Development of chromatographic methods for platelet derived extracellular vesicle isolation and purification

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March 2024

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Tiivistelmä

Tiedekunta: Farmasian tiedekunta

Koulutusohjelma: Proviisorin koulutusohjelma

Opintosuunta: Biofarmasian opintosuunta

Tekijä: Reetta Pusa

Työn nimi: Kromatografisten menetelmien kehittäminen verihiutaleiden solunulkoisten vesikkelien eristykseen

Työn laji: Pro Gradu -tutkielma

Kuukausi ja vuosi: Maaliskuu 2024

Sivumäärä: 49

Avainsanat: solunulkoiset vesikkelit, kromatografia, verihiutale

Ohjaaja tai ohjaajat: Saara Laitinen, Heikki Saari

Säilytyspaikka: ethesis.helsinki.fi

Muita tietoja:

Tiivistelmä:

Solunulkoisten vesikkelien käyttö diagnostiikassa sekä lääkekuljettimina on herättänyt mielenkiintoa viime vuosina, ja aiheesta tehdyn tutkimuksen määrä on ollut jyrkässä kasvussa. Yksi suurimmista solunulkoisten vesikkelien käyttöön liittyvistä haasteista on niiden eristäminen biologisista näytteistä, kuten verestä. Yleisimmän tähän käytettyjä metodeja ovat olleet kokoerottelukromatografia sekä ultrasentrifugointi. Nämä molemmat menetelmät käyttävät partikkelien kokoa ja tiheyttä erotteluperusteina eivätkä ole olleet riittävän tehokkaita erottamaan solunulkoisia vesikkeleitä muista partikkeleista, kuten lipoproteiineista.

Näiden haasteiden takia uusia menetelmiä on pyritty kehittämään erottelun tehostamiseksi. Yksi potentiaalisista menetelmistä on ioninvaihtokromatografia. Kahdesta ioninvaihtokromatografian muodosta tyypillisimmin anioninvaihtokromatografiaa on tutkittu mahdollisena solunulkoisten vesikkelien eristysmenetelmänä johtuen solunulkoisten vesikkelien pinnalla olevasta negatiivisesta varauksesta. Tässä tutkimuksessa selvitettiin toisen ioninvaihtokromatografia-muodon, kationinvaihtokromatografian toimivuutta solunulkoisten vesikkelien eristämisessä.

Tässä tutkimuksessa vertailtiin kahta kationinvaihtokromatografiaa: tyypillistä, vahvaa kationinvaihtajaa (SP Sepharose Fast Flow) sekä lonkeromaista, vahvaa kationinvaihtajaa (Fractogel® EMD SO_3^- (M)). Tämän lisäksi tutkittiin magnesiumin käyttöä eri konsentraatioissa erottelemaan eri lipoproteiinien muotoja solunulkoisista vesikkeleistä.

Lonkeromaisen kationinvaihtajan havaittiin olevan tehokkaampi sitomaan solunulkoisia vesikkeleitä tyypilliseen kationinvaihtajaan verrattuna, kun kokeessa ei käytetty magnesiumia. Nämä vesikkelit voitiin tämän jälkeen eluoida kolonnista natriumkloridin avulla. Magnesiumin käyttö mahdollisti eri lipoproteiinimuotojen erottelun näytteistä ja korkeammat magnesiumpitoisuudet vaikuttivat poistavan merkittävän määrän lipoproteiineja kokonaisuudessaan, mutta johtivat myös alentuneeseen solunulkoisten vesikkelien saantoon.

Nämä tulokset olivat lupaavia ja näyttivät, että kationinvaihtokromatografiaa voidaan käyttää solunulkoisten vesikkelien eristämisessä. Lonkeromainen kationinvaihtaja vaikutti olevan tehokkain erottamaan solunulkoiset vesikkelit lipoproteiineista. Lisää tutkimusta aiheesta tarvitaan, mutta kationinvaihtokromatografia voi tuoda uusia mahdollisuuksia tehokkaaseen, laajamittaiseen solunulkoisten vesikkelien tuotantoon.

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Abstract

Faculty: Faculty of Pharmacy

Degree programme: Master of Science in Pharmacy

Study track: Biopharmacy

Author: Reetta Pusa

Title: Development of chromatographic methods for platelet derived extracellular vesicle isolation and purification

Level: Master's thesis

Month and year: March 2024

Number of pages: 49

Keywords: Extracellular vesicle, chromatography, platelet, isolation

Supervisor or supervisors: Saara Laitinen, Heikki Saari

Where deposited: ethesis.helsinki.fi

Additional information:

Abstract:

The potential of extracellular vesicles (EVs) as diagnostic markers and drug delivery vehicles has been studied increasingly in recent years. One of the challenges in this field has been the isolation of EVs from complex biological fluids such as blood. The methods widely used for the isolation process include for example size exclusion chromatography (SEC) and ultracentrifugation (UC). As these methods use size and density of the particle, the have not been efficient enough in isolating EVs from certain particles such as lipoproteins.

Due to the challenges related to these methods, other isolation methods have been sought to improve the efficiency of EV isolation. One of these methods is ion-exchange chromatography (IEC). From the two forms of IEC, anion-exchange chromatography has been studied more in EV isolation due to the negative net charge on EV particles. However, in this study the functionality and efficiency of cation-exchange chromatography (CEC) in EV isolation was studied as very little research has been done on this method.

In this study, two CEC-resins were studied to define their applicability in EV isolation. A standard strong cation-exchange chromatographic resin SP Sepharose Fast Flow was compared to a strong tentacle-type resin. In addition to this, we studied the possibility to use a magnesium gradient to separate different forms of lipoproteins from EVs through dextran-sulfite precipitation.

Tentacle-type CEC-resin was found to be more efficient in capturing EVs compared to the standard-type resin without magnesium. These EVs could then be eluted from the column with sodium chloride. The use of magnesium gradient allowed the separation of apolipoproteins in the samples. Higher concentrations of magnesium also reduced the number of lipoproteins in the samples altogether but resulted in the loss of EVs as well.

These results were promising and showed that cation-exchange chromatography can be used in EV isolation. Tentacle-type resin seemed to be most efficient in removing impurities and capturing EVs. While more research is needed before these findings can be applied to clinical use, these results prove that cation-exchange chromatography can be used in EV isolation as a new, efficient and up scalable method.

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1 Introduction

Extracellular vesicles (EVs) are membrane nanoparticles formed within and from cells (Yáñez-Mó et al. 2015). EVs are known to be released from most cells in the human body as well as from other organisms, such as bacteria, for example. Their most fascinating function is their ability to transfer genetic information between cells (Valadi et al. 2007). This change of information can be seen in a wide range of cells from prokaryotic to eukaryotic cells and includes for example proteins, nucleic acids and sugars. As well as working as a signaling vehicle between cells, EVs can be involved in the pathogenesis of various diseases. These diseases include different cancers (Xu et al. 2018), autoimmune diseases (Tian et al. 2020) and cardiovascular diseases (Ridger et al. 2017). Due to their functions in cell-to-cell communication as well as their pathophysiological roles, EVs and their possible applications in the treatment and prevention of these diseases is a rising area of research.

Based on their size and method of formation EVs have traditionally been divided into three major categories: exosomes, microvesicles/microparticles and apoptotic bodies as shown in figure 1 (Yáñez-Mó et al. 2015). Exosomes are the smallest of three and are often described as under 150 nanometers in diameter (Bebelman et al. 2018, Doyle and Wang 2019). They are formed by budding inside multivesicular bodies (MVB) from which they are released into the extracellular environment as MVBs fuse with the plasma membrane. Microvesicles on the other hand are generally considered larger in size and range usually between 50-1000 nm in width, the majority of which are however under 300 nm (Doyle and Wang, 2019). Microvesicles differ from exosomes also in method of formation, as they bud directly from the plasma membrane. The third category of EVs are apoptotic particles which are formed from apoptotic cells and are generally largest of the three (Yáñez-Mó et al. 2015).

This division to these three categories has been challenged recently as it is impossible to determine the biogenesis pathway solely based on the size of the

EV (Thery et al. 2018). Therefore, it is recommended to use terms that refer to physical characteristics or biochemical composition, for example. Multiple subpopulations of EVs have been recognized as well, such as exomers and oncosomes (Meehan et al. 2016, Zhang et al. 2018). However, with the currently available methods isolating and characterizing these particles is challenging (van Niel et al. 2018). As the methods develop, it is likely that EVs from specific subpopulations will be studied more often in the future.

Even though EVs were first recognized over 75 years ago (Chargaff and West 1946, Wolf 1967), it was the revelation that EVs could transfer transcriptable RNA between cells (Ratajczak et al. 2006, Valadi et al. 2007) that led to the current surge in EV research. More information about EVs and their diagnostic and therapeutic potential is discovered constantly but their full potential in these fields is still yet to be discovered. While EVs are known to be able to transfer a large variety of cargo, the understanding of the uptake, targeting and regulation of EV cargo and the function of these mechanisms remains limited (Théry et al. 2018, Yates et al. 2022).

Although increasing amount of research data has been published and our knowledge has increased there are still major challenges that EV research is facing. Many of the challenges are related to technologies used in the field and their performance with nanoscale biological material. One of the significant issues is efficient isolation and purification methods of EVs especially from complex biological fluids. Methods that are currently widely used include ultracentrifugation and size-exclusion chromatography (SEC). In 2019 approximately 80% of EV isolations were done using ultracentrifugation as the only isolation method or in a combination with another method (Royo et al. 2020). While majority of EV research is done using these methods, they face some challenges as well. Ultracentrifugation is a widely used and well-known method, but it is not up scalable for larger production as it is laborious and has a low yield of EVs (Coumans et al. 2017). SEC on the other hand is not efficient in separating EVs from complex biological fluids such as blood as many contaminants have

similar characteristics to EVs. The need for new up scalable and efficient methods is high in the EV field and new methods are being developed constantly.

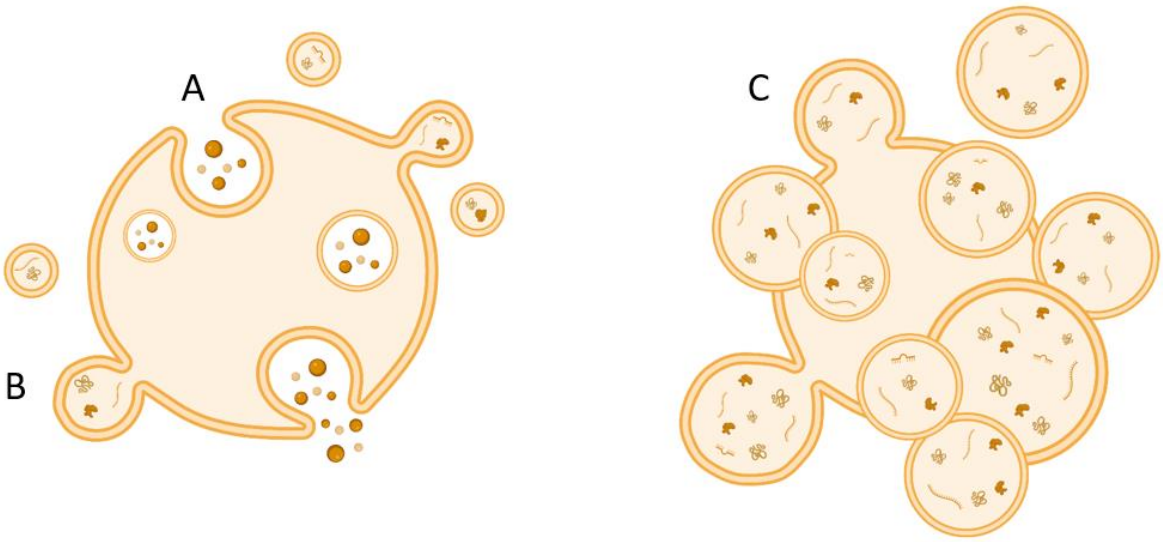


Figure 1. Formation of different subpopulations of EVs. A) Exosomes form within the cells to MVBs from which they are then released. B) Microvesicles pinch directly from cell membrane. C) Apoptotic bodies form as cell goes through apoptosis. Created with BioRender.com

2 Extracellular vesicles in blood

EVs have been discovered in a variety of human biofluids including urine (Pisitkun et al. 2004), saliva (Ogawa et al. 2008) and cerebrospinal fluid (Street et al. 2012). However, an especially rich source of EVs is blood. Majority of blood EVs are either erythrocyte or platelet derived, but EVs originating from other types of cells are found as well. It is estimated that 24-30 % of EVs are platelet derived while 11-38 % are erythrocyte derived (Arraud et al. 2014, Arraud et al. 2016, Headland et al. 2014). Other EVs found include leukocyte derived EVs that make approximately 12 % of EVs found in blood and endothelial cell derived EVs that

make approximately 7 % of blood EVs (Headland et al. 2014). These different forms of EVs are shown in figure 2.

Recognizing and characterizing EV subpopulations and their places of origin can be challenging. Most often this is done using specific markers (Yáñez-Mó et al. 2015). While markers such as CD9 and CD63 are among the most commonly used and are met in a large variety of different EV subpopulations, these do not specify where these EVs originated from. Platelet derived EVs can, however, be recognized based on CD41, a platelet marker. However, not all platelet-derived EVs carry this marker. Similarly, erythrocyte derived EVs can be recognized based on, for example, a CD235a, a glycophorin specific to erythrocytes.

