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Scaled preparation of extracellular vesicles from conditioned media

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ABSTRACT

Extracellular vesicles (EVs) especially of mesenchymal stem/stomal cells (MSCs) are increasingly considered as biotherapeutic agents for a variety of different diseases. For translating them effectively into the clinics, scalable production processes fulfilling good manufacturing practice (GMP) are needed. Like for other biotherapeutic agents, the manufacturing of EV products can be subdivided in the upstream and downstream processing and the subsequent quality control, each of them containing several unit operations. During upstream processing (USP), cells are isolated, stored (cell banking) and expanded; furthermore, EV-containing conditioned media are produced. During downstream processing (DSP), conditioned media (CM) are processed to obtain concentrated and purified EV products. CM are either stored until DSP or are directly processed. As first unit operation in DSP, clarification removes remaining cells, debris and other larger impurities. The key operations of each EV DSP is volume-reduction combined with purification of the concentrated EVs. Most of the EV preparation methods used in conventional research labs including differential centrifugation procedures are limited in their scalability. Consequently, it is a major challenge in the therapeutic EV field to identify appropriate EV concentration and purification methods allowing scale up. As EVs share several features with enveloped viruses, that are used for more than two decades in the clinics now, several principles can be adopted to EV manufacturing. Here, we introduce and discuss volume reducing and purification methods frequently used for viruses and analyze their value for the manufacturing of EV-based therapeutics.

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Abbreviations: AAV, Adeno-associated virus; AEX, Anion exchange chromatography; AC, Affinity chromatography; AMDV, Aleutian mink disease virus; $CaCl_2$, Calcium chloride; CM, Conditioned media; DEAE, Diethylaminoethyl; DSP, Downstream processing; dSTORM, Direct stochastic optical reconstruction; ERK, Extracellular-signal regulated kinases; EVs, Extracellular vesicles; FBS, Fetal bovine serum; FCS, Fetal calf serum; FPLC, Fast protein liquid chromatography; GMP, Good manufacturing practice; GvHD, Graft-versus-host-disease; HDL, High-density lipoprotein; HIV, Human immunodeficiency virus; hPL, Human platelet lysate; HS, Human serum; HSV, Herpes simplex virus; IAC, Immunoaffinity chromatography; IEX, Ion exchange chromatography; IFCM, Imaging flow cytometry; ISEV, International Society of Extracellular Vesicles; LDL, Low-density lipoprotein; LV, Lentivirus; MISEV2018, Minimal information for studies of extracellular vesicles 2018; MoA, Mechanism of action; MSCs, Mesenchymal stromal cells; NaCl, Sodium Chloride; NTA, Nanoparticle tracking analysis; PEG, Polyethylene glycol; PFU, Plaque forming units; PS, Phosphatidylserine; QA, Quaternary amines; RPS, Resistive pulse sensing; SEC, Size exclusion chromatography; sEV, Small extracellular vesicle/exosome-sized EVs (50-150nm); TCID, Tissue-culture infective dose; TEM, Transmission electron microscopy; Tim4, T-cell immunoglobulin domain and mucin domain-containing protein 4; TFF, Tangential flow filtration; UC, Ultracentrifugation; USP, Upstream processing; VLPs, Virus like particles.

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1. Introduction

Extracellular vesicles (EVs) are small membrane-surrounded particles secreted by almost all cell types. They are involved in cell-to-cell communication pathways and play important roles in physiological and pathophysiological conditions in health and disease [1]. During the recent years, the role of EVs in regenerative medicine has been intensively investigated. EVs of various cell types, especially EVs of mesenchymal stem/stromal cells (MSCs), provide therapeutic potential in an increasing number of different disease models [2,3]. Furthermore, MSC-EVs have been already successfully applied to humans [4–7]. Due to the promising results, academic and industrial efforts are taken to qualify EVs for the clinical setting. The translational process provides several challenges regarding the production and the subsequent quality control process [2]. Focusing on MSC-EV products, many of these challenges have been discussed in different white papers [8–11].

EV production processes comprise upstream and downstream processing and subsequent quality control. Upstream processing (USP) refers to all unit operations required to produce EV containing conditioned media (CM), including cell isolation and banking, media preparation, cell expansion to desired quantities, CM harvesting and processing for the interim storage. Downstream processing (DSP) contains all unit operations, i.e., CM clarification, EV purification/concentration and EV polishing, as well as all packing operations, including formulation, filling and finishing. Within this article, we focus on scalable EV DSP unit operations, especially on EV purification and concentration to achieve volume reduction that in principle can be used for the clinical grade production of therapeutic EVs. Notably, DSP procedures are severely impacted by the culture conditions and the media of the USP. Thus, before discussing available purification and volume reducing procedures for EV containing CM, we introduce critical process parameters of the USP, which impacts DSP. Connected to the huge therapeutic potential of EVs harvested from conditioned media of MSCs and to our own research interests [12], at several positions we discuss aspects in the direct context of MSCs and their resulting EV products. Still, most of the discussed issues are representative for other EV products.

2. Strategies for the upstream processing

The supplementation of the cell culture media is one of the decisive points, which probably affects the final EV content of the CM, the starting material of the DSP procedure. Cell culture media contain the nutrition for the cells allowing them to grow and to expand. In principle four different media types exist: I) serum-containing, II) serum-reduced, III) serum-free/xeno-free and IV) chemically defined media. Serum-free media regularly contain alternative supplements such as human platelet lysate (hPL) or other undefined hydrolysates that are not fetal calf serum-derived [13]. Those media can also be termed as xeno-free if they lack any animal components.

The formulation of the media significantly affects the growth kinetics of the cultured cells. MSCs have been historically maintained in fetal bovine/calf serum (FBS/FCS) supplemented media. However, animal components should be - if possible - avoided if cells or their products are considered for the therapeutic use [14–16]. Accordingly, attempts have been made in defining animal component free media (xeno-free media). To this end hPL and human serum (HS) have been qualified as FBS substitutes [13]. Indeed, if prepared appropriately, several studies have demonstrated that hPL supplemented media promote MSC expansion more efficiently than conventional FBS-containing media [13,17–20]. For the production of hPL, platelet concentrates of healthy donors that regularly have exceeded the five-day shelf-life time for their clinical application are stored at −20 °C. Upon freezing and thawing, platelets get destroyed and release their inner components into the outer environment. By centrifugation the solid material is removed. Coupled to the role of platelets in wound healing, hPLs contain coagulation components including fibrin that can induce clot formation [21,22]. To avoid clot formation during processing, following filtration for additional clearance, the supernatants are supplemented with heparin as anti-coagulant. Alternatively, hPLs become depleted for their coagulation ingredients e.g. by calcium chloride ($CaCl_2$)-induced clotting and subsequent removal of the clotted material [23,24]. If not being removed upfront and despite the presence of anticoagulants (e.g. heparin) clotting can occur during concentration processes of ingredients [25,26]. In filtration-based procedures, formed fibrin clots can result in the blocking of filter pores and thus negatively affect DSP.

hPL, either fibrin-containing or fibrin-depleted, is very complex; among others contains donor-derived plasma, lipoproteins including low-density lipoprotein (LDL) and high-density lipoprotein (HDL), EVs, immunoglobulins and high protein amounts [13]. This mixture of components appears as ideal nutrition source for MSCs and other human cell types, e.g. endothelial cells [27,28], but also can provide additional challenges for the DSP. Especially the protein content of the CM can affect DSP technologies. For example, polymer-based precipitation methods regularly depend on higher protein contents as carrier substances. In filtration-based approaches, proteins can build a layer on the membrane which increases the membrane resistance. Although for some applications a controlled layer (cake) formation can be considered as an advantage, for the EV preparation such layers negatively affect purification procedures, especially when pore blocking aggregates are formed during the filtration process [29–31].

Another challenge in using hPL or other primary materials as supplements are inter-donor variabilities [13]. Regularly, animals are sacrificed for the production of animal sera, resulting in the manufacturing of larger batches that can be pre-tested and selected for the desired application. For obtaining larger hPL batches, platelet units from various donors need to be pooled. Although by increasing donor units inter-donor variabilities can be reduced [13], pooled hPL samples provide additional challenges that have been raised by regulatory authorities, i.e. the potential contamination with pathogens especially viruses [25,26,32]. Although viral testing with PCR-based technologies should belong to the standard quality control for blood products [25], such analyses can only detect known viruses, increasing the risk with each other platelet unit to contaminate the whole hPL batch with unidentified pathogens. Aiming to reduce this risk and remaining able to prepare larger hPL batches, virus inactivation strategies are increasingly applied [25,32]. Especially, harsh irradiation procedures have been established to destroy nucleic acids within the samples. Since the procedure might also affect other molecules and destroy EV-associated nucleic acids, viral inactivation could also affect the MSC-supporting features of respective hPL batches. To this end it is worth mentioning that meanwhile several companies sell GMP-compliant, virus-inactivated hPL batches, omitting the need for the time-consuming qualification of self-made hPL units and, although no negative impact on MSC expansion has been observed [33], just leaving the need for confirming the hPL batch's capability to promote MSC expansion and allowing secretion of potent EVs. Even if the virus issue can be solved, it needs to be kept in mind that hPL pools remain undefined and provide unavoidable batch-to-batch variations consequently interfering with the standardization of USP processes.

