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Review

Steric Exclusion Chromatography for Purification of Biomolecules—A Review

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Abstract: Steric exclusion chromatography (SXC) is a purification method that is based on steric exclusion effects from the surface of the target and a hydrophilic stationary phase after the addition of polyethylene glycol (PEG), which leads to an association of the target with the stationary phase without direct binding, such as covalent, electrostatic, and hydrophilic/hydrophobic interactions. The gentle nature of the method has led to an increased focus on sensitive targets such as enveloped viruses with potential for other sensitive entities, e.g., extracellular vesicles and virus-like particles. SXC is related to PEG-mediated protein precipitation, but investigation of further process parameters was crucial to gain a better understanding of the SXC method. After explaining mechanistic fundamentals and their discovery, this review summarizes the findings on SXC from its first reference 11 years ago until today. Different applications of SXC are presented, demonstrating that the method can be used for a wide variety of targets and achieves high recovery rates and impurity removal. Further, critical process parameters for successful process implementation are discussed, including technical requirements, buffer composition, and scalability.

Keywords: steric exclusion chromatography; polyethylene glycol; purification; downstream processing

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1. Introduction

The term "steric exclusion chromatography" (SXC) was introduced by Lee et al. in 2012 [1]. SXC does not refer to size-exclusion chromatography (SEC). This is often subject to confusion, as sometimes the term "steric exclusion" is used as a synonym for size exclusion. SXC is a purification method that has been used to purify a variety of biomolecules. The capture of the target is achieved by the addition of a polyethylene glycol (PEG)-containing buffer to the feed solution and loading the mixture on a hydrophilic stationary phase. The capture mechanism is based on the steric exclusion of PEG from the surface of the target [2]. Before delving deeper into the theoretical fundamentals of the method, let us first take a step back to understand what laid the foundation for the development of SXC.

Precipitation effects were observed in the 1940s, e.g., Cohen et al. observed precipitation of virus after the addition of the polysaccharide heparin, but were still unaware of the underlying principle [3]. The precipitation of proteins was reported in 1964 by Polson et al., reporting that the efficiency of protein precipitation increases with increased molecular weight of the polymer. However, at this stage, no explanation of the precipitating effect could be given [4]. The underlying theory of precipitation by steric exclusion effects of a polymer (dextran, PEG, hyaluronic acid) from a colloid was later investigated by Ogston, Laurent, and colleagues [5–9]. Different polymers have been used in steric exclusion/precipitation studies, for instance, heparin [3], dextran [6], hyaluronic acid [5], or polyethylene glycol [10]. A little bit earlier, Asakura and Oosawa described for the first

time attractive interaction between particles in a solution containing non-adsorbing polymers [11]. This theory, later investigated by Vrij [12], is named depletion interaction and is another fundamental aspect to understand the mechanism of SXC, which is explained in more detail in Section 2. Holistic studies on steric exclusion-mediated precipitation were published by Timasheff and Arakawa in the 1980s [13,14]. On this basis, using PEG as a precipitation agent is a well-known and established method in laboratory applications that offers easy and fast protein purification [15].

A timeline of fundamental theoretical contributions and the development of SXC is depicted in Figure 1.