The composition and cargo of blood-derived EVs can change in different diseases. For example, an increased number of leukocyte and endothelial cell derived EVs has been linked to atherosclerosis (Chironi et al. 2006, Loyer et al. 2014, Ridger et al. 2017). Another possibility is the use of EVs as a biomarker for cancer using blood samples and by recognizing EVs with specific RNA composition (Ogata-Kawata et al. 2014, Tanaka et al. 2013). The use of this method has raised a lot of interest in the field. Before this method can be used as a diagnostic tool, a significant amount of research is still needed. The possibility to use EVs as a disease biomarker is discussed in more detail in chapter 6.1.

While the composition and loading mechanisms of EV cargo remain partially unknown, some mechanisms have been recognized (van Niel et al. 2018). The Endosomal Sorting Complexes Required for Transport (ESCRT) has been recognized as a participant in the selection of protein cargo to the EVs (Colombo et al. 2013). This has been the most studied form of protein EV cargo selection, however other mechanisms still remain (Mateescu et al. 2017). These mechanisms need a significant amount of further research to be understood more comprehensively.

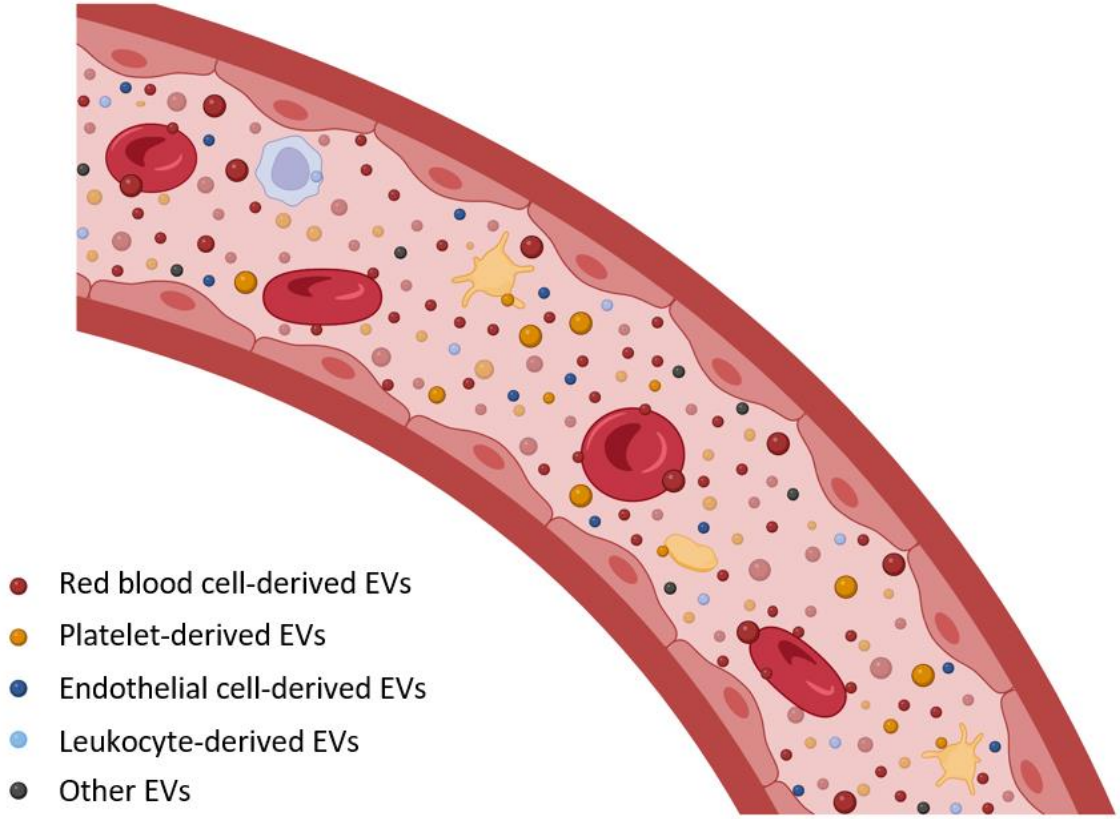


Figure 2. Composition of blood derived EVs. The erythrocyte and platelet derived EVs are the most common forms of EVs in blood. Leukocyte and endothelial cell derived EVs are present as well, and in some cases cancer cell derived or other pathological biomarkers.

2.1 Platelet derived extracellular vesicles

The first EVs recognized from plasma in 1946 by Chargaff and West were platelet derived EVs and later as their origin was determined they earned their nickname “platelet dust” (Wolf 1967). Platelet derived EVs were first recognized as participants of hemostasis and this remains one of the more closely characterized physiological role of EVs (Yu et al. 2018). This participation in coagulation process has been shown by Aatonen et al. in 2012 as it was found that a decreased amount of platelet derived EVs can cause bleeding tendency and

changes in the coagulation process. Platelet derived EVs are as well believed to have a role in immune responses, tissue regeneration and immune responses, among others (Ridger et al. 2017).

2.2 Erythrocyte derived extracellular vesicles

While platelet derived EVs were first to be discovered, erythrocyte derived EVs followed soon after in 1975 (Allan and Michell 1975). They have been recognized to participate in hemostasis together with platelet, leukocyte and endothelial cell derived EVs. Vesiculation of erythrocyte derived EVs is also believed to be a mechanism to control the removal and life span of erythrocytes (Willekens et al. 2008, Rubin et al. 2012).

3 Lipoproteins in blood

Lipoproteins are important lipid carriers in blood forming a significant particle component that challenges blood-based EV research (Takov et al. 2018, Feingold et al. 2021). Lipoproteins can be divided into different classes based on their size, lipid and protein composition: chylomicrons that are 75-1200 nm in size, very low-density lipoproteins (VLDL) are 30-80 nm in size, intermediate-density lipoproteins (IDL) are 25-35 nm in size, low-density lipoproteins (LDL) are 18-25 nm in size and high-density lipoproteins (HDL) are 5-12 nm size. There are approximately 10^{15} lipoprotein particles in each ml of blood of a healthy person (Feingold et al. 2021).

Due to their overlap in size of the EVs and similar physicochemical properties, separating lipoproteins from EVs can be challenging. This is especially significant when size is used as the base of isolation and with VLDL and LDL that are the closest in size to most EVs (Takov et al. 2018, Sódar et al. 2016). Significant is as well the high number of lipoprotein particles compared to those in EVs. While

there are approximately 10^{15} lipoprotein particles in a ml of whole blood, there are only 10^{7}-10^{9} EVs in a similar sample (Coumans et al. 2017).

4 Isolation of extracellular vesicles

While there is a growing interest in EVs and the amount of research done with EVs is increasing, the field faces some major challenges. One significant challenge is the isolation and purification of EVs especially from complex biological fluids such as blood. The size and physicochemical properties of EVs are similar to some other biological particles, such as lipoproteins and viruses. Due to interfering impurities, new methods have been sought in order to isolate high quality EVs with a good yield. Some of the widely used methods in EV isolation are shown in figure 3.

In order to make EV studies more reliable and reproductible and to increase the transparency of the EV research, the international society for Extracellular Vesicles (ISEV) have published guidelines for EV research: “Minimal information for studies of extracellular vesicles” known as MISEV2018 (Théry et al. 2018). These guidelines were first published in 2014 and then updated in 2018.

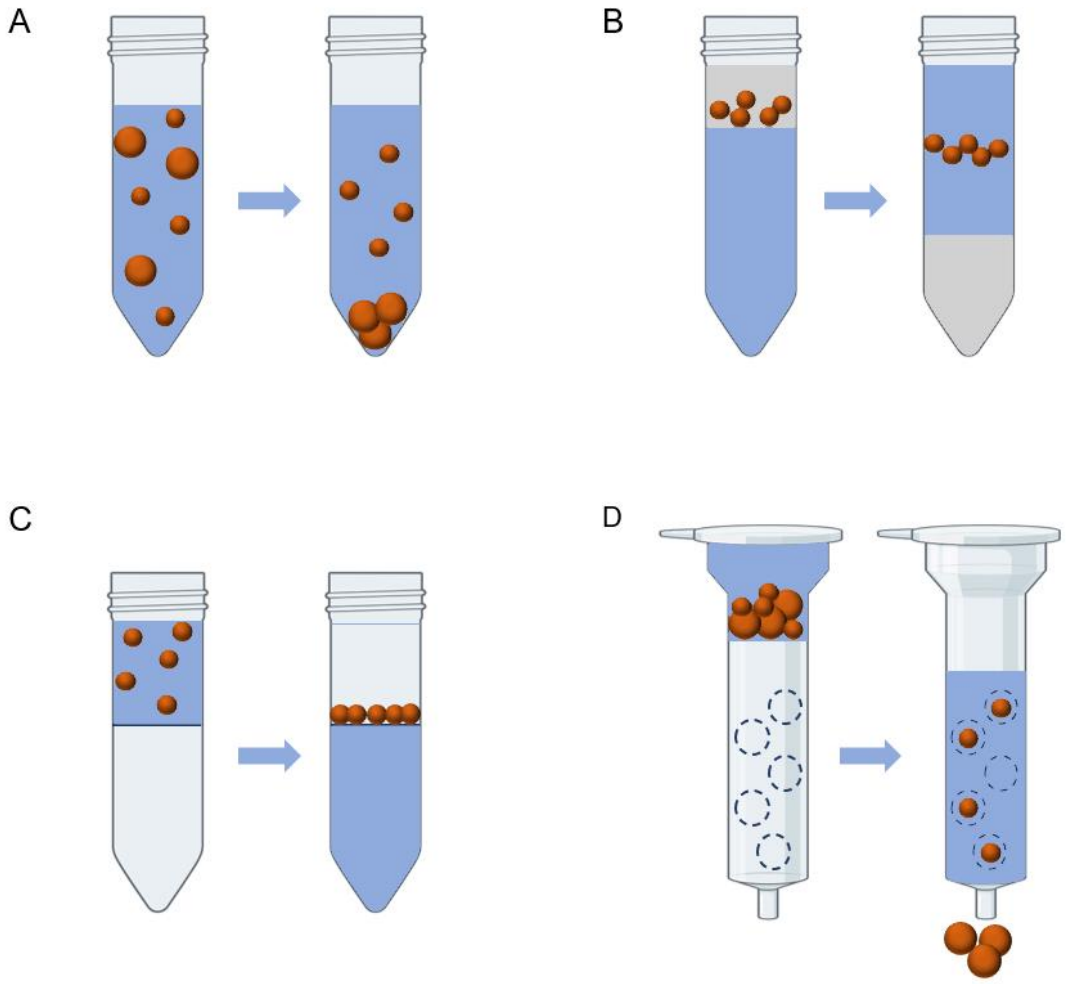


Figure 3. Some of the commonly used EV isolation methods. A) Differential centrifugation separates particles based on their size and density due to different sedimentation speeds in a centrifuge. B) Density gradient centrifugation enhances the differentiation based on density, allowing the degree of purity of the samples to increase. C) Ultrafiltration separates particles as they are pushed through a filter with specific cutoff size. This method can as well be used for concentrating the samples. D) Size-exclusion chromatography isolates particles based on their size by capturing smaller particles in the pores of the matrix as larger pass through the column faster.

4.1 Extracellular vesicle sample preparation methods

The EV sample preparation methods include all steps of the isolation or purification process of an EV sample. While there are multiple possible isolation

methods, no one-size-fits-all solution has been found. The decision of the right isolation method depends on multiple factors: the degree of purity and yield needed, how concentrated the final product should be, whether this isolation needs to be up scalable and applicable for larger scale production and how laborious this method can be, to name a few.

With complex biological fluids such as blood the first step in the sample preparation is often removing the possible cells and other larger particles from the samples (Witwer et al. 2013). This is most commonly done using centrifugation with lower speeds, in which the cells and larger particles sediment at the bottom of the tube. This is often repeated various times. The supernatant can then be collected and used with further isolation methods. One of the challenges, especially with platelet derived EVs (PEV) is the efficiency of platelet removal. Different platelet preparation methods have been found to affect the release of PEV (Zará et al. 2022).

4.2 Differential ultracentrifugation

Differential ultracentrifugation is the most widely used method for isolation of extracellular vesicles and majority of EV research is based on it (Royo et al. 2020). It is based on separating the vesicles from other particles due to differences in their sedimentation speeds. Usually any possible cells and other larger particles are separated from EVs by low-speed centrifugation (< 5000 g) and/or filtration before the ultracentifugation process. Samples are then put through ultracentrifugation with forces as high as 110 000 g or more during which the vesicles pack at the bottom and form pellets. Differentially sedimenting particles can therefore be separated by gradually increasing the centrifugal force. These pellets can then be resuspended with a buffer solution to collect the sample. A useful aspect of ultracentrifugation is that the samples received are fairly concentrated as with many other methods diluted samples can be an issue as it decreases their functionality in the downstream process.

The challenges with ultracentrifugation include the inefficiency in purifying the EVs from particles that are similar in density and size. These include particles such as HDL particles, viruses and aggregated proteins that may be left in the sample and can contaminate it. Ultracentrifugation may also damage the vesicles due to the high g-force and cause them to aggregate which decreases the applications they could be used for in the future (McNamara et al. 2018a, Nordin et al. 2015). The method is as well quite time consuming and laborious and its capacity is limited by the rotor used. Ultracentrifugation is therefore not easily up scalable for larger production.

