proper understanding on the specific binding of antibodies or aptamers on EVs surface can be achieved with the detection of shift in ZP values. It is also applicable to compare the ZP of different sources of EVs, including cell cultures and biofluids, in healthy and diseased conditions. This will allow us to measure the specific ZP of EVs rather than other contaminants present in the sample. It is also important to be aware of the long-term storage effect on the expression of EV surface proteins, which will destabilize their surface characteristics. Our future research will be encouraged by using freshly prepared samples. Ultimately, ZP can be used to predict the surface marker expression of individual EVs as well as to predict the colloidal stability of EVs.

Finally, the results of the FL-NTA study indicated the need to develop and adopt different fluorescent probes for screening the surface nature and distribution of lipid composition within the plasma membrane of EVs. Consequently, this leads to the analysis of the dynamics within the lipid rafts within the lipid bilayer. To study EV heterogeneity and biodistribution, we are indirectly labelling JAr cells with different organelle dyes and visualizing them with the latest FL-NTA. Thus, we will be able to understand the mechanisms involved in EV biogenesis and their trafficking routes. Furthermore, the introduction of antibodies along with the membrane dyes will result in specific binding to EVs. FL-NTA is also capable of detecting EV surface markers and identifying organelle-derived vesicles for a variety of purposes. In this manner, fluorescently labelled membrane-bound vesicles can be quantified with reliable data using FL-NTA.

7. CONCLUSIONS

Study I: Higher concentrations of multivalent ions (Ca^{2+} and Al^{3+}) and anionic (phosphate ions), reduce the negative value of ZP at all EV concentrations examined. The effect of monovalent ions (K^{+}, Na^{+}, and Cl^{-}) on ZP values of EV is minimal. The ZP of JAr EVs is affected at acidic pH and depends on the pH of the phosphate buffer solution. Additionally, detergent (Tween-20) significantly reduces the negative value of ZP. It is evident that EVs need to be characterized systematically using standard ZP measurement protocols.

Study II: There is no significant difference in particle size distribution and zeta potential of blood serum-EVs derived from healthy controls and psoriasis patients (PsV and PsA).

Study III: Increases in the concentration of CMG dye lower the mean size of fluorescent NPs in JAr EVs. The purification method has an impact on fluorescent NP ratios in EVs. Variations in CMG-positive fluorescent NPs is observed in biologically derived EVs. The impact of membrane dye labelling on the ZP of cell-cultured and biological fluid-derived EVs is minimal. The true fluorescent labelling of JAr EVs membrane with membrane dye is revealed following detergent treatment. The Fluorescence NTA exhibits the potential to be utilized as an indicator of EV purity on a routine basis.

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SUMMARY IN ESTONIAN

Ekstratsellulaarste vesiikulite kolloidlahuste omaduste ja määramismeetodite uurimine

Sisukirjeldus

Nanoosakeste (NP) iseloomulikud omadused nagu nende suurus, kuju ja pinnalaeng määravad nende osalemise füsioloogilistes interaktsioonides ja võimaldavad NPde laialdast rakendamist meditsiinis ja teistes valdkondades. Nanoosakesi kasutatakse bioloogias ja kaasaegses meditsiinis näiteks fluorestsentsmarkeritena, ravimite ja geenide transpordiks, aga ka valgu määramiseks ning vähi raviks. Ehkki NPde kasutamine on perspektiivne, võib nende mõju füsioloogilistele süsteemidele olla ka negatiivne, eriti arvestades nende võimalikku toksikoloogist mõju. Selle vältimiseks on NPde kasutamiseks hakatud otsima alternatiivseid võimalusi. Ekstratsellulaarsed vesiikulid (EVd), mida eraldavad stressiseisundis olevad rakud, on membraanseotud NPd. EVd on ohutud ning nende laialdane kasutamine pakub uusi võimalusi, näiteks ravimite kohaletoimetamise süsteemide loomisel. EVde morfoloogia, pinna suuruse ja pinnalaengute iseloomustamiseks on välja arendatud uudsed kaasaegsed meetodid.