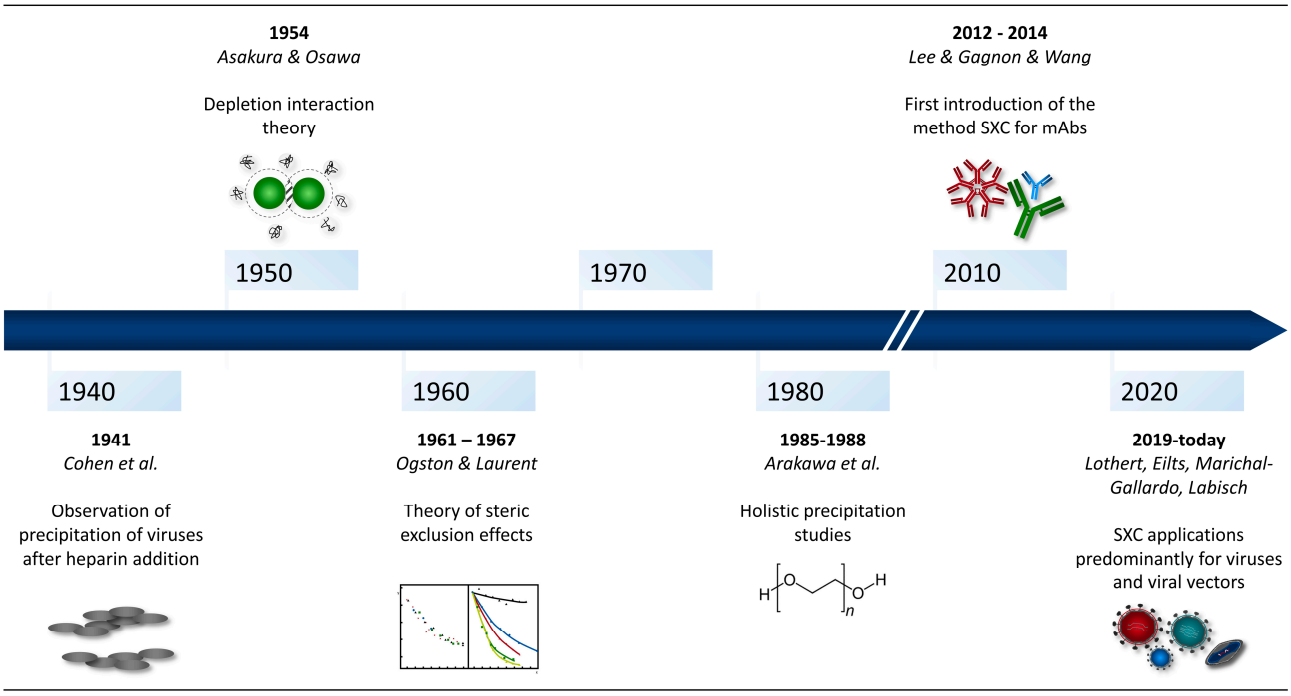

Figure 1. Timeline of the development of steric exclusion chromatography, starting from studies on theoretical principles to the first application and current applications of SXC.

2. Method Fundamentals

The name of the SXC method originates from the steric exclusion effect of non-adsorbing polymers from colloids in a liquid solution that leads to an association of the colloids with a hydrophilic surface that serves as a stationary phase [1]. The term "colloid" is used here as a general expression for all targets purified with this method. The steric exclusion effect described in protein precipitation studies and the attractive force between colloids and the stationary phase in a polymer solution (depletion interaction) are the fundamentals of this method. The mechanism of SXC is shown in Figure 2. In all SXC studies, the polymer PEG was used, but other non-adsorbing polymers resulting in the same effect may be used.

SXC is divided into four steps, as with other classical bind-and-elute chromatography methods: equilibration, loading, wash, and elution. A hydrophilic stationary phase (for further details, see Section 3.1.2) is equilibrated with a PEG-containing buffer. Afterward, the stationary phase is loaded with the colloid-containing feed solution, which is mixed with a PEG buffer, either offline or online (Section 3.2.2). Subsequently, the stationary phase is washed under the same conditions as during the equilibration step, and finally the colloids are eluted with a buffer without PEG [1,16]. An example of an SX chromatogram is shown in Figure 3.