The use of a density gradient such as sucrose or iodixanol in ultracentrifugation can be used to increase the purity of the samples with ultracentrifugation (Ramirez et al. 2018). In this method solutions with increasing densities are used to separate the EVs from other particles. During the ultracentrifugation the particles migrate to their buoyant density layer. These layers can then be collected and used in downstream process or analysis (Hogan et al. 2014).

The use of density gradient during ultracentrifugation has been found to increase the resolution and purity of the samples compared to conventional ultracentrifugation (Van Deun et al. 2014). However, as this method separates particles based on their density, particles with very similar density to EVs may remain in samples (Sódar et al. 2016). These contaminants can include, for example, certain lipoproteins such as LDL (Van Deun et al. 2014). This method is as well time consuming and has low sample capacity and yield of EVs, making it hard to up scale for any large-scale production.

4.3 Chromatographic methods

Chromatographic methods have increased in popularity in the EV field as search for new, more efficient methods of EV isolation has continued. Size-exclusion chromatography (SEC) has become widely used in EV reseach, while ion-exchange chromatography (IEC) has gained popularity as well recently. These

most common chromatographic methods are described in some detail here. The methodologies of cation and anion exchange chromatography are shown in figure 4.

4.3.1 Size exclusion chromatography

Size exclusion chromatography (SEC) is a commonly used method in EV isolation and is often seen as a golden standard in EV research (Coumans et al. 2017). This method is based on the separation of particles due to differences in their size as they pass the porous column matrix. The SEC matrix allows larger particles to pass faster through the column, while smaller particles pass through more slowly because they diffuse into the pores of the matrix more. This allows these different particles to be collected into different fractions.

While SEC is a widely used method for EV isolation, there are some challenges to the use of it as well. One of the most significant challenges is that due to the similar size of other biological particles, such as some lipoproteins, SEC as the only isolation method is not very effective in separating these from EVs. The EVs received after SEC are also diluted and therefore might need some downstream methods for concentrating them in order to be useful in further research as well.

The yield with SEC depends on the fractions collected and the purity of samples needed. With the purest fractions SEC was found to be able to remove 99 % of soluble plasma proteins and over 95 % of HDL (Nordin et al. 2015) If only the purest fractions are collected, this might result in some loss of EVs as well. The efficiency of separation can be increased by using optimal size columns and with careful packaging of columns. A higher yield of EVs can also be received by collecting other than peak fractions as well. This results in an increased number of contaminants as well as more diluted final sample (Böing et al. 2014). The decision of which fractions to collect depends on the degree of purity needed as well as the yield that needs to be received.

Due to the diluted final sample and the fact that SEC can only be used to purify fairly small samples, this method is not up scalable and therefore is not functional in large-scale production. Combining SEC with other methods can be used to enhance the isolation efficiency and result in more concentrated final sample. This may allow SEC to be used in large scale production as well.

4.3.2 Ion exchange chromatography

While SEC is based on the size exclusion of particles, ion exchange chromatography (IEC) separates particles complex mixtures based on their electric charge (Kosanović et al. 2017). IEC methods can be divided into two categories: anion and cation exchange chromatography. These methods and their applicability to EV isolation are described in closer detail in following chapters.

4.3.3 Anion exchange chromatography

Anion exchange chromatography is based on positively charged matrix that is able to capture particles with a negative surface charge. As the net charge of the EV surface is negative, this method has raised interest as a possible method to isolate EVs efficiently from samples (Kosanović et al. 2017, Chen et al. 2018). After the positively charged matrix has captured the negatively charged EVs, they can then be eluted from the matrix by increasing the ionic strength of the buffer and collected in a specific fraction.

Due to the controlled elution phase, anion exchange chromatography results in a highly concentrated final sample. This method is straightforward and can be scaled for larger production (Heath et al. 2018). However, this method may result in a poor yield of EVs due to the strong binding of EVs to the matrix, limiting the functionality of the method in possible future applications and large-scale production. Further development of this method is needed for AEC to meet the yield and purity requirements for clinical applications.

4.3.4 Cation exchange chromatography

In contrast to anion exchange chromatography, cation exchange chromatography is based on a negatively charged matrix that binds positively charged groups. This may seem counter-intuitive to use with EV particles as they have a negative net charge on their surface. However, there are two possible ways this method could be useful in EV isolation. The first of these is positive selection, in which these matrixes may capture the EVs that could then be eluted from the matrix. Even though the net charge of the EV is negative, positively charged groups can be found on the EV surface and this matrix may be able to bind to them. The second option is negative selection in which the matrix is used to catch contaminants with a positive charge while EVs pass through the column in flowthrough (Van Deun et al. 2020).

Little research has been done regarding this method due to its counter-intuitive approach. The effectiveness of this method in purifying EVs needs to be determined as the surface charge is similar in many of the possible contaminants compared to EVs. However, if the method is effective in separating these, this method could be effective in purifying EVs effectively, while still producing a good yield. This method is as well fairly straightforward and could be used with larger sample volumes as well. Therefore, this method would be suitable for large-scale production, such as for clinical applications. However, a significant amount of research is needed to determine the functionality of this method in EV isolation.

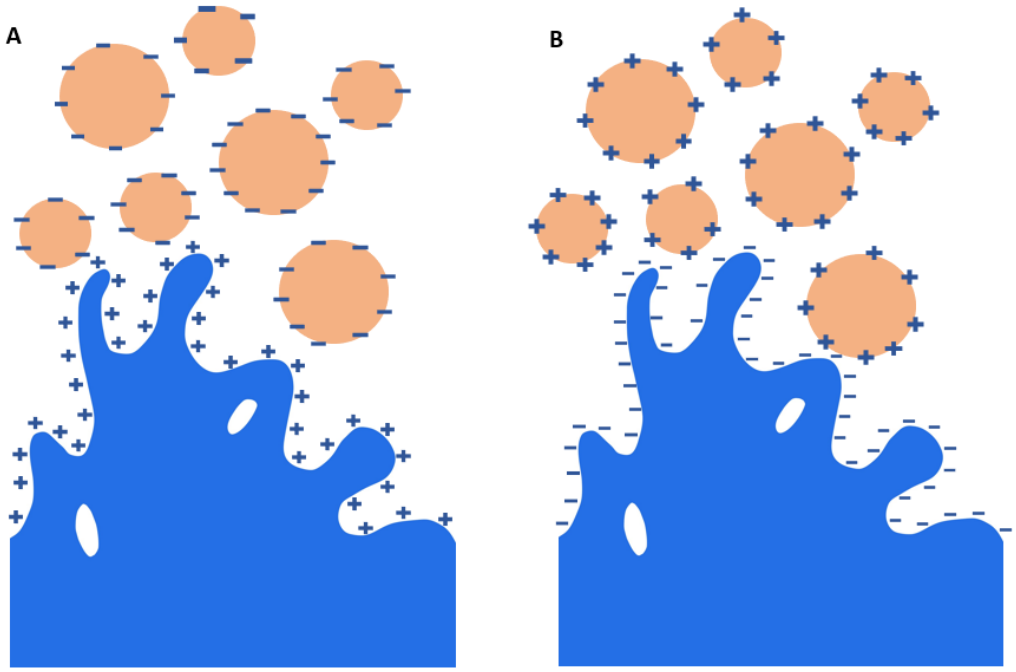


Figure 4. A) The function of anion exchange chromatography. The positively charged matrix forms bonds with the negative charged particles and captures them while other particles pass through the column. B) The function of cation exchange chromatography. Negatively charged matrix binds to the positively charged particles, or in the case of EVs, the positively charged groups on the otherwise negatively charged particle.

4.4 Immunoaffinity isolation

Immunoaffinity isolation assays can be used to isolate EV subpopulations based on their surface ligands. Most typical immunoaffinity assays are based on monoclonal antibodies that bind to a specific EV surface ligand and capture EV subpopulations presenting it. Commonly used affinity-capture beads are coated with antibodies against markers such as CD9 and CD63 (Wiklander et al. 2018). This method is efficient in capturing only those EVs and particles with this specific marker and is therefore a sufficient method in removing contaminants and is one of the best methods to obtain a specific EV subpopulation from a complex biological fluid (McNamara and Dittmer 2020).

Immunoaffinity assays can be useful when studying for example pathological samples where the isolation of EVs with specific target tissue may be vital (Brett et al. 2017, Sharma et al. 2018). However, as no universal EV-marker has been discovered, immunoaffinity assays can only isolate EV subpopulations and therefore with this method a good yield is impossible to achieve, making it not-suitable for any large-scale production (Ramirez et al. 2018).

The elution process of EVs from the affinity-capture beads may as well be challenging with this method. Often in order to detach the EVs from the beads the elution buffers used are often acidic and/or chaotropic which may result in the denaturation of proteins and affect the functionality and integrity of EVs (McNamara et al. 2018b). This may limit the functionality of these EVs in possible downstream processes and analysis.

4.5 Other methods

Ultrafiltration is an isolation method that is based on a filter with specific cutoff size (Lobb et al. 2015, Xu et al. 2015, Xu et al. 2017). As a sample is pushed through this filter, smaller particles pass through the filter while larger particles are retained on the filter from which they can be collected. Besides concentrating EVs, this method can also be used for size-based separation of unwanted contaminants, such as proteins, from EV samples. This method is commonly used with centrifugation and the g-forces of the centrifuge are used to create the pressure needed for the sample to pass through the filter. A pump can be used as well with larger sample volumes.

While this is a fast, simple and efficient method for EV purification, it may be challenging to purify particles that are similar in size to EVs using this method as this isolation method is based on the cutoff size of the filter. Other challenges with this method include the plugging of the filter especially when using complex

biological fluids, such as blood, or with larger sample volumes (Taylor and Shah 2015). This may result in a lowered yield of EVs and in a time-consuming process.

Another isolation method is precipitation (Kim et al. 2015, Konoshenko et al. 2018). In this method a precipitating agent is used to make EVs, soluble proteins and other particles to precipitate and sink to the bottom. This is based on hydrophilic polymers that compete for the solvent with the particles of the samples, in this case the EVs. This results in decreased solubility of the sample particles and these particles precipitate and form a pellet after a centrifugation with at low speed. This pellet can then be resuspended for further analysis or applications. The precipitating agent used depends on the sample in question and in EV research is often polyethylene glycol-based (PEG) agent (Konoshenko et al. 2018).

Precipitation is a simple, straightforward and affordable isolation method and is therefore widely used and many commercial kits are available based for this method. The EV yield with this method is high and it is up scalable for larger production (Konoshenko et al. 2021). However, precipitation is not a specific EV isolation method and contaminants, such as albumin, apolipoproteins and immunoglobulins may be left in the sample (Van Deun et al. 2014). To receive well-purified EVs, other methods are recommended to be used as a combination with precipitation. It is as well notable that the precipitating agent may be left in the final sample and may be incompatible with certain analysis methods, such as electron microscopy.

In addition to being used as isolation methods, both ultrafiltration and precipitation can be used as concentration methods (Kim et al. 2015). With many isolation methods, such as SEC the final product is fairly diluted which may result in difficulties in the downstream process or with analytical methods. Both ultrafiltration and precipitation can then be used after the primary isolation method to receive a more concentrated product. The ideal protocol depends on the

downstream process and final product and choosing the right method is significant.

5 Characterization methods

Another challenge in EV research is the characterization and quantification of EV's. As they are small in size and often in a complex biological fluid, it is hard to detect and separate them from other particles that may be in the fluid (Welsh et al. 2020). Electron microscopy (EM) can often be used to detect single vesicles but is not a high-throughput method. For quantifying and characterization of larger amounts of particles other methods are needed. The field in this area of EV research is constantly developing and multiple methods are emerging.

One of the challenges in detecting EVs is their low refractive index (RI) (van der Pol et al. 2014). RI describes the velocity in which light travels through the sample in question, here EV samples. This is significant when the scatter of light is measured as in some characterization studies described here. As the RI of EVs is low, it has only a small difference to the RI of surrounding buffer in these measurements. This creates an additional challenge as the light scattering is harder to detect.

Yet another challenge is the lack of validated reference material for EVs (Thery et al. 2018). Without a reference material standardizing results between laboratories and instruments is challenging and results may not be reliable (Valkonen et al. 2016). Currently widely used materials such as liposomes and silica beads are not ideal due to their lack of surface markers and high RI. As these differ from the characteristics of EVs, these particles do not reflect the way in which EVs function.

5.1 Flow cytometry

Flow cytometry is a widely used method to characterize the EV population and to measure the concentration of EVs in a sample (Sharma et al. 2018, van der Pol et al. 2010). The detection of flow cytometry is often based on light scattering or fluorescence labeling. With light scattering a laser beam is directed to the sample as the particles are ran through the instrument and the scattering of light is then detected and used to measure the size of the particles passing through the instrument. One of the challenges with this method is the limited ability to detect smaller particles. Often flow cytometry is not effective in detecting particles that are smaller than 600 nm, including a significant amount of EVs.

Fluorescence labeling can be used to detect smaller particles as well. With fluorescence labeling, the markers on the surface of EVs are labeled with a specific fluorochrome-label and the laser beam is then used to excite this label (Arraud et al. 2015). This can be used as a positive marker to show the presence or absence of either specific EV markers or a contaminant (Théry et al. 2018). This method also allows the characterization of EV subpopulations.

5.2 Western blot

Western blot (WB) is a widely used method in cell and molecular biology fields to detect and analyze proteins in a sample (Mahmood and Yang, 2012). This method is widely used in the EV research and can be used to detect EVs and their subpopulations as well as possible contaminants in the samples. As this method is semi-quantitative, some estimates can be made about the amount of marker present in the sample. This is however useful in EV research to estimate the yield and purity of EV sample together with other methods.