Looduslikus keskkonnas on EVd kolloidosakesed, millel on tänu pinnal leiduvatele glükosüleeritud valkudele, lipiididele jt. ühenditele resuspendeerituna summaarne negatiivne pinnalaeng. EVde pinnalaengut saab määrata nende elektroforeetilise mobiilsuse alusel, mis on aluseks ka EVde ζ-potentsiaali (ZP) väärtuse määramisele. ZP väärtust kasutatakse NPde ehk EVde kolloidosakeste stabiilsuse määramiseks lahuses, aga ka NPde ja rakumembraani vahelise esmase kontakti hindamiseks. EVde kolloidosakeste stabiilsus on äärmiselt oluline nende hindamisel erinevates tingimustes kus nad peaksid säilitama oma füsikokeemilised omadused ning kargo. EVde stabiilsuse hindamiseks kasutatakse mitmeid erinevaid meetodeid, kuid siiski puudub käesoleval ajal selleks standardne metoodika. Lisaks on EVde uurimisel oluliseks väljakutseks nende määramine ja kvantifitseerimine.

Käesolevas doktoritöös uuriti EVde kolloidlahuste stabiilsust erinevates tingimustes ning EVde fluorestsentsmarkeriga konjugeerimise mõju EVde füsikokeemilistele omadustele. Mudelsüsteemidena kasutasime inimese kooriokartsinoomi rakkude (JAr) kultuurist ning bioloogilistest allikatest pärinevaid EVsid. JAr päritolu EVde ZP väärtust mõjutavatest teguritest uuriti puhverlahuse kontsentratsiooni, detergendi, ioonse jõu ning lahuse pH mõju. Seejärel võrreldi tervete inimeste ning psoriaasi, nii PsA (psoriaatiline artriit) kui ka PsV (psoriasis vulgaris) põdevate patsientide verest eraldatud EVde ZP väärtusi. Fluorestseeruvate nanoosakeste jälgimise analüüsi (fNTA) kasutades uuriti lipofiilse membraanvärvi CellMask™ Green (CMG) mõju geelfiltratsiooni (SEC) abil puhastatud JAr EVde füüsikalistele omadustele ning kombineerituna tangent-

siaalse voolufiltratsiooni süsteemiga (TFF) ka bioloogilistest kudedest, näiteks veiste follikulaarvedelikust (BFF) ja seemnevedelikust, eraldatud EVdele.

Doktoritöö eesmärgid

Käesoleva doktoritöö peamiseks eesmärgiks oligi uurida EVsid sisaldavate kolloidlahuste stabiilsust mõjutavad tegureid, ning rakendada fluorestseeruvate nanoosakeste jälgimise analüüsi (fNTA) ekstratsellulaarsete vesiikulite määramiseks.

Konkreetsemad töö eesmärgid olid järgmised:

1. Määrata erinevate füsikokeemiliste tegurite nagu puhverlahuse kontsentratsiooni, detergendi, ioonse jõu ning lahuse pH mõju inimese JAr rakkudest eraldatud EVde ZP mõõdetavale väärtusele
2. Iseloomustada psoriaasipatsientide vereseerumist eraldatud EVde füsikokeemilisi omadusi ning võrrelda saadud tulemusi kontrollrühma vastavate väärtustega
3. Uurida lipofiilse membraanvärvi CellMask™ Green (CMG) mõju erinevatel meetoditel isoleeritud JAr EVde ning bioloogilistest vedelikest (veiste follikulaarvedelikust ja seemnevedelikust) eraldatud EVde suurusele, kontsentratsioonile ning ZP väärtusele.

Materjalid ja meetodid

Inimese esimese trimestri kooriokartsinoomi (JAr) rakke kasvatati RPMI 1640 söötmes, mis sisaldas 1% penitsilliini/streptomütsiini, 1% L-glutamiini ja 10% FBS-i, temperatuuril 37 °C niiskes 5% CO2 atmosfääris. Kui rakud saavutasid 80% konfluentsuse, eemaldati konditsioneeritud sööde. Seejärel asendati sööde värske RPMI 1640 söötmega, mis sisaldas 1% penitsilliini/streptomütsiini, 1% L-glutamiini ja 10% EV-depleteeritud veiseloote seerumit (FBS). Rakke kultiveeriti 24 tundi eespoolkirjeldatud tingimustes. Pärast inkubeerimist konditsioneeritud sööde koguti EV-de isoleerimiseks.