Like in hPL supplemented media, MSCs proliferate very well in HS supplemented media and expand quicker than in media containing equivalent levels of FBS [34–36]. However, regarding its complexity and batch-to-batch variations, usage of HS provides comparable issues as that of hPL.

Serum-reduced or serum-free media often contain less protein, which is advantageous for some of the DSP unit operations. If proper adapted – which can be a challenge, since MSCs might initially not expand sufficiently in such media –, the growth performance of many primary MSC types can be as effective as in serum or HS/hPL-containing media. To allow the successful expansion in such media, additional additives, special coatings/treatments of the growth surface and special medium compositions as well as appropriate adjusting strategies are regularly required [37,38]. Consequently, either sophisticated protocols need to be established to allow scaled expansion in such media, or primary MSCs need to be expanded to critical cell numbers in another medium than used for the conditioning of supernatant for the EV production. Although it is a common way in the production of biotherapeutics to separate the production in a cellular growth and production phase [37], both of which use different media that must be well adapted to each other, it complicates USP. Furthermore, with a strategy using different expansion and harvesting media, CM from early passages are lost for the EV production. Although little is known about critical process parameter for the EV production, it can be assumed that everything which affects properties of cells also affect the production, especially the quality, of their EVs. Notably, the tissue source for MSCs (e.g. bone marrow, adipose tissue, umbilical cord, Wharton's jelly), the age and the health status of the donor and the cumulative population doublings of the MSCs have been reported to severely affect the amount and quality of EVs being secreted by MSCs [39,40]. Furthermore, it needs to be considered that primary MSCs are typically polyclonal and during expansion undergo clonal selection procedures which could affect product qualities in stochastic manners [41,42]. Immortalization procedure may help to overcome such hurdles but provide new challenges that will not be discussed here.

The production of EVs can be considered as a reaction of cells on their microenvironment. Consequently, the environment of the cells should be highly controlled during USP. For now, only a few environmental parameters have been investigated. For example, impacts of oxygen concentration (reduction from the oxygen concentration from 21% typically to 2–7%), shear stress or licensing with proinflammatory cytokines (IFN-γ and TNF-α) are reported to increase the quantity and/or quality of EVs that are released by cultured MSCs [43–46]. However, since each target disease may depend on different mechanism of action (MoA) modalities of resulting EV products, general assumptions regarding the quality are hard to meet [11]. Overall, the field has too little experience yet, of how USP strategies affect the EV secretion of MSCs and their qualities. Among others, this is connected to limitations in our current EV characterization abilities (see Box 1). For the scaled production of EV-based therapeutics, it is essential that selected USP conditions promote the release of therapeutically active EVs into the CM. Despite the selection of the right media for MSC expansion and EV harvesting, the type of cell culture vessels, preferably bioreactors (e.g. hollow-fiber reactors and stirred-tank bioreactors), and the growth surface/-matrix has the highest impact on MSCs. The bioreactors can perfectly control physicochemical factors including temperature, pH, shear stress but can also control nutrition by oxygen supply, feeding of metabolites and CM harvesting schemes. The properties of the growth surface including its stiffness, elasticity, biochemical composition, topography highly influence proliferation and functionality of MSCs [10,47]. Thus, it is important to define the optimal or at least feasible scalable USP procedure [48] and to develop EV-specific DSP procedures to the CM obtained from the optimized USP.

BOX 1 : State of the art analyses in EV sample characterization. A critical aspect in the EV field is the evaluation procedure for EV preparation methods. Currently, the *minimal information for studies of extracellular vesicles 2018* (MISEV2018) guidelines of the *International Society of Extracellular vesicle* (ISEV) recommend to characterize obtained EV preparations by particle quantification technologies, e.g., by *nanoparticle tracking analysis* (NTA), which we and others introduced in 2011 as an ''exosome" quantification method [49,50] or by *resistive pulse sensing* (RPS)[51], by quantifying the lipid or protein amount and by providing the ratio of 2 different quantification methods, e.g., the particle per protein amount. Furthermore, the presence of typical EV marker proteins and the absence of contaminants should be evaluated; most frequently, this is performed by Western blots. Image-based methods for the morphological appearance are ideally performed at the single EV level, e.g., *by transmission electron microcopy* (TEM). Finally, functional testing should be performed in appropriate assays [52].

Recently, we introduced *imaging flow cytometry* (IFCM) as a promising method allowing analyses at the single EV level [53]. By the usage of appropriate antibodies, IFCM enables the detection of specific antigens on single EVs and the discrimination of different EV subpopulations [53–55]. Upon analyzing sEV samples that had been prepared from void urine with different small scale sEV preparation methods with NTA, IFCM and Western blot, we learned that IFCM data for the detection of $CD9^+$ sEVs correlated well with semiquantitative Western blot data for the exosomal marker protein TSG101, but not with particle numbers recorded by traditional NTA. The NTA data instead correlated with Western blot intensities of uromodulin (Tamm Horsfall protein), an impurity marker in the urine-EV field [56].

Altogether, the data challenge the accuracy of particle numbers for the evaluation of sEV preparation techniques. In addition to IFCM other 2nd generation EV analysis devices have been developed, such as the plasmon resonance device NanoView [57], the NanoFCM, a flow cytometer designed for sub-micron particles [58] the ZetaView Quatt, an NTA optimized for the tracking of fluorescently labelled particles [59], and the *direct stochastic optical reconstructio*n (dSTORM) device, the Nanoimager [60]. These and other 2nd generation analysis devices are currently used by an increasing number of different research groups. Expectably results obtained with these new generation devices will provide new insights into the EV world and challenge previous procedures and data interpretation. Certainly, several of the current recommendations of MISEV2018 will be outdated soon. Thus, upon studying the literature critical considerations need to be taken of how the accuracy of existing EV preparation technologies were evaluated.

Functional testing of obtained EV products is essential but provides its own challenges that have been comprehensively discussed in a recent white paper we just like to refer to [11].

3. Strategies for the downstream processing

DSP combines several different unit operations to process CM. At first, the CM need to be clarified from remaining cells and larger debris [61]. Typically, this can be performed by centrifugation or filtration. This topic will not be discussed, here. Next, non-EV associated process impurities need to be removed as good as possible and EV must be concentrated in a manner to maintain their functional activities. Eventually, the EVs need to be further purified and polished before they are transferred in applicable doses into their storage containers. Within the following part we mainly focus on concentration/volume reduction and purification methods that already are or theoretically could be applied for the scaled manufacturing of EV products. Aiming to manufacture MSC-EV *off-the-shelf* products, which are sufficient for the treatment of several patients **DSP technologies need to be established that can easily process several ten or hundred liters of conditioned media (Box 2)**. Therefore, a critical step during DSP for the manufacture of MSC-EV products is the reduction of the volume to applicable treatment doses.

BOX 2 : Volume and dosing considerations. An important parameter for setting up and discussing DSP strategies is the volume of CM that needs to be processed for the treatment of a given patient. For now, elaborated experience with MSC-EV administrations in humans is missing and calculation of appropriate MSC-EV dosing mainly can be related to the experience of the MSC field. On average 1–2 $\times$ 10^6 MSCs per kilogram body are systemically applied to patients of various patient cohorts including graft-versus-host disease (GvHD) patients, frequently in up to 3 treatment cycles [62]. In 2011, we treated the first patient world-wide with MSC-EVs. We calculated and defined the MSC-EV treatment dose according to MSC number that was used for the media conditioning. Upon using 48 h MSC-CM harvesting intervals, we justified to include a factor 2 and considered the EV harvest of 48 h CM of 500.000 MSCs as an assumed treatment dose per kilogram body weight. For an 80 kg patient we thus proposed MSC-EV products harvested from CM of 4 $\times$ 10^7 MSCs as an average treatment dose. Considering that MSC-EVs might have a higher turnover rate than MSCs, we decided to apply the MSC-EV product 7 times, each 2nd or 3rd day. Thus, it was calculated that MSC-EV products of 2.8 $\times$ 10^8 MSC equivalents are needed for the considered EV therapy. In 2D cultures we can expand up to 5 $\times$ 10^6 MSCs in a volume of 20 mL. Thus, to also obtain sufficient material for subsequent quality control, it became apparent that more than 1.2-liter MSC-CM had to be processed per treatment cycle of a patient. Indeed, to be on the safe site, we considered to process at least a volume of more than 4 L MSC-CM. This volume, however, hardly can be processed by differential centrifugation procedure as the largest rotors for the final ultracentrifugation (UC) procedure are limited to processing volumes of less than 400 mL. At that time, we had used an optimized polyethylene glycol precipitation (PEG) procedure followed by UC; here, the volume is reduced following a PEG precipitation at low-speed centrifugation allowing the simultaneous processing of larger volumes (we processed 2 L in one run) [4,63]. Escalating MSC-EV dose administration and clinical scoring of the patient implied that the dose calculation was appropriate for the successful suppression of the GvHD symptoms of the patient [4].