5.3 Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) is a widely used method to evaluate the size, size distribution and concentration of nanosized particles in a sample (Dragovic et al. 2011). NTA is based on measuring the Brownian motion of particles. This motion is caused by the molecules of the solvent surrounding the particles that create random movement of the particles. As smaller particles move faster compared to larger particles, the velocity of each particle can be used to measure the size of the particle.

This motion is measured by illuminating the sample with a laser beam and tracking the scatter of light the particles create. Each particle, including extracellular vesicles, scatter the light and can be detected with a microscope. Based on the Brownian motion of the particles, the translational diffusion coefficient can be estimated and by using this, the hydrodynamic diameter of the particles can be calculated by using Stokes-Einstein equation (Equation 1) when the temperature and viscosity of the surrounding solvent are known.

While NTA is a widely used method and essential tool in EV research, it has some disadvantages as well. This method assumes that each particle is spherical which might not always apply to the actual shape of each particle and this may affect the results regarding particle size and size distribution. Other particles that resemble EVs in size can as well be detected and mischaracterized as EV's which may be misleading when interpreting the results. One significant group of particles that can be mischaracterized as EVs are lipoproteins as especially LDL and VLDL are similar to EVs is size.

Another challenge with NTA is variety of sensitivity in NTA instruments. As the sensitivity and detection limit of instruments may vary the comparison and reproducibility of results can be a challenge (Welsh et al. 2020). The detection limit can vary and may cause the results to be unreliable. NTA instruments are often able to accurately measure particles with size range of 50-1000 nm but

smaller particles can be undetected and with larger particles the Brownian motion can be too slow to tract accurately (Dragovic et al. 2011, van der Pol et al. 2010).

$$d(H) = \frac{kT}{3\pi\eta D} \qquad \text{(Equation 1)}$$

Equation 1. Stokes-Einstein equation where d(H) is hydrodynamic diameter, k is Boltzmann's constant, T is temperature, η is viscosity and D is translational diffusion coefficient.

6 Future challenges and prospectives

The possibility to use EVs as diagnostic tools and in therapeutics to treat different diseases has been an area of interest in the EV field for a while. As stated before, EVs are known to carry cargo, including protein and RNA, in their lumen and to participate in the cell-to-cell communications. These properties make them an especially interesting area of study. The possibilities and current advancements of the EV field are described in this chapter.

6.1 Extracellular vesicles as biomarkers

EVs offer a huge opportunity for the advancement of diagnostics and have been therefore a significant area of EV study. One of these possibilities is to use EV cargo, specifically (mi)RNA in the EVs, to detect cancers from plasma or urine samples (Fais et al. 2016, Ogata-Kawata et al. 2014, Tanaka et al. 2013). This method would allow faster and easier way to detect cancers and use screening as a method to catch cancers in an early stage.

While the use of EVs as biomarkers is a fascinating area of research, this field still faces many challenges before it can be applied to diagnostics in full-scale. A reliable and standardized method for characterization of EVs is fundamental for this to be possible (Coumans et al. 2017). Clinically relevant information that

would need to be easily accessible include size distribution, concentration, cellular origin and morphology of EVs (van der Pol et al. 2010).

As of now, one of the major challenges is the lack of validated reference material. Without this material it is hard to validate the results received from different laboratories and instruments. Currently some laboratories have obtained the International Organization for Standardization (ISO) standard 15189 as standardization tools for EV analysis (Ayers et al. 2019). While these are good tools, for clinical applications other methods to standardize and validate EVs are much needed and currently being developed.

6.2 Extracellular vesicles as therapeutic agents

Another clinical application of EVs is their use as drug carriers. As EVs are known to be able to carry cargo including proteins and RNA, the possibility to use them as vehicles for drug delivery has raised interest in the EV field. The possibility to use EVs to target drugs to, for example, cancer cells is among the possibilities that are currently studied in the EV field (Murphy et al. 2019). Another interesting possibility is to use EVs as vehicles to pass through the blood-brain barrier for drugs that would not otherwise be able to do this due to their physicochemical properties. Some promising results have been obtained with this method (Banks et al. 2020).

As EVs are natural components of blood, the advantage of this method is it's known biocompatibility and safe elimination routes (Muzykantov 2010). This is especially interesting when studying EVs isolated from blood, as blood transfusions are a well-known practice with possible side effects and challenges acknowledged.

Some successful studies of using innate EVs as therapeutics have been published as well. For example, EVs secreted from different cell types, such as platelets, T-cells and mesenchymal stem cells have been found to have pro-

regenerative and anti-inflammatory functions (Johnson et al. 2021, Asemani et al. 2022, Bruno et al. 2017). There are many ongoing studies with the use EVs therapeutically as of now and the number of studies using EVs as therapeutic agents has been increasing constantly (Wiklander et al. 2019). While EVs are studied widely and more information is obtained constantly there is still a number of factors and mechanisms to be understood before EVs can be used to their full potential in the field of therapeutics.

As more clinical applications are currently being developed and the interest in finding therapeutic applications with EVs in increasing, the need for effective and up scalable purification protocols in increasing. Without new up scalable isolation protocols any large-scale production of EVs for future therapeutic purposes will not be sustainable.

7 Aim of the study

As the demand from blood donations is based on the clinical demand for red blood cells, not all other therapeutic components of collective whole blood are utilized to their full extent. Especially buffy coat fractions and platelet concentrates produced from whole blood form a side stream of potential source for novel use as their demand is not so high in the clinical use as of now. As the interest of Finnish Red Cross Blood Service is to use donated blood for the benefit of the patients, it is interested to find new ways to utilize these side streams more efficiently. As blood products are known to be a rich source of EVs, Blood Service has an interest in finding new ways to investigate and use EVs from these products. However, as stated before, the purification process of EVs from complex biological sources, including blood, has remained a challenge in the field of EV -research.

One of the most significant challenges in the purification of EVs from blood-based products has been separating EVs from lipoproteins and other contaminants that resemble the physical characteristics of EVs, such as large protein complexes.

Due to these similar characteristics, commonly used methods such as size-exclusion chromatography (SEC) have not been proven to be effective enough in separating these components from each other, resulting in samples with contamination of these particles (Coumans et al. 2017). Another widely used method ultracentrifugation increases the purity of the samples but may result in aggregation and is not an up scalable method. The purity of EV samples can be increased by combining different methods, but this faces challenges as well as it increases the purification time and complicates the up scaling methods for large scale production. For example, immunoprecipitation is effective in purifying the samples from contaminants but as there is not a universally applicable marker for EVs, they are only able to isolate EV subpopulations, resulting in significantly decreased yield of EVs (Ramirez et al. 2018).

In addition to being effective in separating EVs from contaminants, these methods should be able to produce EVs with a good yield and preferably in a concentrated form. With some widely used methods, such as SEC, the final product is quite diluted and therefore would need alternative concentration methods to be easily applicable in further research (Coumans et al. 2017). These methods should be applicable for larger-scale production without being laborious and time-consuming. While the final product with SEC is diluted, one of the advantages of SEC is that it meets other requirements for larger scale production and is easily up scalable.

An emerging area of interest in the isolation and purification of EVs has been IEC as shown. The main focus in this research has been on isolation based on anion exchange chromatography (Heath et al. 2018). As the negative charge on EV surface on seems to be strong and universal, this method could be a possible solution for the simultaneous purification and enrichment of EVs. The method is easy to perform and could easily be up scaled for larger production of EVs. This method, however, has some challenges to it as well shown to be having poor EV recovery as bound EVs are challenging to elute from the resin. Removing anionic

impurities such as albumin or nucleic acids may be challenging as they bind to the resin similarly to EVs.

Another option in the use of IEC is cation exchange chromatography (Van Deun et al. 2020). As opposed to anionic exchange chromatography, this method is based on positively charged particles and domains binding to the negatively charged resin. Even though the net surface charge of EVs is negative, the surface structure is complex and includes positively charged regions that may result in EVs binding to the resin. Alternatively, cation exchange may be used for negative selection in EV purification specifically focusing on the removal of lipoproteins. Relatively little research has been done in the EV field using this method, which is why this method is studied further in this project.

In this study the functionality and applicability of cation exchange chromatography in the isolation and purification of EVs from platelet products was studied using both positive and negative selection approaches. The focus of this study was on the removal of lipoproteins as they are one of the most significant challenges when isolating EVs from blood products due to their overlap in size. The efficacy of cationic exchange chromatography was studied using a conventional surface-coated strong cationic exchanger SP Sepharose Fast Flow and comparing it to a strong cationic exchanger with tentacle-type resin, Fractogel EMD SO^{3-} (M). The differences of these resins are presented in figure 5. In this tentacle-type resin the cationic exchanger groups are bound to long polymer chains attached to the resin surface. These tentacles are theorized to be more effective in binding EVs compared to conventional cation exchangers.

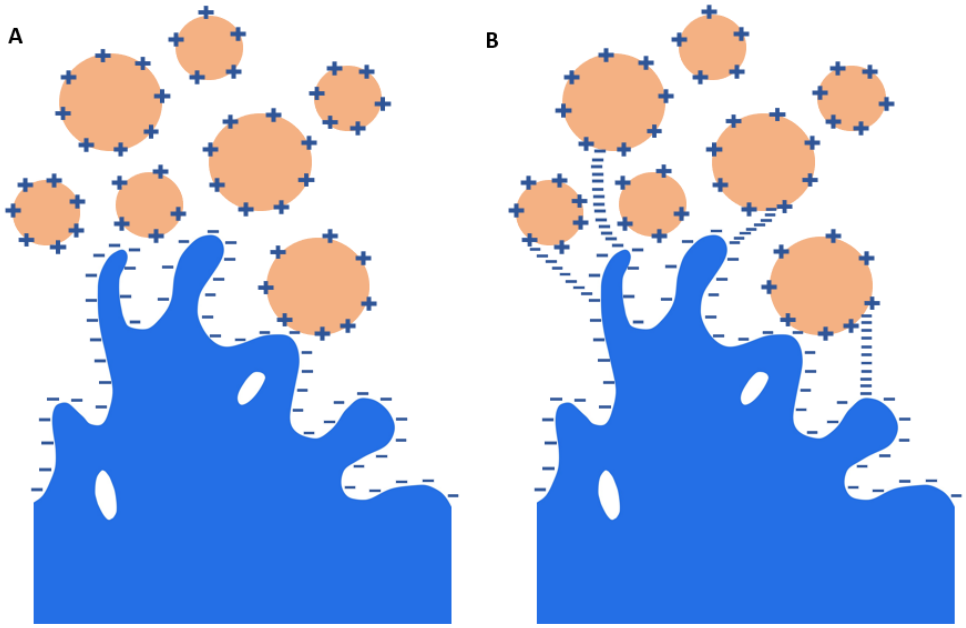


Figure 5. A) The function of a conventional cation exchange resin. The negatively charged matrix binds to the positively charged particle and captures them. These particles can then be eluted from the matrix and collected as fractions. B) The function of a tentacle-like cation exchange resin. The negatively charged matrix has negatively charged polymer tentacles that are able to reach more particles compared to the conventional cation exchange chromatography.

As lipoproteins have been successfully separated using magnesium mediated cation exchange chromatography (Hirowatari et al. 2005 & Hirowatari et al. 2002), the possibility of using this process in enhancing the isolation process of EVs and lipoproteins was studied. Dextran sulfite precipitation has previously been used in differential precipitation of lipoprotein classes from plasma samples. This precipitation of lipoproteins is based on divalent metal cations such as Mg^{2+} or Mn^{2+}, which form a bridge between the lipoproteins and sulfite groups of dextran sulfite. As the functional cation exchanger in strong cation exchange chromatography is a sulfate group, the use of Mg^{2+} in enhancing the separation of EVs and lipoproteins was studied as a possible method to enhance this separation.

In this study the functionality of these two resins in the separation of EVs and lipoproteins were compared as well as the effectiveness of both positive and negative selection of EVs. The positive selection in this case was studied by comparing how efficiently the negatively charges resin was able to bind to the groups with positive charge on the EV surface compared to the efficiency in which they bind to the lipoproteins. The negative selection was studied by comparing the amount of EVs passing through the column and comparing that to the amount of lipoproteins passing through the column in the flowthrough.

In addition, we screened different Mg^{2+} concentrations in running conditions to determine whether the Mg^{2+} -mediated cation exchange could be used to enhance the differentiation of these particles and at which concentrations this isolation is most effective. The Blood Service currently has chromatographic methods to differentiate HDL from EVs efficiently based on their size, however these methods have not been efficient enough in separating LDL, VLDL and chylomicrons from EVs. As blood donation is happening in post-prandial (i.e. well-fed persons) situation and donors are not fasted, especially chylomicrons pose a challenge in nanosized particle purification. Therefore, the focus of this study is in separation of these particles from EVs.

8 Materials and methods

8.1 Preparation of the samples

In this study standard leukocyte-reduced platelet concentrates derived from buffy coats of four anonymous ABO- and RhD-matched whole blood donations as accepted by the Finnish Supervisory Authority for Welfare and Health were used to study the efficacy of these isolation methods. Each concentrate had passed the quality control studies at the Blood Service and was cleared for clinical use. These platelet concentrates were 3-5 days old.