Uuringutes I ja III kasutatud EVd eraldati JAr rakkude konditsioneeritud söötmest. EVde puhastamise protsessis eemaldati konditsioneeritud söötmest esmalt järjestikuse tsentrifuugimise teel (4°C juures) rakud, rakufragmendid ning apoptootilised kehad. Saadud supernatant kontsentreeriti Amicon Ultra tsentrifuugi (10kDa) abil täiendavalt 500 µl-ni, millest EVde isoleerimiseks kasutati benchtop SEC kolonne.

Eraldatud EVde iseloomustamiseks kasutati nanoosakeste jälgimise analüüsi (NTA), skaneerivat ja transmissioon elektronmikroskoopiat (vastavalt SEM ja TEM) ning Western blot analüüsi. EVde ZP väärtuse määramiseks kasutati ZetaView NTA instrumenti. Uuringus I iseloomustati fosfaatpuhvri kontsentratsiooni mõju (0,01; 0,1 ja 1,0 mM puhverlahuses) nii 0.03% Tween-20 juuresolekul kui ka ilma detergendita. Samuti uuriti kloriidide (10 mM NaCl, KCl,

$CaCl_2$ ja $AlCl_3$) mõju EVde ZP väärtusele erinevatel pH väärtustel (pH= 4,0; 7,0; 10,0).

Uuring II läbiviimiseks kasutati 12 psoriaasihaige ning kontrollrühmana 12 terve inimese vereseerumit, mida tsentrifuugiti sarnaselt JAr konditsioneeritud söötmega. Viimasel tsentrifuugimisel saadud supernatant kontsentreeriti 500 µl-ni ning pandi qEVoriginal/70nm SEC kolonnidesse. SEC abil puhastatud EVd resuspendeeriti 1,0 mM fosfaatpuhvris (pH 7,4), misjärel määrati NTA seadme abil nende suurus ja ZP väärtus ning võrreldi psoriaasipatsientide ja kontrollrühma tulemusi. BFF ja seemneplasma proovid tsentrifuugiti järjestikku kasutades võrreldavaid tsentrifugaaljõude. Saadud kontsentreeritud supernatandist eraldati EVd kasutades benchtop SEC kolonne. Uuringus III uuriti CMG-märgistuse mõju erinevate meetoditega eraldatud JAr EVde mõõdetavale suurusele, kontsentratsioonile ning ZP väärtusele. fNTA abil uuriti samu parameetreid bioloogilistest vedelikest eraldatud EVdele, mis olid märgistatud CMG-ga. CellMask™ Green (CMG) on lipofiilne värvaine, mida kasutatakse EVde membraanide märgistamiseks.

Tulemused

SEM ja TEM analüüsid kinnitasid EVde tüüpilist tassikujulisi ja sfäärilist morfoloogiat. SEM-i ja TEM-i abil leitud EVde keskmine diameeter (~120 nm) langes hästi kokku NTA analüüsil saadud tulemustega. Western blot analüüsi tulemused näitasid EVde pinnal leiduvate markerite CD9, CD63 ja CD81 ning JAr EVde puhul ka valgu HSP70 olemasolu.

EVde ZP väärtus nihkub fosfaatpuhvri kontsentratsiooni kasvades vähem negatiivsete väärtuse poole. Samuti nihkus ZP väärtus vähem negatiivsete väärtuse poole detergendi lisamisel. Seejuures sõltus Tween-20 mõju oluliselt fosfaatpuhvri kontsentratsioonist, kuid ei sõltunud EVde kontsentratsioonist. ZP väärtus muutus oluliselt vähem negatiivseks ka kolmevalentse Al^{3+} ning kahevalentse Ca^{2+} juuresolekul, samas kui monovalentsed ioonid Na^+ ja K^+ seda ei mõjutanud. ZP väärtus vähenes lineaarselt ioonse jõu suurenemisel. Lahuse pH väärtuse suurenemisel EVde ZP negatiivne väärtus suurenes. Happelisemate ja aluselisemate pH väärtuste korral oli EVde ZP väärtuse muutus tulenevalt puhvri kontsentratsiooni muutusest suurem kui neutraalse pH väärtuse juures.