Certainly, USP and DSP as well as the administration route will affect dosing of future MSC-EV products. Expectably, higher amounts of given MSC-EV products will be required for systemic than for local applications. Eventually also the numbers of applications can be reduced. Indeed, Warnecke *et al.* applied EVs from approx. 2–4 $\times$ 10^6 MSCs ones to successfully treat a patient receiving an intracochlear transplant [7].

4. Volume reduction by ultracentrifugation

Classically, EVs are prepared by differential centrifugation [64]. Here, a series of different centrifugation steps progressively remove cells, larger debris and larger particles including larger EVs as clarification procedure. The principle of the method is the separation of components based on their different sedimentation speed in a liquid, driven by centrifugal forces (*g*-forces). The sedimentation speed depends on the size and density of sedimenting components and the viscosity and density of the solvent [65]. Smaller EVs, especially exosome-sized EVs (sEVs) but also byproducts, e.g. protein aggregates and lipoproteins that might contribute to or antagonize the EVs therapeutic effect [10], are precipitated in a final, volume-reducing UC step. sEVs typically start to sediment at 70–80,000 × *g* forces [66]. Applied centrifugal forces for EV preparation typically vary between 100,000 to 120,000 × *g* [67,68]. Notably, the sedimentation speed also depends on the rotor type, especially on its minimal and maximal radius, its maximum speed, its maximum *g*-force and the total pathlength sedimenting components have to pass. Since commercially available ultracentrifuges can hardly process more than 400 mL liquid in one run, differential centrifugation procedures are limited in scalability. Furthermore, an increasing number of studies reports negative impacts of UC on the integrity and functionality of prepared EVs. We and others observed that UC results in aggregate formation of EVs and other components [53,69,70]. Thus, UC is not a real option as volume reducing method for the manufacturing of clinical MSC-EV products and other methods need to be considered. Thus, there is the need for the therapeutic EV field to establish alternative methods for the EV preparation that allow processing of much higher volumes of CM than they can be processed in differential centrifugation procedures.

To this end, DSP methods might be adopted from other research areas, especially from virology. Enveloped viruses and EVs share some common features including size, molecular composition and their assembly pathways [71–74]. Like enveloped viruses, sEVs are surrounded by a lipid, protein containing membrane. Moreover, comparable to virus, EVs can bind to the plasma membrane of other cells and enter them by fusion or endocytosis [71]. Consequently, well-established DSP techniques being used for GMP-compliant scaled virus production are candidate technologies for the scaled EV preparation [30,75].

In the following part we discuss DSP techniques which are well established for the production of different viruses frequently used as gene therapeutic vectors or within the vaccine field (Box 3). Due to their higher similarity to EVs, we focus on preparation technologies for enveloped viruses including gamma retroviruses, lentiviruses (LVs) and *Herpes simplex* viruses (HSV). However, we will also consider preparation techniques for non-enveloped viruses (adenoviruses and adeno-associated viruses) that according to our understanding provide merits for the EV preparation but that until now have sparely been used for the preparation of enveloped viruses.

BOX 3: Viruses used in gene therapeutic approaches share some common features with sEVs.

EVs share several features with enveloped viruses including human immunodeficiency virus (HIV), gamma retroviruses and DNA containing human *Herpes simplex* viruses (HSV). Amongst others, these viruses and EVs are surrounded by a protein-associated phospholipid bilayer membrane. Derivatives of the HIV became increasingly popular as gene transfer vehicles in gene therapeutic approaches and more recently for the production of CAR-T cells [76,77]. Also, the DNA-containing human HSVs are used as gene therapeutic vectors, especially for the delivery of episomal DNA [78]. Cells producing enveloped viruses also release membrane surrounded virus-like particles (VLPs). The VLPs of enveloped viruses are vesicles that resemble aspects of the viruses, e.g. they can contain viral capsids, but lack other viral components such as the viral genome, consequently they are not infectious [79,80].

Although within a comparable size range, non-enveloped viruses are differently composed than enveloped viruses and EVs. They are not surrounded by a membrane and are just encapsulated by proteins with self-assembling properties. Non-enveloped viruses frequently being used for the production of vaccines or for transfer of DNA are adenoviruses and adeno-associated virus (AAV) or their VLPs, respectively [81–83].

5. DSP methods for viruses that have been or can be adopted by the EV field for the volume reduction and EV purification from clarified, conditioned media

Based on size, density, surface charge and molecular composition, different methods have been established allowing preparation of viruses or VLPs for the clinical application that in parts have already been adopted by the EV field. Basically, three different strategies are used that will be described in more detail: i) polymer-based precipitation strategies, ii) filtration-based strategies including size-exclusion and iii) affinity chromatography-based strategies. These strategies are frequently used as standalone technologies but can also be combined to improve the purity and concentration of obtained products. Upon evaluating the methods for the EV preparation, important parameters are the concentration, recovery and purification factors as well as the biolog-

ical activity of obtained products. While the EV field is largely lacking valid analysis technologies and quantitative functional assays allowing accurate evaluation of the EV concentration before and after processing (Box 1), in the virology field, biological activities can be quantified by deciphering the number of viral particles e.g. as plaque forming units (PFU) or as median tissue culture infective dose required for infecting 50% of the cells within an assay ($TCID_{50}$) (Box 4). Consequently, until analysis methods within the EV field have been improved, experience from virology can be considered as helpful orientations in selecting appropriate DSP methods.

BOX 4 : Advantages in virus containing sample over EV sample characterization. Virus-containing samples are regularly characterized by determining the number of infectious virus particles in given samples, e.g. by PFU and/or the tissue-culture infective dose ($TCID_{50}$) [84,85]. These methods are biological readouts depending on the maintained infectivity of prepared viruses. Thus, the accuracy of DSP techniques can be analyzed and compared more reliable than those for the EV preparation. Quantification of biological activities in contrast to particle numbers, presence of molecular markers and/or numbers of vesicular like structures warrant functionality of prepared viruses. For now, biological single EV assays do not exist. The functionality of obtained EV samples can only be analyzed in bulk assays not allowing EV quantification. Of note, this functionality may also depend on sample components being co-purified with the EVs [10].

6. Polymer-based precipitation technologies

Polymers like PEG are hydrophilic, largely inert polyether available with different polymer chain lengths and thus with different molecular weights. By efficiently binding water molecules, their addition to aqueous solutions/suspensions reduces the solvent availability and can increase the concentration of solved compounds beyond critical concentrations resulting in their precipitation. It is also discussed that in relation to the size of their hydrodynamic diameter, other components can be sterically excluded from the solvent, larger components at lower PEG concentrations and smaller components at higher PEG concentrations [86,87]. For simplicity it can be assumed that PEG binds so much water that originally solved components become insoluble and precipitate.

The precipitation effectivity is influenced by different factors, e.g. the molecular weight of the PEG, the salt concentration and the pH value of the final mixture [88–92]. The higher the molecular weight of the PEG, the more it reduces the solubility of solved compounds. Ions and pH values affect electric charges of solved compounds and thus their interactions with PEG and the solvent. Depending on the compound its solubility can be increased or decreased by the presence of specific ions in a pH value-dependent manner [88,93,94]. Precipitated compounds can be sedimented at low *g*-forces or isolated by filtration. Thus, PEG precipitation allows volume reduction and enrichment of compounds without the need of any UC step and can be scaled to several liters.

According to these characteristics, PEG precipitation is a traditional technology for the concentration of various viruses [95–98]. Recoveries of up to 100% of the infectious viruses and concentration factors of more than 100-fold have been reported [99,100]. Maybe at first confirmed by the commercial companies, e.g. the vendors of Exoquick©, PEG derivatives can effectively be used for the EV precipitation [101,102]. As mentioned before, with the need to process several liters for the preparation of MSC-EVs, we compared the precipitation efficacy of PEGs with different molecular weights, PEG 6000, 8000 and 20000. Indeed, upon using serum-supplemented media and addition of 75 mmol/l NaCl, all PEGs were able to precipitate EVs at various concentrations. Coupled to the relation of the viscosity of PEG stock solution and the molecular weight, PEG 6000 was selected as the PEG of choice [63]. Maximal EV yields were obtained at final PEG concentrations of $\geq$ 10% and following $\geq$ 8 h incubation. With increasing PEG concentrations, however, more protein was co-precipitated, consequently we decided to use PEG 6000 at a final concentration of 10%. Following volume reduction, subsequent UC allowed significant reduction of the co-precipitated protein content [63] (Fig. 1). Even though EV products obtained with the optimized PEG precipitation method from cell culture supernatants are purer in terms of the estimated particle numbers per protein amount than related EV products obtained by differential centrifugation procedures, PEG-prepared EV products contain several non-EV associated byproducts [63]. Still, MSC-EVs prepared with the optimized protocol were successfully applied to the aforementioned human GvHD patient and to several animal models [4,42,46,103–108]. At the example of an ischemic stroke mouse model, we confirmed that MSC-EVs prepared with this protocol conferred comparable therapeutic effects than their applied parental MSCs, implying that co-precipitated molecules do not negatively affect the functionality of the prepared MSC-EVs [103].