First, the plasma of the platelet products was removed using protocol shown in figure 6. The platelets were centrifuged at 3220 g for 30 minutes, after which the supernatant was discarded, and the cells were resuspended in PBS. This was repeated three times after which the suspension was left for cold activation at +4 °C overnight. The following day the suspension was centrifuged twice using the same settings as the previous day and the supernatant was collected for further studies. The supernatant was divided to 5 ml samples to which magnesium chloride was added in final concentrations of 0,05 M, 0,1 M, 0,2 M and 0,4 M. In addition, a sample without magnesium chloride was prepared as well. Samples were filtered with 0.22 µm filter before continuing to chromatography.

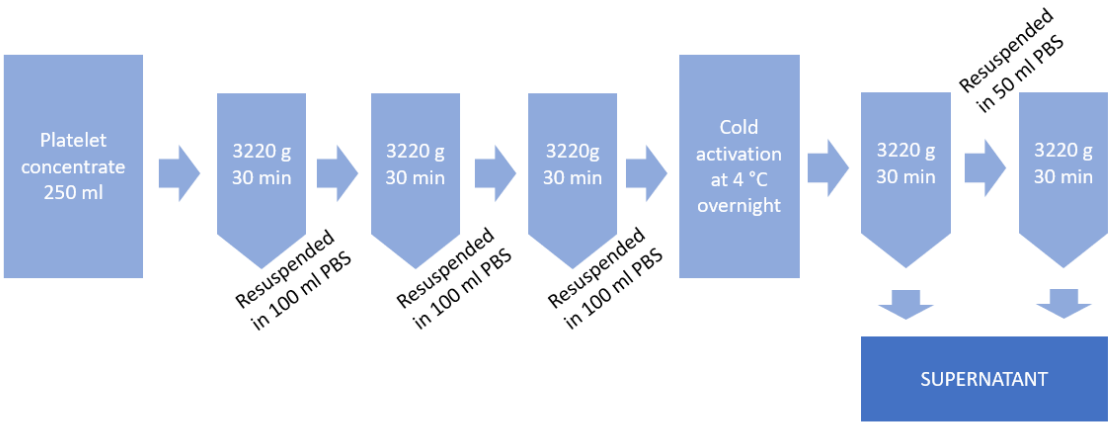


Figure 6. Isolation process of the platelet concentrates. Samples were isolated by centrifuging three times at 3220 g for 30 minutes during the first day. The samples were left at +4 °C overnight for cold activation after which they were centrifuged twice with the same settings and the supernatant was collected.

8.2 Chromatographic methods

Each sample with the volume of 5 ml was ran at 1 ml/min with a peristaltic pump through either Fractogel® EMD SO_3^- (M), a strong cationic ion-exchange column with a tentacle-like structure, or a SP Sepharose Fast Flow column, a standard strong cationic ion-exchange column. The binding buffer with both matrixes was PBS with corresponding magnesium chloride concentration to each sample. With

each sample, 10 ml of flowthrough was collected after which the column was eluted using 0.5 M sodium chloride (NaCl) and 10 ml of the eluate was collected. The composition of samples is shown in figure 7. After collection each flowthrough and eluate sample was concentrated using ultrafiltration with Amicon® Ultra – 15 10K centrifugal filters.

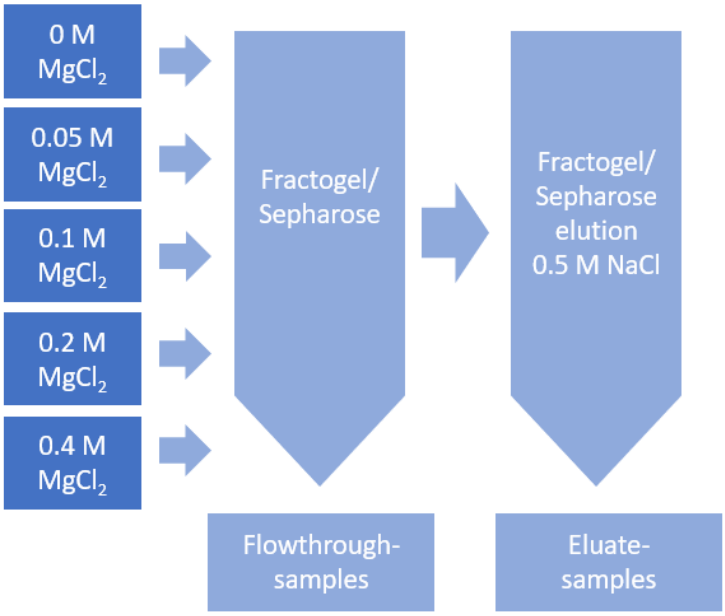


Figure 7. The isolation process using Fractogel® EMD SO_3^- (M) or SP Sepharose Fast Flow. Mg^{2+} was added to samples in four concentrations: 0,05 M, 0,1 M, 0,2 M and 0,4 M before the run through colunms.

8.3 Sample analysis

The protein concentration of each sample was measured using Pierce™ Bicinchoninic Acid Protein Assay Kit. The procedure was done following the manufacturer's instructions with BSA protein standards. Following the BCA reaction, the sample absorbance was measured using at 562 nm.

Lipoprotein and extracellular vesicle presence in the samples was analyzed using Western blotting. Each sample was fixed to the same volume of 20 µl in order for

the lipoprotein and EV concentrations to be easily comparable. 5 µl of 4x Laemmli was first added to each sample and the samples were then heated for five minutes at 100 °C. The samples and a Precision Protein Standard with a volume of 5 µl were loaded to the wells of a 4-20% Mini Protean TGX gel. The samples were run through the gel first at 40 V for 10 minutes and then at 200 V for approximately 35 minutes. The proteins were transferred to a PVDF/nitrocellulose membrane using Bio-Rad Trans-blot Turbo Transfer System. The membranes were blocked for 1 hour at room temperature using 3% BSA in TBS-T and cut into strips according to the corresponding molecular weight of the analyzed antigens before adding the primary antibodies. To measure the V/LDL lipoprotein presence, an ApoB antibody (Medix biochemical #100261, 1:1000, in 3% BSA, TBS-T) was used. For the extracellular vesicles, the following primary antibodies were used: CD9 (BD Pharmingen, #555370, 1:1000, 3% BSA, TBS-T), CD41 (Beckman Coulter, #sz-22, 1:10 000, 3% BSA, TBS-T) and CD63 (BD Pharmingen, #556019, 1:1000, 3% BSA, TBS-T). The membranes were left at the antibody solution to bind at +4 °C overnight.

The following day the membranes were washed 3 times for 10 minutes each wash using TBS-T. A secondary antibody (1:500 goat anti-mouse-IgG-HRP (Bio-Rad, #170-6516) and 1:10000 StrepTactin (Bio-Rad, #161-0380) TBS-T) was added and left to incubate for 1 hour at room temperature. After the incubation the membranes were washed with TBS-T for 3 times for 10 minutes each wash and for 5 minutes using TBS. Clarity™ Western ECL Substrate (Bio-Rad, #170-5060) was added to illuminate the bands on the membrane. The images were analyzed, and the band intensity was measured using ImageJ software. The intensity of each band was compared to the intensity of the band indicating the starting material of each blot. The percentual averages and standard deviations from these samples were calculated.

8.4 Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was used to measure particle concentration and size distribution. A ZetaView instrument (Particle Metrix, Inning am Ammersee, Germany) was used with 488 nm laser. Samples were diluted in milli-Q water to meet 40-200 particles per frame. 11 positions and sensitivity of 85 were used for the measurement.

9 Results

9.1 Extracellular vesicle yield

The total yield of EVs were first estimated by measuring the total particle number of samples isolated with SP Sepharose Fast Flow by using nanoparticle tracking analysis (NTA). The total particle number of each sample is shown in figure 8 and the size distribution of particles in the flowhtough samples are shown in figure 9 and the distribution of eluate samples are shown in figure 10. The particle concentration was higher in flowthrough samples compared to eluate samples throughout the experiment. The highest concentration can be seen in higher magnesium flowthrough samples with concentrations of 0,1, 0,2 and 0,4 M Mg^{2+} where the concentration of particles in ml was at the highest over 10^{11} particles in ml. This was close to, and at certain samples even higher, than the total particle count in the starting material.

The size distribution indicates that the particles found in the samples were fairly small, most under 400 nm. While these may indicate the presence of EVs, many other particles, including lipoproteins, fall into this size category as well. Therefore these results cannot be used to determine whether these particles measured with NTA are in fact EVs. As starting material is expected to have more lipoproteins compared to samples isolated with cation exchange chromatography, the NTA results cannot be used as a comparison of the EV yield. While flowthrough samples with high magnesium concentration seem to produce higher results, it needs to be taken into consideration that the magnesium in the sample may as well form aggregates with the buffer as magnesium phosphate salts are generally

insoluble in water, that seemingly increases the number of particles in the sample. Because of this, NTA by itself is not reliable enough method to estimate EV yield and other methods are needed to receive conclusive results.

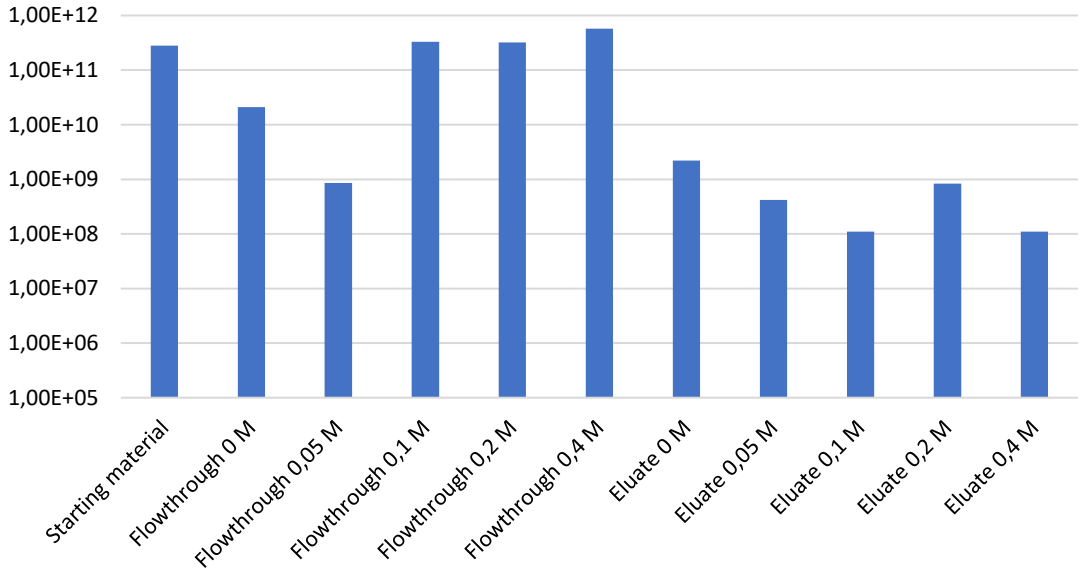


Figure 8. Total particle number of samples isolated with Sepharose SP Fast Flow measured with nanoparticle tracking analysis. On the left the result of starting material. The following samples show the results of flowthrough samples starting without magnesium and the from lowest to highest Mg^{2+} concentration. The eluate samples show the results from after the samples were eluted from the column with 0,5 M NaCl in the same order as flowthrough samples.

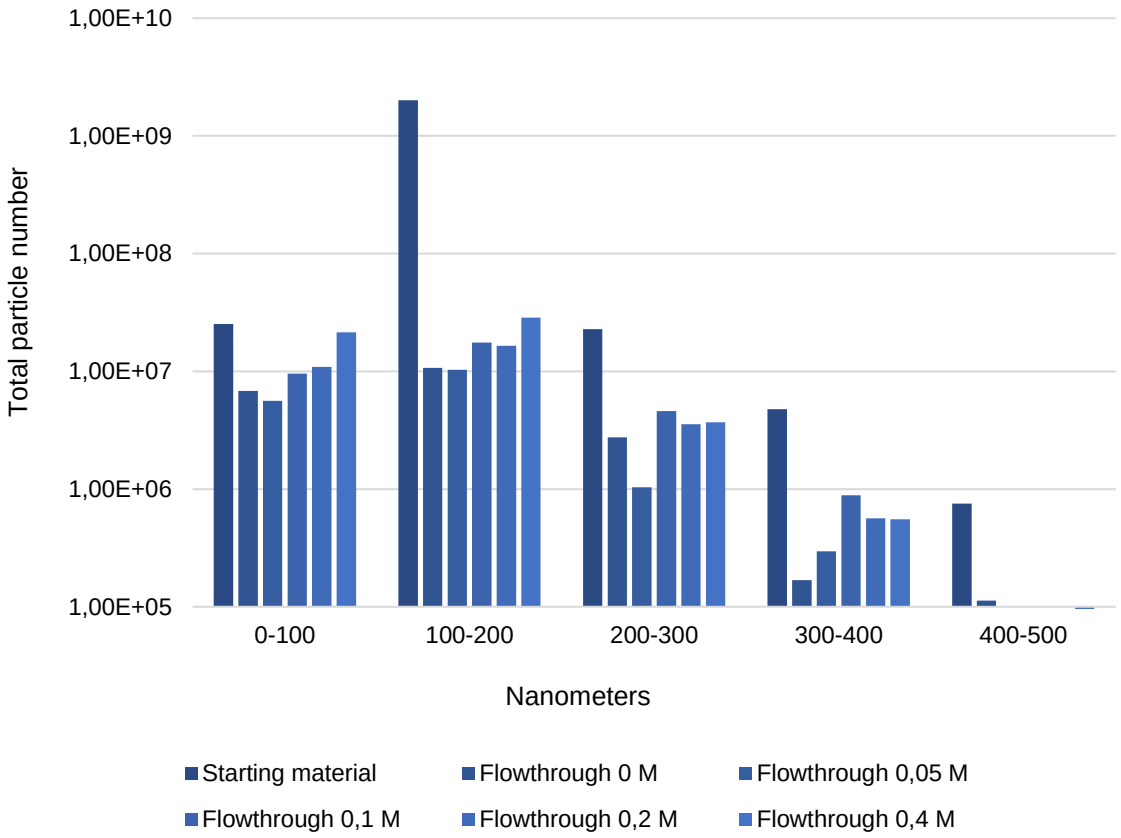


Figure 9. Size distribution of flowthrough samples isolated with SP Sepharose Fast Flow and measured with nanoparticle tracking analysis. The number of particles is highest in starting material in each size category.