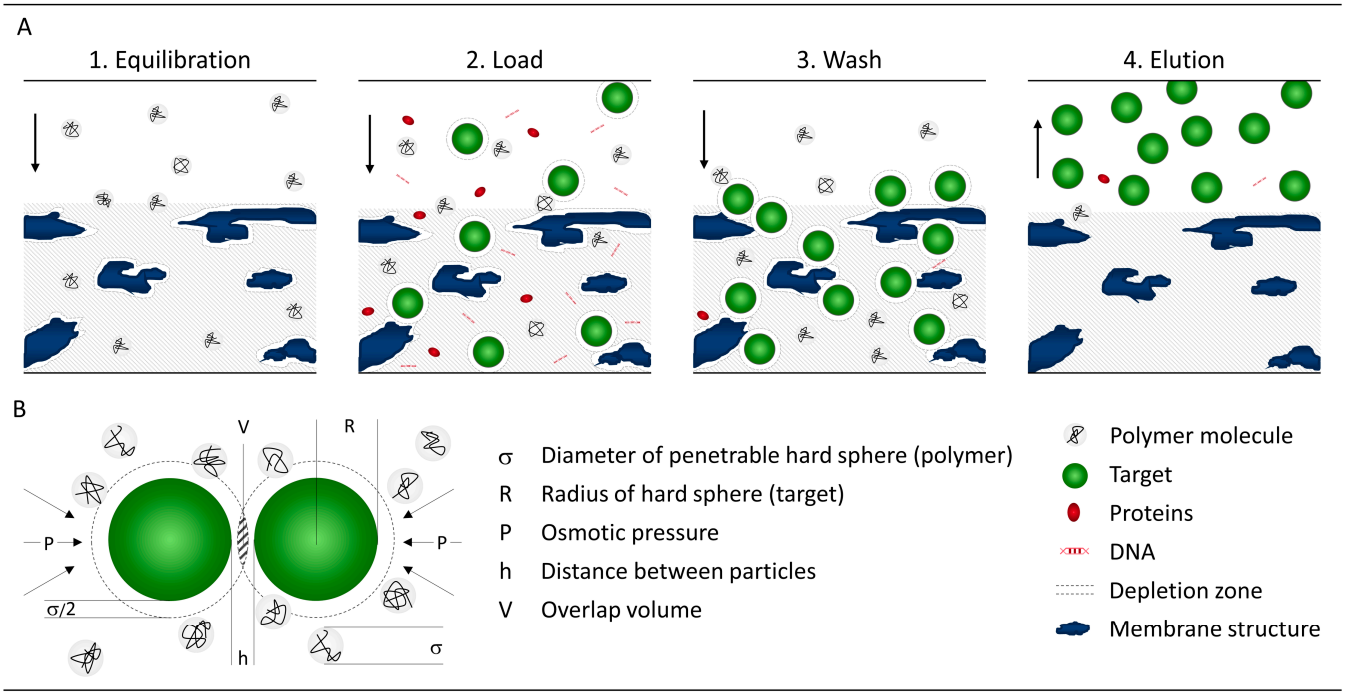

Figure 2. Principles of SXC. (**A**) Steps of SXC: equilibration of stationary phase with PEG-containing buffer. Next, the feed solution mixed with PEG buffer is loaded and the association of the target with the stationary phase occurs. A wash step with a PEG-containing buffer follows to wash out the remaining impurities before final elution without PEG. (**B**) Depletion interaction between two hard spheres in the presence of PEG. Arrows indicate flow direction. Figure adapted from [16,17].

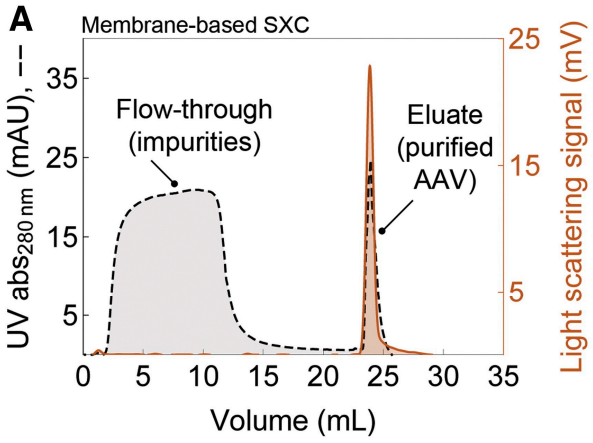

Figure 3. Example chromatogram of SXC purification of AAV2 with a liquid chromatography system comprising UV and light-scattering signal. Light-scattering signal detects AAV particles. Figure extracted from [18].