According to our understanding, PEG precipitation procedures are not much more scalable than up to the few 10-liter level. Notably, the success of the PEG precipitation procedure highly depends on the protein content of the starting material. If the protein content is very high, e.g. in pure plasma samples, the amounts of components being co-precipitated with EVs is massively increased. Oppositely, EVs cannot effectively be precipitated with the described protocol from CM with low protein contents (own observations). If required, for the adjustment to low protein conditions, salt and/or PEG concentrations could be increased towards the critical point EVs start to precipitate again. Another disadvantage of the PEG procedure is that for now, PEG procedures require numerous manual handling steps on open systems which increases contamination risks. Altogether, we like to conclude, even though PEG precipitation in combination with subsequent purification procedures, e.g. UC/filtration, allows manufacturing of medium-scaled EV products, it is not the right technology for the production of EV products at the industrial scale.

7. Filtration-based-methods

In contrast to centrifugation procedures, filtration-based methods might be scalable for the EV production to the industrial level. Basically, there are two filtration principles that can be used for the concentration and purification of particles in the size range of viruses, VLPs and EVs, i) dead-end filtration and ii) tangential flow filtration (TFF). In dead-end filtration, the liquid to be filtered (the feed) is applied to a membrane or a filter cartridge that retentates particles being larger than the filter pores. Driven by *g*-forces or pressure, liquids and compounds smaller than the filter pores pass the membrane. Retentates form a filter cake which can also retentate compounds being smaller than the filter pores, thus, negatively affecting the purification of the retentates (Fig. 2). Although easy to operate, membrane fouling such as filter cakes and clogging of the filter pores decreases the filtration capacity over time. Coupled to its low costs dead-end filtration is frequently used at laboratory scale. At a larger scale, dead-end filtration is rather used for the clarification of feed liquids than for the preparation of its therapeutic particles [29].

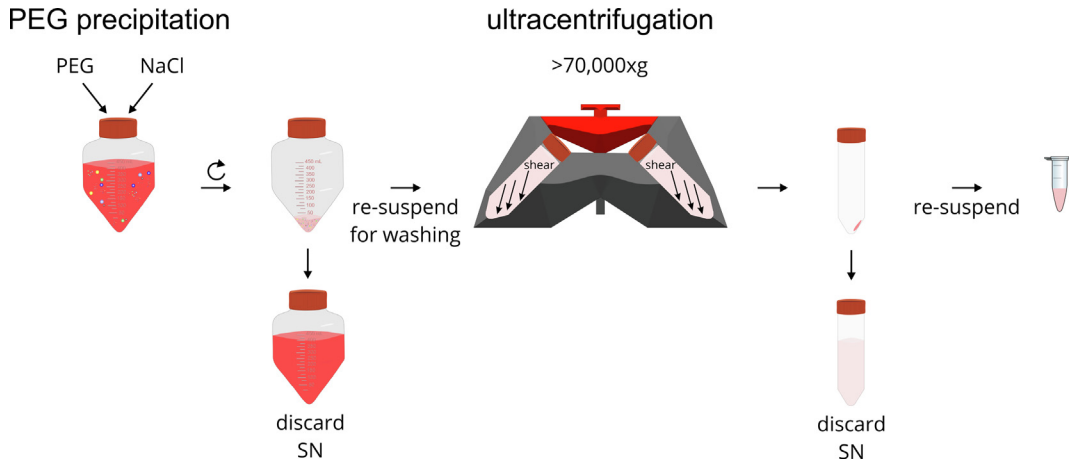

Fig. 1. EV enrichment by PEG precipitation. PEG in the presence of the right salt (e.g. NaCl) and protein concentration results in the co-precipitation of EVs and some byproducts that can be pelleted at low g-forces. In a polishing process a proportion of impurities can be removed by a subsequent washing and ultracentrifugation step.

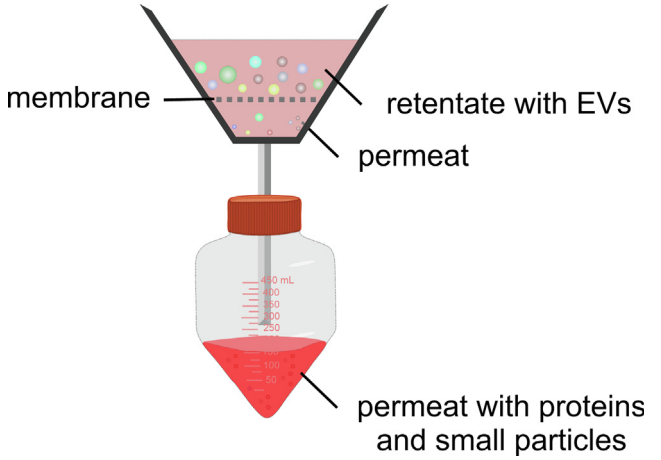

Fig. 2. Principle of dead-end filtration. Driven by *g*-forces or pressure, the liquid of the feed and compounds smaller than the pores can pass filter membranes. Compounds being larger than the pores remain in the retentate and form filter cakes that can also retain compounds being smaller than the pores.

In contrast to dead-end filtration, in TFF, also named cross-flow filtration, the feed flows with low pressure over the membrane surface thus reducing layer and cake formation as well as minimizing shear stress forces. In TFF devices, feeds are regularly circulated by pumps through a closed system containing a reservoir and the filter-module, most commonly hollow fiber units or membrane-containing cassettes. Upon passing the filter units, which are available in different materials with different filter pore sizes, a certain percentage of the feed passes through the pores including a proportion of the compounds being smaller than the pores. Thus, over time, the feed volume gets continuously reduced and the retentate depleted of pore-passing compounds (Fig. 3). Consequently, particles being larger than the pores become concentrated over time [109]. In addition, to the capability in concentrating particles being larger than the pores, TFF is also used for diafiltration. Here the original solvent of the feed can be gradually exchanged to another one, e.g. exchanging the CM liquid with a buffer of choice [110].

Coupled to its potentially longer lifecycle times, its reduced membrane fouling capacities and the low applied shear stress forces, TFF is broadly used in the industry, both for the clarification of feed liquids as well as for preparation and purification of

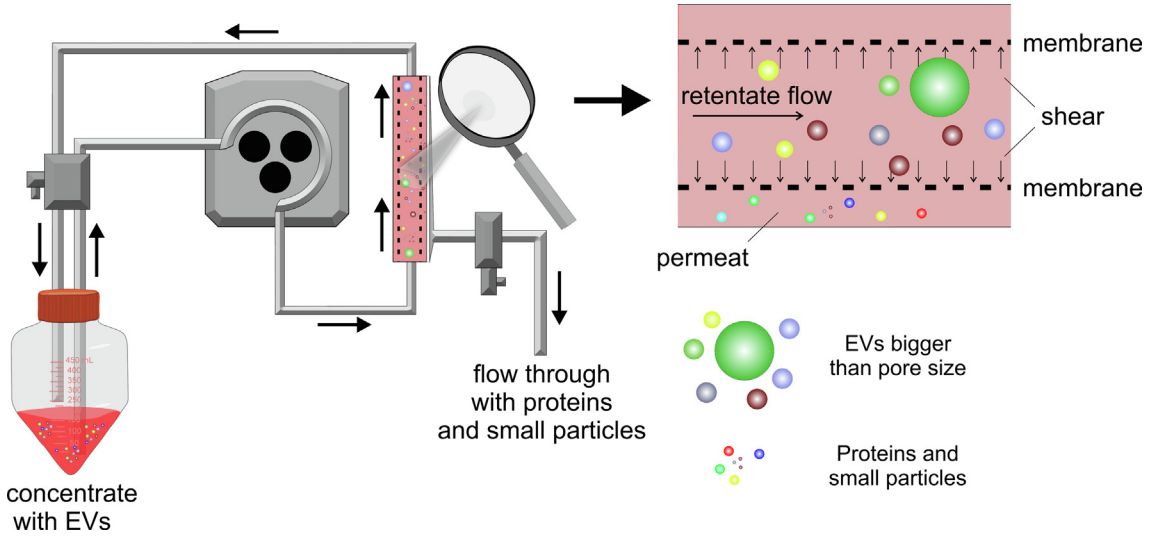

Fig. 3. Principle of the tangential flow filtration for retentate collection. The TFF device is composed of a peristaltic pump, a tubing system and a filter cartridge. Driven by the peristaltic pump sample feeds circulate in the tubing system. Upon passing the filter cartridge liquid and compounds being smaller than the pore size of the filter cartridge pass the pores resulting in the concentration and purification of compounds being larger than the pores in the retentate.

particles, and for diafiltration processes. It has already been used in 1994 for the concentration of retroviral vector particles and allowed recovery rates of more than 90% [111]. Combined with subsequent UC, and using starting volumes of 2 L, TFF was able to enrich infectious HIV-1-based lentiviral vectors up to 1,800-fold and recovery rates above 90% [112]. Today, TFF can be considered as a traditional method for virus preparation, frequently being used in combination with other DSP technologies [113].