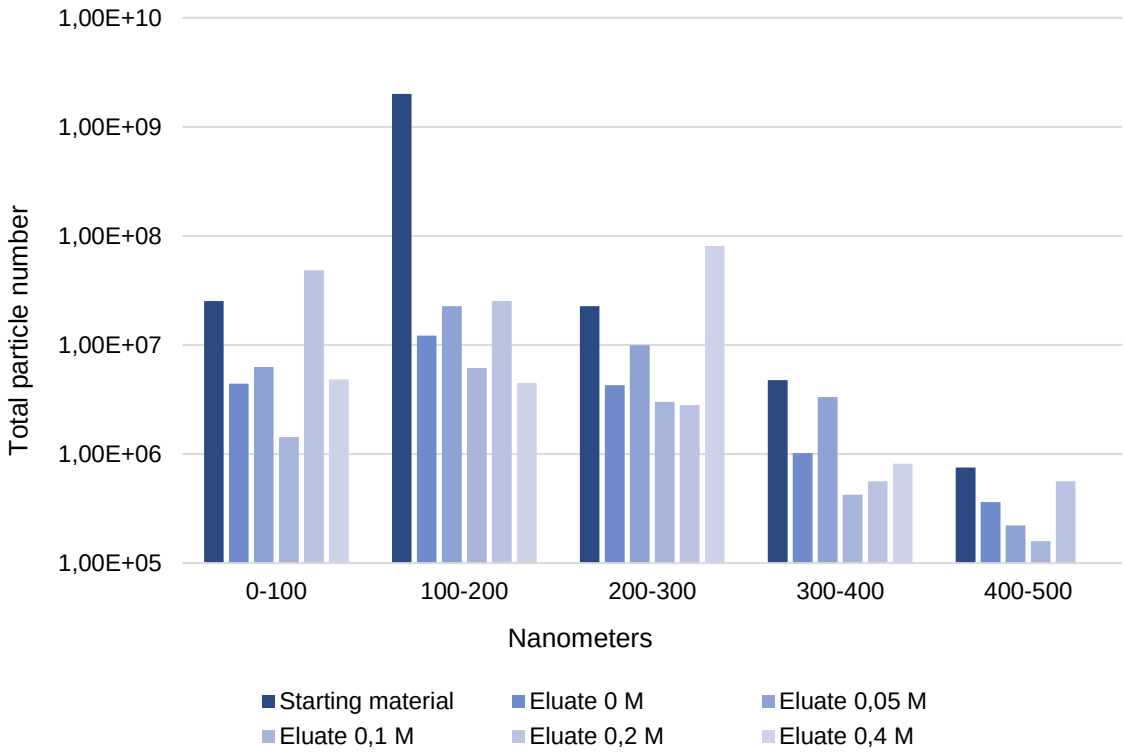


Figure 10. Size distribution of eluate samples isolated with SP Sepharose Fast Flow and measured with nanoparticle tracking analysis.

As no conclusive results of the total EV yield can be obtained from the NTA, these samples were analyzed by using western blot as well. Representative image of the western blot of samples ran through Fractogel EMD SO_3^- (M) are shown in figure 11A and the representative image of samples ran through SP Sepharose Fast Flow are shown in figure 11B. The average intensity of the bands indicating EV markers and the standard deviation of the four replicates of samples isolated with Fractogel are shown in figure 12. These results were compared to the band intensity of starting material to estimate the yield of EVs. Similar results from samples isolated with SP Sepharose Fast Flow are shown in figure 13. In some samples the standard deviation is high, however the results are fairly consistent throughout the experiment.

The CD63 marker seen at approximately 50 kDa showed a decrease in the intensity of the band in flowthrough samples isolated using Fractogel without Mg^{2+} and with Mg^{2+} concentration of 0,05 M. However, with concentrations of 0,1 M

and 0,2 M the intensity of band increased again. With the Mg^{2+} concentration of 0,4 the intensity of the band decreased again and is approximately 40% of that of the starting material. With samples isolated using SP Sepharose Fast Flow, this similar phenomenon could be seen as the band intensity decreased slightly without Mg^{2+} and with concentration of 0,05 M. The band intensity increased slightly with Mg^{2+} concentration of 0.1 M and decreased again with higher concentrations. These were, however, very slight differences and the band intensity stayed higher with every concentration of Mg^{2+} compared to samples isolated with Fractogel.

The marker indicating CD9 presence seen at 25 kDa differed between the two columns. With samples isolated with Fractogel the band intensity decreased significantly in the flowthrough samples even without any addition of Mg^{2+} and is approximately 40% of that of the starting material on average. The band intensity increased slightly with lower Mg^{2+} concentrations and decreased again with higher concentrations. This cannot be seen in the flowthrough samples isolated with SP Sepharose Fast Flow, where the band intensity decreased steadily as the Mg^{2+} concentration increased, resulting in approximately 10% of EVs with this marker passing through at the concentration of 0,4 M.

One significant difference between the matrixes could be seen at the eluate samples with CD9 markers without Mg^{2+} and with Mg^{2+} concentration of 0,05 M. With samples isolated using Fractogel, a significant amount of EVs could be eluted from the matrix using NaCl. The intensity of these bands could reach over 80% of the intensity of the starting material. This phenomenon could not be seen with samples isolated using SP Sepharose Fast Flow. With this matrix, virtually no EVs seem to elute from the matrix with NaCl. However, as seen at the flowthrough samples, a higher percentage of these passed through the column at the flowthrough compared to samples isolated with Fractogel. This seems to indicate that the tentacle-like structure of Fractogel was in fact more efficient in capturing EVs and that these EVs could then be eluted from the matrix using

NaCl. At higher Mg^{2+} concentrations the CD9 and CD63 markers showed that no EVs seem to elute from either matrix with NaCl.

With both Fractogel and SP Sepharose samples, the intensity of the band indicating CD41 presence was the most consistent throughout the experiment as can be seen in figures 8A and 8B. While it is possible that EVs with CD41 marker are present in these samples, the consistency may also be caused by some other process, such as other antibodies derived from the residual plasma in the starting material at the same size-range binding to the same secondary antibody used in this experiment. As the number of other markers indicating EV presence have more fluctuation, it may indicate that the bands indicating CD41 presence are in fact caused by other antibodies. Therefore these results need to be viewed critically.

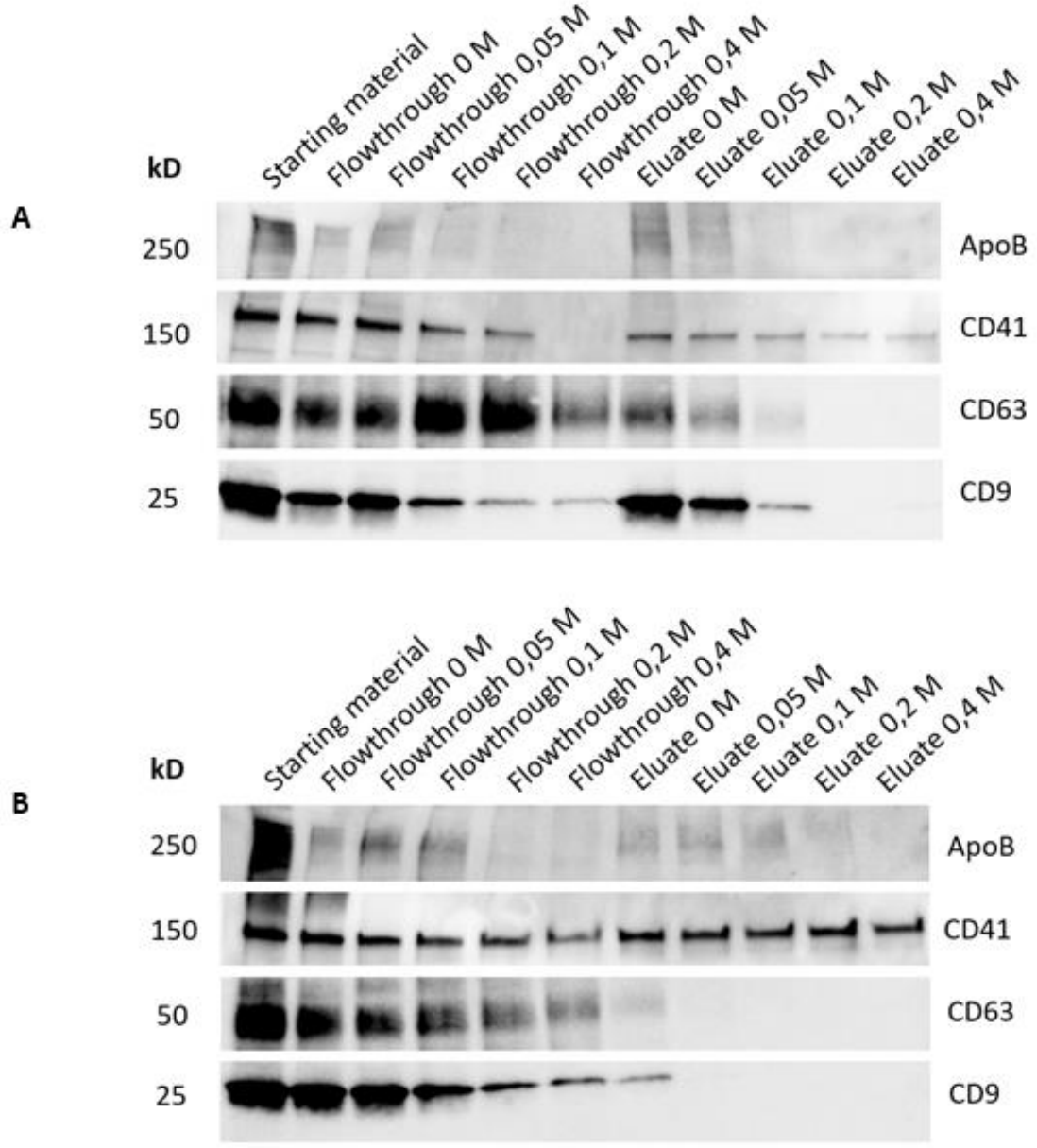


Figure 11. A) Representative western blot images from EV isolation using Fractogel. On the left, the size of the protein is being showed. Markers indicating ApoB presence shown at the size of 250 kDa, markers for CD41 at 150 kDa, markers for CD63 at 50 kDa and markers for CD9 at 25 kDa. The sample on the left shows results from starting material and the following samples from left to right the results of flowthrough samples from lowest to highest Mg^{2+} concentration. The samples eluated with NaCl are shown in the right with samples from lowest to highest Mg^{2+} concentration. B) Representative western blot images from EV isolation using SP Sepharose Fast Flow. The same markers and similar samples are shown in the same order as before.

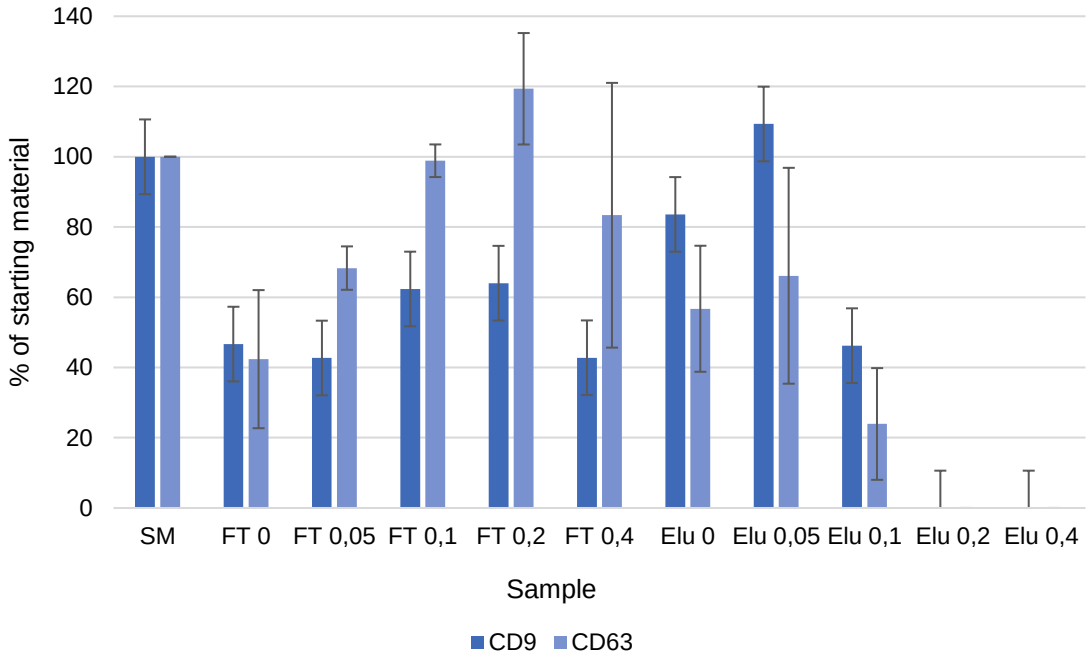


Figure 12. The western blot results with EV markers of samples isolated with Fractogel. The average intensity of each band was calculated and compared to the intensity of the starting material. The average percentage of each sample as well as standard deviations are shown. SM indicates the amount of starting material. Flowthrough (FT) samples are shown on the left from lowest to highest Mg^{2+} concentration and eluate samples on the right in the same order.