When PEG is added to a colloid-containing solution, a zone around the colloid is formed that contains a lower PEG concentration than the bulk solution. This zone is called the depletion zone or PEG-deficient zone. The depletion zones are also formed at the stationary phase. The formation of these PEG-deficient zones is based on the steric exclusion of the polymer molecules from the surface of the colloids (or stationary phase), because the zone is not accessible to the center of gravity of the polymers, which are arranged in a random coil structure and can be seen as penetrable hard spheres [17]. According to [14], the depletion zone was reported to have approximately a thickness of the radius of the penetrable hard sphere and contains an excess of water molecules. The steric exclusion of

the PEG molecules from the colloid's surface leads to a loss of conformational entropy of the polymer chains, which in turn creates a thermodynamically unfavorable increase in free energy [2,17]. The excess in free energy promotes the physical reorganization of the viral vectors in order to lower the free energy of the system. During loading, random encounters of a colloid and the stationary phase or of a colloid with another colloid occur. When a colloid is close (distance < PEG molecule diameter) to another colloid or the stationary phase, PEG cannot penetrate the gap. This creates negative osmotic pressure, resulting in weak attraction. Moreover, when the depletion zones overlap, the excluded volume is reduced, and excess water is transferred from the depletion zone to the bulk solvent. By the transfer of water to the bulk solvent, the PEG concentration is reduced, and PEG molecules can occupy a larger volume. The free energy of the system is reduced and thus stabilizes the association of the colloids with the stationary phase or with one another [17]. The capture mechanism does therefore not include direct chemical binding [1] and can be described rather as a thermodynamically driven reversible association of the colloids and the stationary phase. For dilute polymer solutions, the depletion potential depends on the polymer concentration and molecular weight [19]. The selective purification of the colloids is achieved by the size difference between the colloids and the impurities. PEG is preferentially excluded from large colloids over small colloids. It was observed that large targets precipitate at smaller PEG sizes and lower final PEG concentrations, while small targets require higher concentrations of larger PEGs [6,7,20]. Hence, SXC is to some extent a size-specific method for a certain colloid size, and the ideal PEG buffer composition in terms of PEG concentration and molecular weight depends on the size of the colloid to be purified. The association is simply reserved by applying a PEG-free buffer during elution. The success of the process seems to depend mainly on the PEG size and concentration, as well as other buffer compositions as described in more detail in Section 3.2. In addition, flow velocity plays a crucial role, as will be discussed in more detail in Section 3.3.

The SXC method should be clearly differentiated from other chromatography methods, such as size-exclusion chromatography (SEC) and hydrophilic interaction chromatography (HILIC). SEC separates molecules based on their retention time, thus size, in a porous bead matrix without any association/binding of the target to the stationary phase. HILIC, similarly to SXC, uses a hydrophilic stationary phase, but for HILIC organic solvents are used in the mobile phase to promote interaction of hydrophilic targets with hydrophilic stationary phases separating molecules based on their hydrophilicity. In contrast, for SXC, PEG is used, which is a hydrophilic polymer that leads to the steric exclusion of large colloids and the stationary phase from its center of gravity and a capture mechanism based on depletion interaction, as described above.

3. Process Parameters and Optimization of SXC

3.1. Technical Requirements

3.1.1. Equipment

Technical requirements for SXC are similar to other chromatography techniques. A controlled flow rate is required with several buffer inlets and preferentially a dynamic mixer located downstream of the pumps providing mixing of two buffers (PEG buffer with a non-PEG buffer of choice or PEG buffer with feed solution containing the target to be purified). Alternatively, a loop for loading (applies only for a small scale) can be used. The importance of buffer mixing is discussed in more detail in Section 3.2.2. Additionally, pressure sensors are required, and depending on the target, a UV detector or multiangle light-scattering device could be useful to monitor target product capture and elution. In the published studies, commercially available chromatography systems providing these technical requirements have been used, except for one study that used starch-coated magnetic nanoparticles as a surface for antibody capture mixed with PEG and collected the particle-associated antibody in a magnetic field [21]. Under certain circumstances, it may not be necessary to use a chromatography device if the sample is mixed externally with

PEG and loaded manually onto a stationary phase. However, the flow rate plays a decisive role, as discussed in more detail in Section 3.3, and can be better regulated with a pump.

3.1.2. Stationary Phases

When it comes to the selection of stationary phases, there are several possibilities. SXC is not restricted to a certain chromatography media, but any hydrophilic porous material may serve as a stationary phase. The stationary phases used for SXC are mainly monoliths and membranes, but various materials of these stationary phases are conceivable. Stationary phases and their characteristics are listed in Table 1.