More than a decade ago, the TFF technology was adopted to the EV field [114]. According to its advantages it is increasingly used for the EV preparation for GMP- and non-GMP compliant EV products [40,76,115–120]. To our best knowledge, for the manufacturing of biotherapeutic particles, GMP-compliant TFF devices are available from five different companies, i.e. Repligen (former Spectrum Labs), Merck, Pall Corporation, Sartorius and Cytiva Lifesciences (former GE Healthcare). The TFF devices differ in their design including that of their filtration and control units. Systems are available as manually operated small scale (<100 mL feed volume) to fully automated industrial scale devices (>1000 L feed volume).

As mentioned before, even membrane fouling is reduced in TFF systems compared to dead-end filtration, fouling can also affect TFF performances, especially if protein-rich media are used. Upon using pore sizes being smaller than the viruses or the EVs that should be enriched, membrane fouling results in the reduction of the filtration speed and the retention of impurities that are smaller than the pores. Regularly this reduces the purity of the preparations and is considered as disadvantageous. However, depending on the EV product and its application, EV co-concentrated compounds might also importantly contribute to its therapeutic activity [10]. To avoid filter cake formation and reduce membrane fouling, backflush operations can be performed, in which the direction of the flow is briefly reverted from the filtrate towards the feed resulting in the at least partial removal of filter cakes and the prolonging of possible processing times [121]. Provided, activities in therapeutic EV products are basically EV associated and do not depend on smaller co-purified compounds, TFF appears as a useful technique for the manufacturing of therapeutic EV products. For obtaining optimal TFF results, parameters must be adjusted individually for each kind of CM [30,122]. According to our own experience several liters of CM with low protein contents can be filtered by TFF, while high protein content CM quickly result in cake formation and membrane fouling severely affecting the purity of concentrated EVs. For the optimization of the process, the pore size is a critical parameter. For allowing optimal depletion of impurities, pore sizes are ideally chosen as large as possible, but they should be small enough to efficiently retain most of the EVs of interest. For protein pure CM we obtained good results with 750 kDa filter cartridges (unpublished).

8. Chromatography-based methods

Chromatography-based methods exist for more than 160 years and are used in various manners for the separation of ingredients of multi-compound mixtures [123]. Amongst others, different chromatography-based techniques have been used for the virus preparation and for the preparation of EVs. Chromatography is based on the interaction of two phases: a stationary and a mobile phase. The mobile phase carries the mixture to be separated, either in a gas or a liquid phase, along or through a stationary phase. Coupled to different affinities of the stationary phase to specific ingredients of the mobile phase, ingredients are fractioned during chromatographic processes [124–126]. For the preparation of viruses, mainly three different stationary materials are used, monoliths, membrane adsorbers and packed beds of porous beads [127]. The most important difference is that mass transport of monolith and membranes completely differ from that of porous beads. Mass transport means the way how solutes are carried to and from the material surface [128]. Packed beds are typically columns filled with a defined packing material, regularly porous resins [129]. Packed-bed chromatography rely almost exclusively on diffusive mass transport. Diffusion is generally slow, and with increasing molecular sizes of compounds to be fractioned (e.g. viruses and EVs) may become dramatically slow [110,130]. Furthermore, larger particles cannot enter the pores of the stationary phase, thus reducing the overall binding capacities of the stationary phase. Membranes and monoliths rely on convective mass transport which is not limited by the molecular size of the compounds to be fractioned. In addition, the binding capacity and resolution of stationary phases are largely independent of flow rates. Compared to pack-bad chromatography, membrane and monolithic stationary phases without increasing shear stress forces, thus, allow 10–20 times higher flow rates than pack-bed chromatography. In addition to economic reasons, short processing times provide essential advantages for labile products including EVs and viruses.

Monolith materials are hardened polymers forming a solidified sponge-like structure. A multitude of pores and tubes increase the surface area significantly, thus, providing a huge interaction zone between the mobile and stationary phase [131]. Membrane adsorbers are composed comparable to microfiltration membranes but provide affinities to selected ingredients of the mobile phase. Like the monolith chromatography, adsorbent membrane chromatography can be scaled to the industrial levels. Compared to the volume of the stationary phase, monoliths provide larger surfaces than adsorbent membranes. However, monoliths and membrane adsorbers are prone for clogging. Thus, for obtaining good results, initial media clarification is advisable, alternatively monoliths or membranes with larger pore sizes should be chosen (up to 6 μm) [128].

For the preparation of viruses, regularly virus-containing CM or pre-prepared viruses stored in selected buffers are applied to the mobile phase. Coupled to the different principles of how viruses can be prepared from the mobile phase, different chromatography principles are used. The most frequently used chromatography types for virus purification are anion exchange chromatography (AEX), affinity chromatography (AC) and size exclusion chromatography (SEC), which will be discussed in more detail in the following sections.

9. Anion-exchange chromatography (AEX)

AEX is as an ion exchange chromatography (IEX) in which a cationic stationary phase binds negatively charged ingredients of the mobile phase. Usually, the stationary phase is integrated into capsules or columns. The cationic groups of the stationary phase are chosen according to the experimental needs, i.e. high recovery or high purity, with high, low or middle ionic strength binding capacity. Quaternary amines (QA) are representatives for a strong binding affinity and diethylaminomethyls (DEAE) for AEX materials with weaker binding affinities [132,133]. After binding the desired particles and washing off low-bound impurities, bound particles are eluted with an appropriate elution buffer. Frequently anion containing buffers are used, whose anions compete with the binding of the negatively charged compounds of the original feed [134]. Upon increasing the anion concentration within the elution buffer over time (gradient elution), bound compounds can be gradually eluted and thus fractioned; with less negatively charged impurities eluting earlier than those with higher negative charges, e.g. the desired particles (Fig. 4). Alternatively, acidic buffers can be used for the elution of the bound compounds. Protons applied with

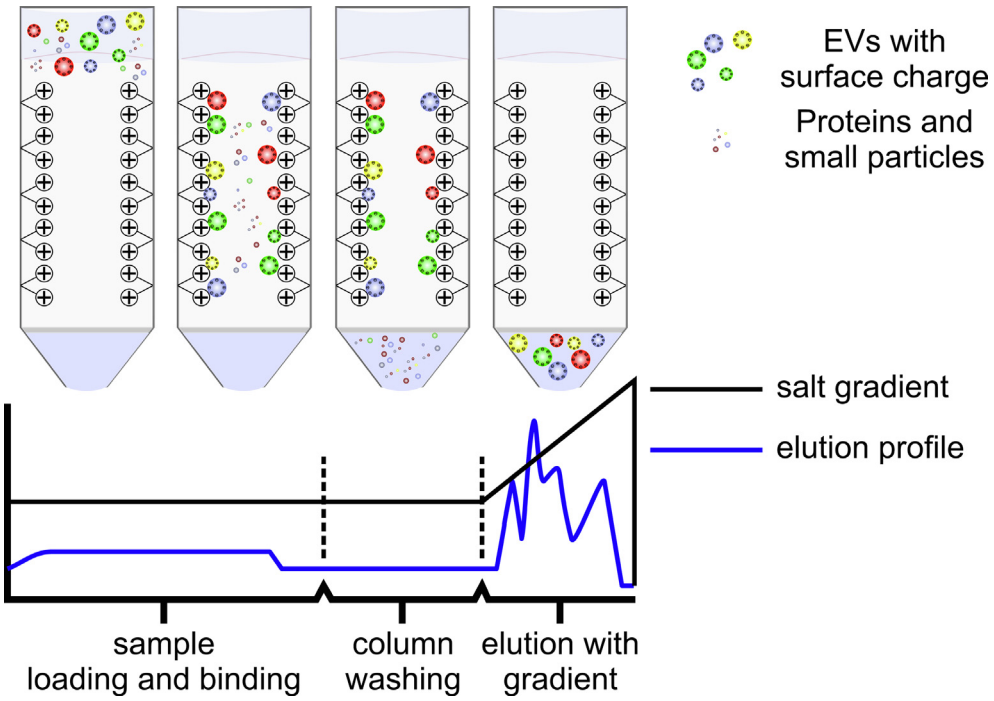

Fig. 4. Principle of anion exchange chromatography. The stationary phase of AEX capsules/columns contains cationic molecules (+). Negatively charged compounds of the mixture loaded to the mobile phase, e.g. EVs in cleared CM, bind to the cationic groups and are separated from the non-binding ingredients. Bound compounds are regularly eluted by gradually increasing the salt concentration or gradually lowering the pH value of the applied elution buffer, respectively.

the elution buffer can neutralize anionic site-groups and thus interfere with their ability to bind to the positively charged stationary phase. Depending on the pH value of the buffers and the pKi of the bound compounds, they can be gradually eluted. Of note, massive pH shifts can negatively affect the compound solubility and result in their precipitation [125,135].