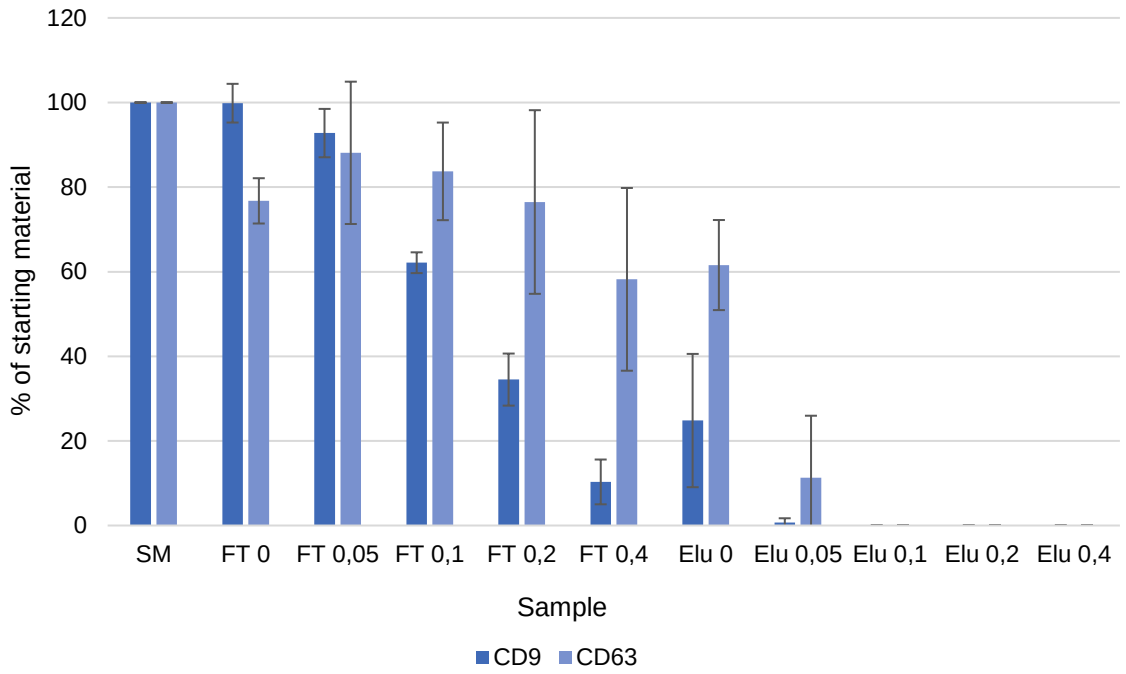


Figure 13. The western blot results of isolation with SP Sepharose Fast Flow. The band intensity was calculated and compared to the starting material. The average percentage of each sample is shown as well as standard deviations of each EV marker. SM indicates the amount of starting material. Flowthrough (FT) samples are shown on the left from lowest to highest Mg^{2+} concentration and eluate samples on the right in the same order.

9.2 Purification of extracellular vesicles

One of the most significant challenges in purifying EVs is their separation from lipoproteins, especially LDL and VLDL as they are similar in size and other physicochemical properties. The efficacy of Fractogel EMD SO_3^- (M) and SP Sepharose Fast Flow in separating these was studied using western blot. A general picture of the ApoB presence can be seen in figures 11A and 11B. These images do not, however, show the composition of the two isoforms of ApoB, ApoB48 and ApoB100. The presence of these two isoforms can be seen in figures 14A, showing the presence of these markers in samples isolated with Fractogel, and 14B, showing them with samples isolated using SP Sepharose Fast Flow.

A closer look in the effectiveness of purification of the two isoforms of ApoB, ApoB48 (chylomicrons), shown at 250 kDa, and ApoB100 (VLDL and LDL), shown at 550 kDa, showed significant differences between the two isoforms. With both matrixes, the isolation process without Mg^{2+} resulted in the removal of ApoB100 from the flowthrough samples but did not remove ApoB48 or decrease the intensity of the band indicating ApoB48 presence.

An addition of Mg^{2+} to the concentration of 0,05 M resulted in the ApoB100 passing through the column in the flowthrough as well as the ApoB48 in samples isolated with SP Sepharose Fast Flow. However, this phenomenon could not be seen as clearly in samples isolated with Fractogel EMD SO_3^- (M). With this matrix, the addition of Mg^{2+} to the concentration of 0,05 resulted in significantly decreased presence of both ApoB48 and ApoB100. However, both bands were still visible, yet weaker compared to the samples isolated with SP Sepharose Fast Flow.

At Mg^{2+} concentrations of 0,1, 0,2 and 0,4 M the SP Sepharose Fast Flow was effective in removing ApoB48, but markers indicating ApoB100 were present in all samples. However, with samples isolated using Fractogel, the intensity of both bands indicating ApoB presence seemed to increase at the concentration of 0,1 M Mg^{2+}. An addition of Mg2+ to the concentrations of 0,2 M and 0,4 M resulted in the removal of ApoB48, while band indicating the presence of ApoB100 was still present. While these stronger Mg^{2+} concentrations were more effective in removing both forms of ApoB from the flowthrough, it is noteworthy that the EV yield was lowest at these concentrations as well.

Figures 11A and 11B show that some ApoB is eluted from the matrix of Fractogel without Mg2+ and with 0,05 and 0,1 M. This indicates that the tentacle-like structure of Fractogel captured some lipoproteins as well as EVs. However, the intensity of the bands is decreased which indicates that some lipoproteins remain in the matrix. With SP Sepharose Fast Flow, some ApoB particles could be seen

in the elute samples without Mg^{2+} and with low Mg^{2+} concentrations as well. The low intensity of these bands indicates that the amount eluted is fairly small.

While the presence of other possible contaminants were not studied using western blot, some results can be seen by studying the image of SDS-PAGE gel. Figure 15A shows the image of a gel with samples isolated using Fractogel and figure 15B shows similar samples from isolation with SP Sepharose Fast Flow. With both images the intensity of bands present decreased as the Mg2+ concentration increased in the flowthrough and eluate samples. With Fractogel an increase in eluted proteins could be seen at samples without Mg^{2+} and with low Mg^{2+} concentrations. This is most likely due to the tentacle-like structure of Fractogel as similar peak cannot be seen at samples isolated using SP Sepharose Fast Flow.

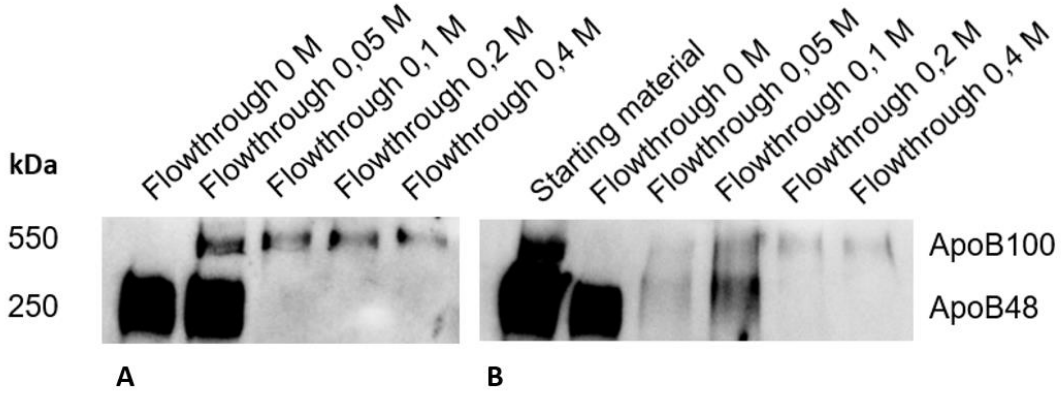


Figure 14 A). ApoB48 and ApoB100 composition of samples isolated using SP Sepharose Fast Flow. B). ApoB48 and ApoB100 composition of samples isolated using Fractogel EMD SO_3^- (M).

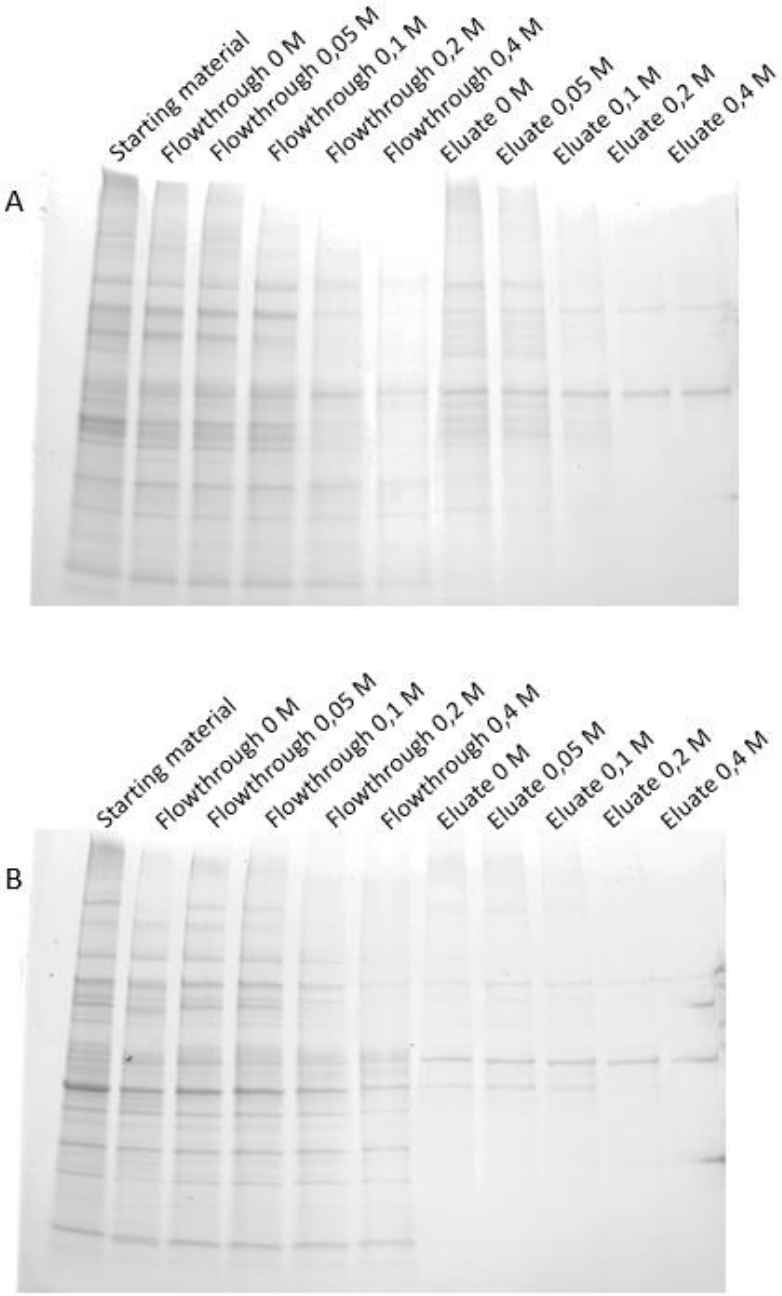


Figure 15 A) SDS-PAGE gel images of samples isolated using Fractogel EMD SO_3^- (M). B) SDS-PAGE gel image of samples isolated using SP Sepharose Fast Flow.

10 Discussion

Both Fractogel EMD SO_3^- (M) and SP Sepharose Fast Flow were found to be effective in decreasing the amount of LDL, VLDL and chylomicrons from the samples. However, the binding patterns of ApoB48 and ApoB100 showed a difference in the function of the corresponding lipoprotein forms: chylomicrons (ApoB48) and V/LDL (ApoB100). Without Mg^{2+} both resins seemed capable to remove LDL and VLDL but were not effective in removing chylomicrons from flowthrough samples. An addition of Mg^{2+} in concentrations of 0,05 M and 0,1 M resulted in the reduction in chylomicrons, however LDL and VLDL were present in the flowthrough samples.

While the total amount of ApoB was lowest in flowthrough samples with Mg^{2+} concentration of 0,2 and 0,4 M with both resins, with these concentrations the EV yields decreased significantly as well. The number of other contaminants seemed to decrease as well with these Mg^{2+} concentrations, as was seen in figures 15A and 15B. While these concentrations may be effective in purifying samples, the decreased yield may result in issues with future studies and prospective. The balance between the yield required and degree of purity needed can be challenging to achieve.

An interesting difference between the two resins can be seen when analyzing the eluate samples. These samples show that the EV markers CD9 and CD63 are present in eluate samples of Fractogel without and with lower Mg^{2+} concentrations while with samples isolated with SP Sepharose Fast Flow, these markers are nearly absent in all eluate samples containing various concentrations of Mg^{2+}. This indicates that Fractogel is able to capture EVs more efficiently with its tentacle-like structure compared to conventional strong cationic exchange chromatographic resins. This is a promising discovery that hopefully may increase the possibilities of cation-exchange chromatography in EV isolation.