Uuriti ka ZP väärtuse muutumist psoriaatilise artriidi patsientide ning tervete inimeste vereseerumist eraldatud EVde korral. TEM analüüsi kasutati seerumist saadud EVde morfoloogia ja suuruse määramiseks, kusjuures TEM-I abil määratud EVde keskmine diameeter oli kooskõlas osakeste suuruse mõõtmise tulemustega, mis saadi NTA analüüsil.

Tervete ja psoriaasihaigete EVde ZP väärtuste võrdlemiseks suspendeeriti EVd 1,0 mM fosfaatpuhvris. Leiti, et eri rühmade EVde suurusjaotus ei erinenud oluliselt ning osakeste keskmine diameeter oli vahemikus 80–120 nm. Oluliselt erinevust ei olnud ka NTA analüüsil saadud psoriaasipatsientide PsA ja PsV ZP väärtuste vahel.

Uuringus III vaadeldi fluorestsentssondide, nt. membraanvärvide EVdega sidumise mõju EVde füsikokeemilistele omadustele. CMG membraanvärvi kasutamisel täheldati JAr EVde puhul heterogeenset suurusjaotust. Lähtudes fluorestsents (flNP) ja scatter (t-NP) režiimis tehtud mõõtmiste tulemustest, olid kontroll EVde ja CMG-märgistatud EVde suurused vahemikus ~30–400 nm.

CMG kontsentratsiooni suurenemisel nihkus CMG-märgisega JAr EVde suurusjaotus väiksemate osakeste poole, samuti vähenes osakeste keskmine suurus. CMG kontsentratsiooni muutumine põhjustas samuti flNPde kontsentratsiooni erinevuse. Lisaks esines erinevusi fluorestsentsmärgisega osakeste osakaaludes erineva suurusega fraktsioonides.

Nii fl kui t-NP osakeste keskmine suurus oli oluliselt suurem kui EVde puhastamiseks kasutati nii TFF ja SEC meetodit. Vastupidiselt eeltoodule, ainult SEC abil puhastatud JAr EVde fl-NPde ja t-NPde keskmine osakeste suurus ei muutunud. EV proovide märgistamine CMG värvaine abil näitas, et SEC abil puhastatud JAr EV fl-NP-del oli CMG –positiivsete NPde osakaal oluliselt suurem (~29%) kui TFF+SEC abil puhastatud JAr EVdel. ZP mõõtmised näitasid, et ainult SEC-ga puhastatud JAr EVde fI-NPde ZP nihkus oluliselt negatiivsemate väärtuse poole kui vastav kontroll (t-NPd). T-testid ei näidanud ZP väärtuste olulisi erinevusi JAr EVde fl- ja t-NPde vahel, mis olid isoleeritud TFF+SEC abil (p = 0,183).

Uuringu III üks eesmärk oli ka kindlaks teha, kas EVde allikas omab mõju CMG-märgisega EVde suurusele, kontsentratsioonile ning ZP väärtusele. BFF EVde fl-NPde puhul täheldati osakeste suuruste suurt hajuvust (~33 kuni 380 nm), samas kui seemneplasmast pärinevate EVde fl-NPde suurus oli vahemikus ~50 kuni 380 nm. EV proovide märgistamine CMG-ga näitas, et BFF EV fl-NPdel oli oluliselt kõrgem CMG-positiivsete NPde protsent (~60%) kui seemneplasmast pärinevatel EVdel (~18%). ZP väärtus nihkus BFF EVde fl-NPde korral oluliselt vähem negatiivsete väärtuste poole kui t-NPde korral ($p \leq$ 0.05). T-testid ei näidanud olulist erinevust (p = 0,353) seemneplasma fl-NP ja t-NP ZP väärtuste vahel.