Traditionally, AEX used resins as stationary phase [136], however, coupled to the discussed advantages of monoliths and membrane adsorber, mainly cationic monoliths and membrane adsorbers are used for the virus and VLP preparation [132,133,137,138]. AEX chromatography can be controlled and automatized by fast protein liquid chromatography (FPLC) units, run in a closed system and performed in a GMP compliant manner [117]. Regularly, DSP procedures for the preparation of active virus or VLPs take only a few hours and allow recovery rates of more than 50% [117,132,139–141].

At physiological pH values, EVs have a negative zeta-potential [142,143] and thus are predestined for the purification with AEX chromatography. Coupled to the similarity of enveloped viruses and EVs, established AEX chromatography strategies can serve as a blueprint for the EV preparation and facilitate the establishment and optimization for appropriate EV preparation protocols [117,144,145]. AEX can be scaled to industrial level. A critical parameter aside clogging issues is the nucleic acid content of the applied feed. Nucleic acids are negatively charged and have higher cationic affinities than EVs and thus can reduce the EV-binding capacity of the stationary phase [131]. Thus, it might become mandatory to remove nucleic acids enzymatically (e.g. Benzonase treatment) from feeds, e.g. clarified CM, before AEX can be performed, as it is standard for industrial virus preparations [133]. For creating an optimal enzymatic reaction milieu, buffers can be exchanged by diafiltration before EV purification with FPLC controlled AEX [146]. Another critical parameter is the applied pressure normally EVs are purified under low or medium pressure to avoid particle damage by high shear stress [115]. Furthermore, the salt concentration of the elution buffer or its pH value may affect the quality of prepared EVs [147]. Even if EV qualities are not negatively affected by the elution buffer, buffer exchanges by diafiltration processes might be required to replace the elution buffer with an appropriate storage buffer [110,134].

To best of our knowledge first approaches that prepared EVs via AEX were reported in 2016. The group of Alois Jungbauer used monolithic AEX for the separation of EVs and HIV-1 gag VLPs from CM of gag-expressing CHO cells. With their strategy they were able to deplete most impurities from both, the obtained EV and the VLP fraction. Despite Benzonase treatment, DNA was recovered in the EV fraction that also contained several EV markers. According to their results the AEX method performed at the laboratory scale is much more effective than density gradient centrifugation [133]. The group of Darwin Prockop used resin-based AEX for the preparation of EVs from supernatants of MSCs raised in a protein-free medium in the 1-liter scale. Connected to the clarification of the CM by 2,500 × *g* centrifugation, the mean particle size within the obtained EV fraction was above 200 nm. It contained detectable amounts of CD63 and CD81 but not of CD9. Furthermore, prepared MSC-EVs with reported recovery rates between 73% und 81% were able to reduce cognitive impairments in a traumatic brain injury mouse model [144]. Furthermore, a couple of other groups has reported the usage of AEX for the EV preparation [117,145,148–151]. According to our own experience AEX is a very promising technology for the preparation of therapeutic EVs at the industrial level. As discussed before, it might require a Benzonase pretreatment of the feed that as in our case can be performed in a two-step diafiltration procedure in GMP compliant TFF devices [146].

10. Affinity chromatography (AC)

Methods based on AC are widely used since 1968 to isolate diluted antigens, enzymes and viruses [152,153]. AC is based on the specific and reversible interaction between compounds of interest and their immobilized interaction partners. In

immunoaffinity chromatography (IAC) specific antibodies, Fab fragments, nanobodies or aptamers are coupled to the stationary phase to specifically capture compounds containing corresponding antigens [154–157]. AC can also be performed with immobilized molecules that specifically interact with molecules on the compound to be purified, e.g. immobilized molecules may act as ligands or receptors for molecules being present on the compounds to be purified. Upon specific binding of selected compounds, AC is highly selective and allows efficient purification of considered compounds [126,158,159] (Fig. 5).

In virology, IAC was at first used in 1973 for the isolation of Aleutian mink disease virus (AMDV); here, antibodies prepared from serum of chronically infected mink were coupled to activated Sepharose 4B columns. Extracts of infected tissues were loaded to the columns and following extensive washing, captured viruses were effectively eluted either with 0.75 M NaCl or 0.2 M glycine HCl [160]. In 1998, an IAC protocol for the purification of recombinant AAV vectors was established; here, monoclonal antibodies directed against AAV-2 capsids were immobilized on an activated HiTrap-Sepharose column. After optimization of the procedure, approximately 70% of the initial infectious particles could be eluted with 2.5 M $MgCl_2$ in PBS, 50 mM Tris-HCl at pH 7.0 [161]. Connected to restrictions to selected AAV variants and the huge antibody purification costs, this technology was not developed any further, instead commercially available sepharose columns loaded with nanobodies recognizing outer shell proteins of AAVs are used [162]. Nanobodies represent a class of antibody fragments consisting of a single monomeric variable domain derived from heavy chain antibodies from camelids [163]. Compared with conventional antibodies nanobodies provide some unique advantages, amongst others they can easily be produced recombinantly in prokaryotic systems [164]. Beyond the usage for the AAV and the initial AMDV purification we are not aware of any IAC-based strategy that had been used for the preparation of enveloped viruses.

In contrast, IAC provides several advantages for the preparation of defined EVs. Indeed, immunocapturing became an attractive method for the preparation of EVs including that of selected EV subtypes. At first immunocapturing of EVs was described in 2001, here, EVs containing different HLA-antigens were captured by magnetic beads loaded with different anti-HLA antibodies. Following washing without any elution procedure, bead-EV aggregates were analyzed flow cytometrically [165]. Based on the same principle the technique has been and is frequently used for EV analyses. For example, the Théry-lab separated different EV subtypes with anti-CD9, CD63 and CD81 antibody loaded magnetic beads and following Tween mediated lysis performed proteomic downstream analyses and thus characterized different EV-types in more detail [166]. Also based on a beat capturing principle a commercial product of Miltenyi can effectively be used to unravel EV heterogeneities in various body liquids and cell culture CM without releasing the EVs from the aggregates [167–169]. For now, we are not aware of any published results that immunoaffinity captured EVs have been eluted from the stationary phase for elaborated functional analyses. Commonly, immunocaptured EVs are either analyzed as bead-aggregates in flow cytometry or lysed for omic downstream analyses. Only in a few studies EVs that had been immunocaptured in microfluidic devices, have been eluted from the solid phase by harsh conditions, e.g. by treatment with a glycine-HCl pH 2.2 or with a 8 M Urea, 50 mM ammonium bicarbonate buffer, both of which might negatively affect the integrity of EVs [170,171].

A commercial system for the small scale IAC preparation of EVs is provided by the German company IBA-Lifescience; here, streptamers of low affinity Fab fragments with increased avidity are loaded to the stationary phase of agarose columns. After binding and washing of the EVs originally included in the feed, by the addition of biotin, streptamers are disaggregated and EVs released. Due to their low affinity, Fab fragments dissociate from the EVs leaving them finally in an unlabeled manner (www.iba-lifesciences.com/strep-tag/exosome-isolation). Even though this principle provides scaling potential, coupled to the high costs of antibodies, IAC is currently not really considered as an attractive method for the scaled EV production. Eventually as for AAV, in the future, recombinant nanobodies might allow development of affordable scaled IAC processes.