Based on these results there appears to be two mechanisms of binding of EVs and lipoproteins with the cationic exchange chromatography. The first of these is the negatively charged resin binding directly to the domains with a positive charge. This phenomenon can be seen with lower magnesium concentrations. During the elution phase these bonds seem to break and result in the elution of the EVs from the column. This is more strongly seen when using Fractogel EMD SO_3^- (M) compared to the SP Sepharose Fast Flow as there is a significant difference in band intensity between the two. This is in line with assumption that the tentacles in the matrix of Fractogel bind more efficiently to the positively charged domains of the vesicles as they can reach them better.

The second binding mechanism seems to be magnesium dependent. This mechanism can be seen to increase the strength of binding as magnesium concentration increases. The exact mechanism behind this binding is unclear, but it is most likely carbohydrate or phospholipid based. This results in strong binding of the vesicles to the matrix that the elution with NaCl does not break. This can be seen clearly with magnesium concentrations of 0,2 and 0,4 M and with both matrixes, as EVs or lipoproteins do not seem to pass through the column in either Fractogel or SP Sepharose Fast Flow samples.

As neither EVs nor apolipoproteins elute with NaCl when bound to the matrix with higher magnesium concentrations, other options to elute them should be considered. As Mg^{2+} has a strong counterion selectivity, there are limited options to break this bond. One of these options is using higher concentrations of magnesium, which may allow these bonds to break. As the strength of the bonds differ based on the phospholipid composition of each particle, using this in a gradient elution might allow the lipoproteins and EVs to elute at different magnesium concentrations. This could be used to separate these particles into different fractions and to increase the purity of EVs. Another option to break these bonds is to use ethylenediaminetetraacetic acid (EDTA). EDTA binds to magnesium and may result in the elution of lipoproteins and EVs, ideally in different fractions.

Deciding on the purification protocol depends highly on the properties of the final product in need. Three different possible purification protocols were created based on these experiments and are shown in figure 16. The first option, shown as protocol A, begins with the use of SP Sepharose Fast Flow to remove LDL and VLDL from the samples. After collecting the flowthrough, these samples would then be run through Fractogel, resulting in EV's binding to the matrix of the column while contaminant passing through. EVs could then be eluted using NaCl. This method would be able to remove V/LDL from the samples but leave chylomicron contamination. This process is straightforward and easy to operate and would most likely result in quite well purified EVs with a decent yield.

With the second isolation protocol option, shown as protocol B, first the LDL and VLDL are removed from the samples using SP Sepharose Fast Flow without Mg^{2+}. Mg^{2+} would the be added to the collected flowthrough to the concentration of 0,05 M after which the sample would be ran through the SP Sepharose Fast Flow column again, hopefully resulting in the reduction of chylomicrons. Either Fractogel EMD SO_3^- (M) or SP Sepharose Fast Flow could then be used to capture the EVs to the column by adding a Mg^{2+} concentration of 0,4 M. The EVs could then be eluted from the column with gradient elution by increasing the magnesium concentration and collecting the eluate in fractions.

This method would result in well purified EV samples. These samples would be quite concentrated, as the elution process can be controlled so that the fractions of interest are collected. The yield with this method could as well be higher than with protocol A as the binding to the column is more efficient with high concentrations of Mg^{2+}. The downside of this protocol is the complexity and length of the purification process as well as high magnesium concentration in final product.

The third option, protocol C, would be to simply use either of the columns for the purification process with a magnesium concentration of 0,4 M. This would result

in binding of the EVs as well as lipoproteins which possibly could then be separated from each other with gradient elution. A challenge with this method would be the separation of all types of lipoproteins from the EVs. If the separation resolution is sufficient, this method would result in well purified EVs with a good yield. This method would as well be simple and straightforward and would not be as laborious as the methods described before. The advantages and disadvantages of all three protocols are shown in table 1.

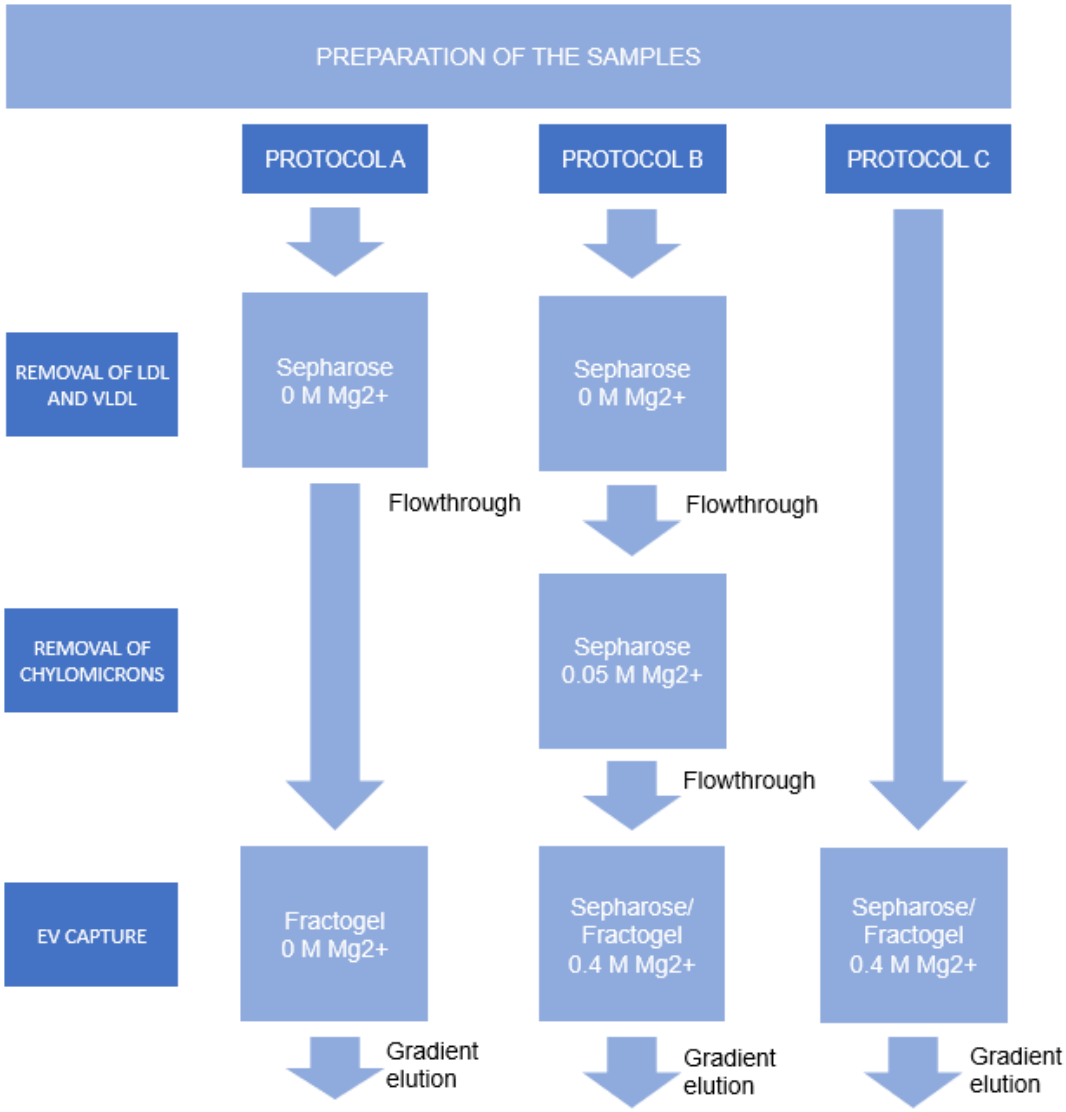


Figure 16. Three possible purification protocols with cationic exchange chromatography. A) Protocol combines Sepharose SP Fast Flow and Fractogel EMD SO_{3-} (M) without Mg^{2+}. B) In this protocol the V/LDL and chylomicrons are

first removed without and with low Mg^{2+} concentrations and the samples are then run with high Mg^{2+} concentration to bind them and eluted with gradient elution. C) this protocol uses gradient elution as the only separation method.

Table 1. The three possible purification protocols with cation exchange chromatography and the expected advantages and challenges

Method	Purity	Yield	Work
Protocol A	Good degree of purity	Moderate yield	Fairly simple and straightforward
Protocol B	Very good degree of purity	Good yield if EVs can be eluted efficiently	More complicated and laborious method
Protocol C	Degree of purity unclear	Good yield if EVs can be eluted efficiently	Fast and straightforward

The decision of which purification method is ideal depends on the degree of purity and yield needed and any further applications of the product. The use of high concentrations of Mg^{2+} may as well be impractical in large scale production. These methods could as well be used for purifying the samples themselves or as a pre purification method before other methods. The Blood Service does have other methods to purify HDL and other contaminants from EVs but these methods are not discussed in close detail here due to proprietary reasons. However, Fractogel EMD SO_3^- (M) and SP Sepharose Fast Flow can be seen as potential methods to be used to purify LDL, VLDL and chylomicrons from the samples as these have proven to be the most challenging with other purification methods available at the moment.

Both of these methods are up scalable and could be used to isolate EVs from samples with larger volumes. The dynamic binding capacity of EVs has not yet been measured with these columns, but it has not been a challenge thus far. It is known that the binding capacity Fractogel EMD SO_3^- (M) is 130 mg of proteins/ml of resin and the binding capacity of SP Sepharose Fast Flow is 70 mg of

proteins/ml. They are therefore expected to be able to bind larger amounts of EVs and contaminants and are expected to have the capacity to purify EVs from other complex fluids such as plasma. All the described methods are as well applicable to the FRC Blood Service production facilities, which is one of the fundamental demands for the purification methods.

The effect of magnesium in either the future applications of these EVs and any further purification methods needed have to be taken into consideration as well. Especially when Mg^{2+} is used to eluate the samples from the resins the concentrations of magnesium may be high and therefore affect the future applications of these EVs. Purifying the samples from magnesium may therefore be necessary in some applications as magnesium is known to bind and precipitate with many components. Another aspect that needs to be taken into consideration is the effect of magnesium to the pH value of the samples. As magnesium may decrease the pH of a solution, this may affect the binding and stability of EVs and other analytes. This needs to be studied in the future to see whether it has an effect on the final product.

Another challenge is resulting in a concentrated enough product for it to be functional in further research and other possible future applications. The collected flowthrough and eluate samples in this study were fairly diluted and would need other concentration methods before further applications. However, gradient elution and more selective fraction collection and other purification methods can lead to more concentrated product, as modern chromatography systems can be used to select the distinct elution peak for collection. Ultrafiltration was used in this study to increase the concentration of the product but this and other concentration methods may harm the EVs and decrease the functionality in possible future applications.

Most of the analysis in this study was done using western blot which is a semi quantitative method. While the results in this study were consistent, this method alone does not result in conclusive results and therefore other methods to analyze

samples should be considered. Flow cytometry and ELISA are widely used methods that may be able to give additional information on the sample concentration and composition.

While NTA is a widely used method in EV research as well, it has significant challenges and limitations. As it is not able to characterize particles and recognize EVs specifically, it may create misleading results if samples contain contaminants with similar size. In this study one of the biggest challenges related to NTA is the similar size of lipoproteins to the EVs. NTA is not able to separate these from each other, which creates challenges in the interpretation of the results. Therefore, other methods are needed to verify the results.

Few studies regarding cation exchange chromatography in EV isolation have been made. The combination of SEC and cation exchange chromatography has been studied by van Deun et al. (2020). This study found the combination of these methods to be effective in purifying the EVs from both ApoA100 and ApoB48. With these promising results, this is another option to study further in the EV isolation. Combining cation exchange chromatography with other isolation methods may help to solve the challenges of using this method alone. In addition to SEC, other possible methods to combine with could be ultrafiltration or precipitation.

As anion exchange chromatography has also been found to be effective in EV isolation (Kosanović et al. 2017, Heath et al. 2018). Therefore, this is another reasonable option to consider in the future. A comparison between cation and anion exchange chromatography is needed to fully understand the differences in purity and yield in the final products received from these methods. IEC results in a concentrated final product and straightforward to use. The challenge with this method is the elution from the matrix, as EVs can bind to the column tightly.

As EV research moves towards more clinical applications, the need for effective purification methods with a good yield and up scalable properties increases. In

this study, the use of cation-exchange chromatography showed promising results for the isolation process. However, little research has been done thus far and more studies and development of this process needs to be done before it can be used to produce clinical-grade EVs.

11 Conclusions

In this study, the comparison of the two different types of cation exchange-chromatography found differences in their function and applicability in EV isolation. Fractogel, the tentacle-type cation exchange chromatography, was found to be more effective in capturing EVs and isolating them from blood products compared to the standard, smooth Sepharose SP Fast Flow without and with low magnesium concentrations.

The use of Mg^{2+} gradient in the sample allowed the purification process to be more specific in removing certain lipoproteins from the samples. Higher Mg^{2+} concentrations were effective in removing lipoproteins but resulted in a loss of EVs as well. Therefore, combining different magnesium concentrations could be used to modify the process to reach the ideal outcome.

Cation-exchange chromatography seems to be a functional EV-isolation method by itself or in a combination with other methods. As this method is fairly straightforward, up scalable and modifiable to the needs of EV isolation, it is promising new way to produce EVs even in higher quantities. However, as little research has been done on this method altogether, it still requires further research before it can be used in future applications, such as in the production of clinical-grade EVs.

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