Fluorestsentsmärgise kinnitumise tagamiseks EV membraanile töödeldi fl-NTA mõõtmistel EV membraane nende lüüsimiseks NP-40 detergendiga (0,5%). Selle tulemusena vähenes tNPde kontsentratsioon JAr EVdes märkimisväärselt. Fluorestseeruvate NPde kontsentratsiooni vähenemine oli samuti silmatorkav, sest nende kontsentratsioonid vähenesid pärast detergendiga töötlemist alla fNTA avastamispiiri.

Arutelu

JAr EVde füsikokeemiliste omaduste iseloomustamine NTA ning SEM ja TEM abil kinnitas osakeste heterogeenset suurusjaotust. SEM- ja TEM-mõõtmiste tulemused korreleerusid hästi osakeste suuruse mõõtmisega NTA meetodi abil. Western blot analüüs kinnitas, et EV pinnamarkervalgud olid nii puhastatud JAr EVdes kui ka vastavates raku lüsaatides. Lisaks saab EVde negatiivset pinnalaengut määrata ZP mõõtmiste abil.

1,0 mM fosfaatpuhvri kõrge juhtivus võib mõjutada EV pinnal asutavate ioonide jaotumist. Söötme kõrgem juhtivus võib mõjutada ka elektrilise kaksikkihi (EDL) stabiilsust, sest vähendades elektrostaatilisi tõukejõude EVde vahel ja põhjustades vähem negatiivseid ZP väärtusi, mõjutab see EVde kolloidosakeste stabiilsust.Tween-20 lisamine fosfaatpuhvrile võis põhjustada detergendi molekulide adsorptsiooni EVde pinnale ning kolloidlahuste stabiilsuse vähenemist. Üldiselt näib, et madala kontsentratsiooniga detergendilisandiga fosfaatpuhvris on EVde ZP väärtus stabiliseeritud tänu mitteioonsetele interaktsioonidele, mis takistavad EVde vahelist tõukumist.

Nagu eespool mainitud, võis Tween-20 lisamine viia Tween-20 molekulide adsorbeerumiseni. On pakutud, et polüvalentsete ioonide ZP väärtuse vähenemine on tingitud elektrilise kaksikkihi vähenemisest, EVde puhul põhjustab ZP väärtuse vähenemist kõrgema valentsusega ioonide suurenenud seondumine. Elektrilise kaksikkihi kokkusurumine ümber EVde pindade soodustab NP-NP interaktsioonide tõttu veelgi NPde agregatsiooni, mida näitab ZP väärtuse vähenemine. pH väärtuse vähenemine põhjustas samuti ZP väärtuse vähenemise ning selle suurenemine ZP väärtuse suurenemise. Saadad tulemuste põhjal on EVde nanomeditsiinilisteks ja terapeutilisteks rakendusteks soovitav EVde iseloomustamiseks kasutada nende füsikokeemilisi omadusi. Üldiselt näib, et EVde ZP väärtus on madala kontsentratsiooniga puhverlahustes stabiliseeritud tänu Tween-20 mitteioonsetele interaktsioonidele, mis takistavad EVde vahelisi steerilisi interaktsioone.

Uuringus II iseloomustasime ja võrdlesime psoriaasipatsientide (PsA ja PsV) ning tervete kontrollrühma vereseerumist eraldatud EVde füsikokeemilisi omadusi. Ehkki varasemalt on leitud, et EV osakeste suurusjaotus ja ZP väärtus on seotud nende päritoluga, ei leitud käesolevas uuringus kontrollrühma ja psoriaasihaigete vereseerumist eraldatud EVde suurusjaotuse ja ZP väärtuste vahel olulist erinevust. EV kolloidosakeste stabiilsust võib kaudselt mõjutada ka nende pinnaomaduste muutumine pikaajalisel säilitamisel. Viimane võib olla ka põhjuseks, miks ei tuvastatud uuritud rühmade vahel ZP väärtuse erinevusi.

Uuringus III vaadeldi EVde CMG membraanvärviga märgistamise mõju erinevate meetodite abil eraldatud JAr EVde ning erinevatest bioloogilistest vedelikest eraldatud EVde suurusele, kontsentratsioonile ja ZP väärtusele. CMG kontsentratsiooni suurenemine põhjustas fl-NPde keskmise suuruse vähenemise. Lipiidide jaotus EVde membraanis on väga erinev. Värvaine madalal kontsentratsioonil fluorestseeruvate NPde kumulatiivsete suurusvahemike keskmiste osakeste suurus ei muutu.