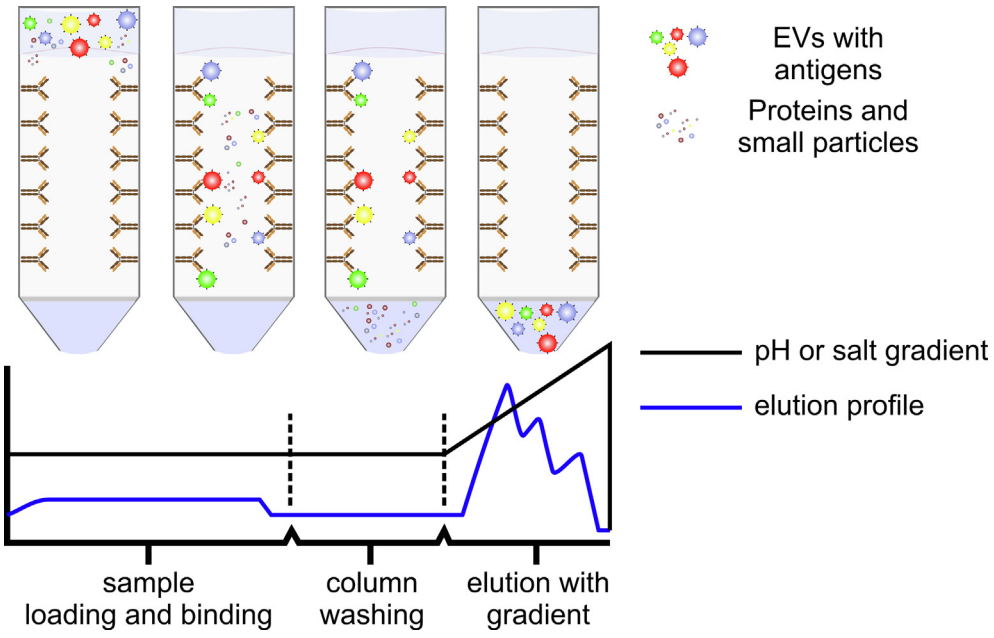

Fig. 5. Principle of affinity chromatography. The stationary phase of AC beads/ matrices/ columns is coupled with antibodies, antibody fragments or other ligand agents. Specific compounds of the feed bind to the molecules on the stationary phase. Washing steps remove weakly retained molecules. Bound compounds are eluted by appropriate buffers.

As mentioned before other AC principles are available and have been used for virus and EV preparation. A frequently used AC method for the purification of different types of viruses, including AAV-2, γ-retrovirus, lentivirus and HSV, is heparin-based AC [172–174]. Here, stationary phases with immobilized heparin are used. Heparin is a highly sulfated, negatively charged linear polysaccharide belonging to the family of glycosaminoglycans. It specifically interacts with various molecules and is used as cationic exchanger in ion exchange chromatography or in AC as ligand for heparin binding molecules [175–177]. In addition to many soluble molecules, heparin binds several virus- and EV-associated molecules. Consequently, for the preparation of viruses and EVs protein clearance with appropriate operations is highly recommended [178]. Using heparin AC, 53% of the original lentiviral particles of the feed were recovered in concentrated eluates (elution buffer: 0.35 M NaCl) that were largely depleted for the vast majority of contaminants [179].

After observing that heparin inhibits cellular EV uptake [180,181], the Maguire-Lab used heparin-coated agarose beads for the EV preparation from cell culture CM and from plasma. Following elution with 2.15 M NaCl in PBS overnight at 4 °C the RNA content and some proteins of the prepared EVs were analyzed [182]. Columns packed with a Capto Heparin resin were used to separate EVs and VLPs from pre-purified EV/VLP samples by applying a linear salt gradient, 120 to 2000 mM NaCl. Eluted EVs and VLPs were subsequently used for molecular downstream analyses [178]. For now, we are not aware that any of the Heparin prepared EVs were analyzed at the functional level. Although the method might be scalable for the functional EV preparation and unless synthetic Heparin is used, its qualification as a GMP-compliant EV preparation technology is challenged by the fact that Heparin is regularly prepared from pigs and may contain unidentified pathogenic contaminants [177].

Another AC method is based on phosphatidylserine (PS) which is a component of the cells' plasma and EV-membrane. In living cells, PS is localized in the inner membrane leaflet and only detectable in the outer membrane leaflet when cells become apoptotic. Despite the observation of Alain Brisson's group that only a small proportion of plasma EVs can be labelled by Annexin V, it is broadly assumed that EVs contain PS in their outer membrane leaflet [183]. In addition to Annexin V, the T-cell immunoglobulin domain and mucin domain-containing protein 4 (Tim4) binds to PS in a Ca^{2+}-dependent manner on apoptotic cells and on EVs [184,185]. Upon using Tim4-Fc fusion protein-loaded magnetic beads for the small scale preparation, PS^{+} EVs that can easily be eluted by EDTA containing elution buffers, have been captured, purified and applied to molecular analyses [185]. Functional analyses had not been performed. Meanwhile required components are sold by Fujifilm (labchem-wako.fujifilm.com/us/category/01118.html). Due to the Ca^{2+}-dependent binding of EVs to the stationary phase, the system provides several advantages. Provided sufficient amounts of Tim4-FC fusion proteins can be produced with reasonable costs, the system appears scalable. However, it remains controversy whether therapeutically active EVs contain PS on their outer membrane leaflet or whether like living cells functional EVs retain PS just on the inner membrane leaflet.

There might be other concepts for AC which we have missed to discuss. According to the current state of the technology, some of the AC technologies appear scalable. However, we are not aware that any of the principles has been indeed used for the scaled EV purification. Until now, high costs for production of AC stationary phases and challenges in the elution of bound EVs limit the applicability of AC for the scaled EV production.

11. Size exclusion chromatography (SEC)

In SEC, also deciphered as gel filtration, the stationary phase is a porous material that allows smaller but not larger compounds of the feed to enter the porous system. While the liquid and compounds that are larger than the pores pass the material, pore-entering compounds are retarded and are eluted in a delayed man-

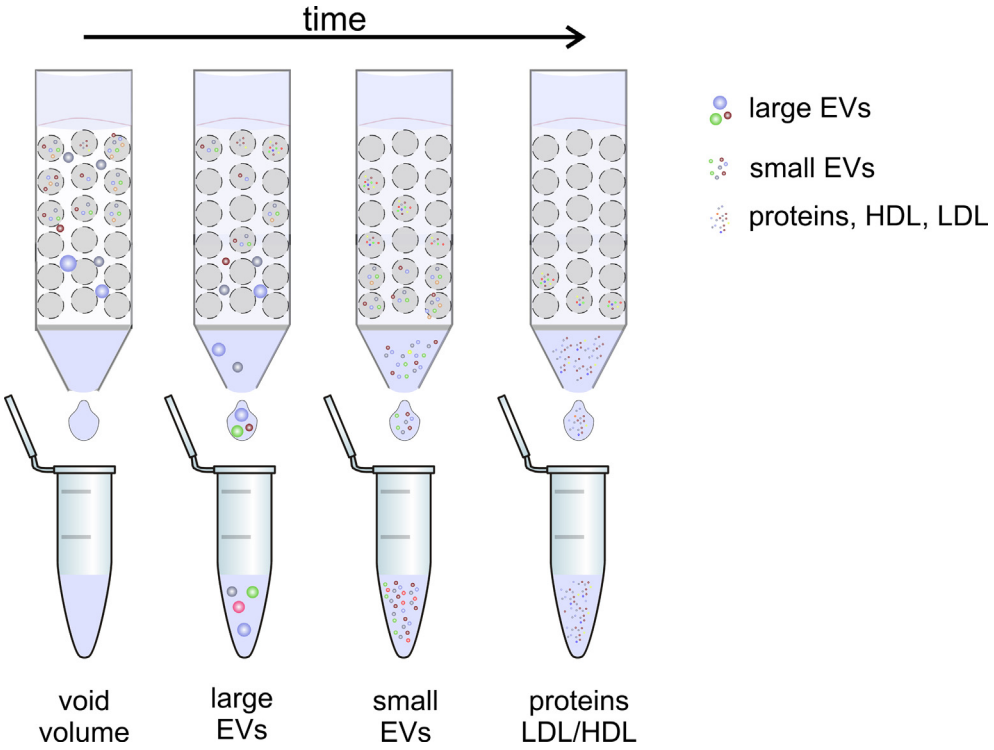

Fig. 6. Principle of size exclusion chromatography: The feed is applied to the columns filled with a porous stationary phase. While smaller compounds enter the pores and are retarded in their flow, larger components pass the stationary phase without being retarded and thus are eluted before pore entering compounds.

ner (Fig. 6) [186]. At first, SEC was used for separation of proteins in 1959 [187]. Since then, SEC with organic polymers or inorganic silica matrices has been used for protein, antibody and virus purification [188–192]. Most frequently agarose-based gel filtration matrices are used, commonly Sepharose with exclusion limits ranging from 7.7 nm up to 400 nm [75,193,194]

SEC is traditionally used for the purification of viruses. While it allows very effective purification it is not a volume reducing method and rather dilutes obtained samples. Accordingly, SEC is commonly used as a polishing operation for volume-reduced compound preparations with insufficient purities. Thus, if implemented in the DSP procedure it is a rather late unit operation [30,110]. For example, a combination of ultracentrifugation with SEC has been used for the preparation retroviral particles for gene therapeutic approaches with a recovery rate of 19% and an estimated purity of 90% [192]. Depending on the DSP strategy, volume reducing operations can also be implemented following SEC, e.g. ultrafiltration, a specific dead-end filtration type [192].

Since 2014, for the preparation of viruses and larger biomolecules a novel SEC type is used, the Capto™ Core. It is a new agarose bead-based flow through resin available with different pore sizes (Capto™ Core 400 and Capto™ Core 700), whose beads contain an inert shell surface and an adsorbing core with octylamine ligands that bind and retentate core entering molecules [195–197]. It has originally been used for the purification of egg-based influenza virus and is increasingly implemented in viral purification procedures with reports that it helps to purify viruses as efficient as Cesium Chloride density centrifugation [195,198].

In the EV-field, SEC has been used for the EV preparation right from the beginning [49,199–202]. Upon describing a one-step method for the preparation of EVs from primary body liquids, SEC-based EV preparation methods became more popular within the last few years [203]. It has been reported that compared to UC EV preparations, SEC EV preparations reveal a higher EV yields and improved EV integrities [204]. Coupled to this, the ability in inducing extracellular-signal regulated kinases (ERK) signaling in endothelial cells of SEC prepared cardiomyocyte progenitor cell-derived EVs was increased in comparison to UC prepared EVs of the same CM [205].

SEC has been also considered for scaled EV production processes [206–208]. Following TFF, we for example have used Capto™ Core-based SEC for the purification of EV products with yields of 70–80% and purities comparable to UC [206]. Very recently, a protocol has been published combining ultrafiltration and SEC using Superose 6 prepacked XK 26/70 SEC columns. EVs from 500 mL UF clarified CM of pooled umbilical cord blood-derived mononuclear cells were SEC purified and their functional activities approved in a wound healing assay [207]. Although these results qualify SEC as a promising unit operation for the scaled EV production, we are not aware of any report in which SEC has been implemented in an EV preparation strategy that starts with several liters of conditioned media as it would be required for the systemic treatment of a given patient (Box 2). Thus, it remains a challenge to further scale these processes. In this context it is worth to mention, based on the same principle as for Capto™ Core, novel materials are developed that might further improve purification capabilities of SEC-based methods in the future [209].

12. Method dependent challenges and future perspectives

Most of the described EV preparation methods in principle can be performed under current GMP compliant conditions. As discussed, a critical factor for the handling of any EV preparation strategy is the volume reducing operation. Traditionally, the volume reduction for the preparation of EVs from EV containing liquids was performed by ultracentrifugation. Here, the maximal processing volume is below 500 mL per run and therefore far from the need for the production of EV-based therapeutics. Polymer precipitation such as PEG precipitation procedures allow volume reduction at low *g*-force centrifugation and coupled to the fact that low-speed centrifuges can process much higher volumes than ultracentrifuges, polymer precipitation methods became an option for the production of EV-based therapeutics [63]. Although we are preparing MSC-EVs with an optimized PEG protocol, and applied obtained MSC-EVs successfully to a patient and various animal models [4,42,46,103–108], we are critical whether centrifugation-based methods, although being scalable into industrial level e.g. disc stack centrifuges, are the best option. Here, TFF and AEX appear as promising methods, as both unit operations proceed large volumes fast and cost effective in industrial levels. SEC is a polishing rather than a volume reducing operation and affinity chromatography even scalable might be too expensive (Fig. 7).

Although several reports have successfully used TFF and AEX techniques at small scale, scaling to industrial levels is challenged by several factors, most rely on upstream preparation methods. Challenges connected to the media, especially the protein content of the original media and the presence of free-nucleic acids in CM have been discussed. We think that these issues will be solved in the near future. Beside donor variability on general, other critical components are batch-to-batch consistencies that depend on the

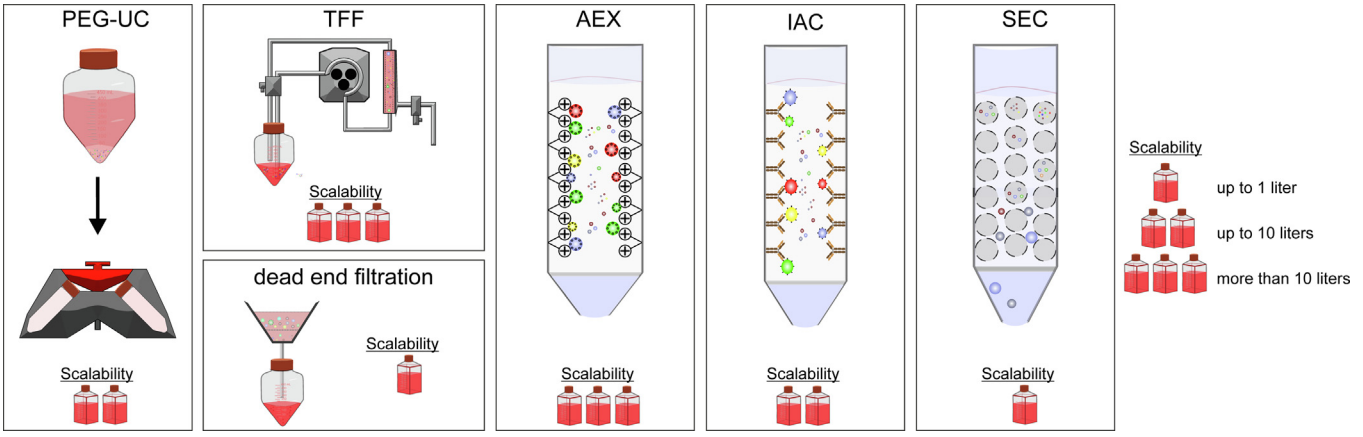

Fig. 7. Scalability assessment: The scalability of a method is represented symbolically by one flask for the lowest and three flasks for the highest scalability. SEC and dead-end filtration are considered small scale, PEG-UC and IAC intermediate and TFF as well as IEX for high scalability.

biology of the EV producing cells, e.g. cell aging and stochastic processes during their expansion. Such biological parameters may significantly affect the product quality. According to our understanding robust processes for primary cells are hard to achieve. In this context some of the experiences from the MSC field are worth to be discussed.

Commercially produced MSCs have been applied to aGvHD patients in different phase III clinical trials: while an early clinical trial failed to show efficacy [210,211], a later one-armed clinical trial on pediatric aGvHD patients showed positive effects of the MSC therapy [212,213]. Despite the positive results of the later trial, the FDA did not approve the respective MSC product, remestemcel-L, for the treatment of pediatric aGvHD patients in the United States of America. According to their understanding, the potency of the product, which derives from bone marrow of varying donors, is not appropriately tested for its potency und thus, impacts of product heterogeneities not sufficiently controlled (https://www.fda.gov/media/140988/download). It is an important goal for cell therapeutic manufactures to control such processes with a tight and automated process strategy that can effectively react on different cellular properties (e.g. different growth rates) during the process. However, for EVs it might be easier to use immortalized producer cells; while it is critical to administer immortalized cells to patients, EV of immortalized cells appear less critical; in contrast to the cells, EV lack endogenous expansion potentials. Thus, product variabilities might be reduced to a minimum, if immortalized cells are used for the EV production as long as they fulfil the required product characteristics, i.e. their metric parameters [10], and contain the expected potency. However, potency testing provides its own challenges that we recently have comprehensively discussed in a white paper [11].

For EVs prepared from primary MSCs and as tested in ischemic stroke and GvHD mouse models as well as in a multi-donor mixed lymphocyte reaction assay, we indeed observed inter-donor as well as batch-to-batch variations of EV products derived from the same donor MSC stock [42,104]. For now, we lack any information of how critical functional batch-to-batch variations are for EV products manufactured from CM of immortalized cells. Expecting that the risk can be reduced but not mitigated, it is highly recommended to really understand the process, determine the critical process parameters and include sufficient *in process* controls in the USP and DSP processing to become able to identify any variations in the production procedure as early as possible and not just at the end of the process. Of course, any kind of industrial EV production must be free of mycoplasma, viral, bacterial, or fungal contaminations. We see the whole manufacturing as an interplay where all steps should go hand in hand: USP procedures influence DSP procedures, so both should be developed in parallel; valid process analytics are needed to identify critical process parameters etc. For sure there will not be one unique process for EVs, but as better we understand the EV manufacturing, we will find common principles which will allow the establishment of robust platform technologies for EV USP and DSP.

13. Conclusion

EVs provide many novel therapeutic opportunities. However, aside of optimizing USP and DSP technologies there are many more challenges to address for successfully translating EVs into the clinics [2]. We can profit immensely from the knowledge that has been collected for virus production processes, especially for that of enveloped viruses, which share several features with EVs. Here, we have introduced several promising DSP technologies that might be used as unit operations for the production of EV-based therapeutics. Aside many critical aspects one needs to keep in mind, *the process defines/is the product* that is, any process parameter being altered may affect the metrics and the potency of the EV product [9,10,214]. Therefore, it is always key to confirm the product's potency in appropriate read outs before selecting a method for the production of EV therapeutics [11].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Disclosures

BG is a scientific advisory board member of Innovex Therapeutics SL and Mursla Ltd. and a founding director of Exosla Ltd.

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