Ainult SEC või TFF+SEC abil puhastatud JAr EVde võrdlemine näitas, et SEC abil puhastatud JAr EVde fl-NPde CMG-positiivsete NPde protsent (~29%) oli mõnevõrra kõrgem kui TFF-SEC abil puhastatud EVdes (~26%). Veelgi enam, TFF+SEC abil puhastatud JAr EVde fl-NPde suurus oli suurem võrreldes ainult SEC abil puhastatud EVde suurusega. Lisaks oli SEC abil puhastatud JAr EVde ZP väärtus negatiivsem kui TFF+SEC abil puhastatud EVdel, mis näitas et need agregeerusid vähem.

Selle uuringu üks eesmärk oli ka uurida EVde eraldamiseks kasutatava allika mõju CMG-märgisega EVde suurusele, kontsentratsioonile ja pinnalaengule. On huvitav märkida, et bioloogilistest vedelikest eraldatud EVdel olid suuremad erinevused märgistatud osakeste suhetes kui rakukultuurist eraldatud EVdel. Suurematele erinevustele võisid kaasa aidata lipiidide struktuur ja koostis, samuti rasvhappeahelate permutatsioonid, erinevate rühmade polaarsus jne. Need tegurid võivad põhjustada ka erinevaid ZP väärtusi BFF ja seemneplasmast eraldatud EVde korral. Kokkuvõttes võib öelda, et EVde allikas võib mõjutada CMG-positiivsete fl-NPde osakaalu.

Nagu selgus detergendiga töödeldud fl-NPde uurimine NTA fluorestsentsrežiimis, olid uuritavad NPd ümbritsetud lipiidse kaksikkihiga. fNTA meetodit on võimalik kasutada heterogeensete EV proovide kvaliteedi kontrollimiseks ja proovide võrdlemiseks.

Kokkuvõte

Mitmevalentsete katioonide (Ca^{2+} ja Al^{3+}) ning anioonide (fosfaatioonid) kõrgemad kontsentratsioonid vähendasid EVde ZP negatiivset väärtust kõikidel uuritud EV kontsentratsioonidel, samas kui ühevalentsete ioonide (K^+, Na^+ ja Cl^-) mõju oli minimaalne. Samas EVde ZP negatiivne väärtus lahuse pH väärtuse suurenedes kasvab. Lisaks vähendas EVde ZP negatiivset väärtust oluliselt detergendi (Tween-20) lisamine lahusele. Läbiviidud uuringud näitasid, et EVde iseloomustamiseks tuleb kasutada standardiseeritud ZP väärtuse mõõtmise protokolle. Uuringu II tulemused näitasid, et psoriaasipatsientide (PsV ja PsA) ning kontrollrühma vereseerumist eraldatud EVde suurusjaotuses ning ZP väärtuses ei olnud olulisi erinevusi. Selgus, et EVde märgistamiseks kasutatava CMG värvaine kontsentratsiooni suurenemine vähendas JAr EVdes fluorestseeruvate NPde keskmist suurust. Fluorestseeruvate NPde suhtele EV proovides omab olulist mõju EVde puhastamiseks kasutatav metoodika. Bioloogilistest vedelikest saadud EVde puhul täheldati CMG positiivsete fl-NPde varieerumist. Samas membraanvärviga märgistamise mõju ZP väärtusele oli minimaalne nii bioloogilistest vedelikest kui ka rakukultuuridest eraldatud EVde puhul. JAr EVde puhul ilmnes tõeline fluorestsentsmärgistus pärast detergendiga töötlemist. Ülaltoodut kokku võttes võib väita, et ZP väärtust saab kontrollitud tingimuste korral kasutada nii märgistamata kui märgistatud EVde iseloomustamiseks. Lisaks on fNTA potentsiaalne tööriist EV preparaatide puhtuse rutiinseks kontrollimiseks.

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