When analyzing the stationary phases for SXC, it can be observed that cellulose membranes were predominantly used, either in a stabilized or non-stabilized form. The membranes from Cytiva and Sartorius were punched and either assembled manually into appropriate stainless-steel housings [18,22–29] or overmolded with an injection molding machine [16]. Ready-to-use membrane modules for SXC are commercially available from ContiVir offering CaptuVir™ modules [30]. However, the modules have not yet been used in any published study, which makes it difficult to evaluate their performance. In addition to that, to date, there is no product portfolio of cellulose membrane modules available offering a whole scale-up concept for SXC, which currently limits the implementation of the method, but this may change in future when such a product portfolio becomes available.

Table 1. Stationary phases used for SXC.

Stationary Phase	Size	Pore Size	Supplier	Reference
CIMmultus® OH monolith	0.34 mL, 1 mL [1]	1.3 µm [2], 2 µm	Sartorius BIA Separations	[1,22,31]
Polyacrylamide monolith	0.83 mL [3]	10–100 µm	Self-fabricated	[21]
Whatman® regenerated cellulose membrane filters	13 mm or 25 mm diameter, 10; 15, or 20 layers	1 µm	Cytiva	[18,22–28]
Hydrosart® stabilized cellulose membrane	13 mm diameter with 10 or 15 layers; 25 mm diameter with 5 layers	3–5 µm	Sartorius	[16,25,29]
Glass fiber filters	13 mm diameter, 15 layers	1.6 µm	VWR	[29]
Ultipor® Nylon polyamide membrane	13 mm diameter, 15 layers	5 µm	Pall	[29]
Starch-coated magnetic nanoparticles	-	-	Chemicell	[2]

[1] Column volumes available up to 8000 mL; [2] 1.3 µm pore size is available for a column volume of up to 80 mL, and a 6 µm pore size is also available; [3] calculated based on the Methods section.

Monoliths are also used as a stationary phase. Commercially available monoliths are from Sartorius BIA Separation, which indicate on the product website the use of the CIMmultus OH monolith for the capture of large solutes in the presence of polyethylene glycol [32]. These monolith modules are available for different process scales up to a column volume of 8 L, offering a potential scale-up solution. When taking the publishing date of the SXC studies into account, a pronounced trend can be noticed from the use of monoliths (in four out of five studies between 2012 and 2018) towards the use of membranes (in nine out of nine publications between 2019 and 2022) for SXC applications.

The use of membranes and monoliths can be explained by the size of the target that is purified by SXC. Especially in recent publications, large viruses were the main target (Section 4). This also implicates the preferred use of membranes and monoliths, as these chromatography matrices have large pores and the mass transport is based on convection [33–35], whereas mass transport is diffusion-based with resins [36,37]. Moreover, the pores of resins are fairly small and therefore not suited for large targets, such as enveloped viruses [38,39]. Even for small targets, resins might work less efficiently, since the viscosity of the PEG buffers results in reduced diffusive transport according to the Stokes–Einstein relation [40]. Stationary phases with large pores offer higher permeability

and lower back pressure [33,34]. This is advantageous when applying viscous PEG buffers to avoid reaching the pressure limit of the devices.

3.2. Buffers

3.2.1. PEG Size and Concentration

Polymer concentration and chain length are frequently discussed factors in the optimization of an SXC process. As briefly mentioned in Section 2, the hydrodynamic radius of a polymer molecule (referred to as a penetrable hard sphere) has a decisive influence on the association of a target with the stationary phase (Figure 2). Sin et al. confirmed the importance of the hydrodynamic radius as an influencing variable by comparing branched-chain and linear PEG molecules in protein precipitation applications. The branching lowered the yield of the precipitation compared to linear molecules, unless there were similar hydrodynamic radii [15]. However, in SXC studies the chain length or molecular weight (in Daltons) is often used to compare different PEG sizes. To date, only linear polyethylene glycol has been used as a polymer for SXC. PEG is an inert, non-adsorbing, and hydrophilic polymer, which makes it suitable for pharmaceutical and biological applications [41]. Due to an inversely proportional effect of polymer hydrophobicity on the effectiveness of steric exclusion, higher-order molecules such as polypropylene glycol would be less effective for SXC [1]. Back in 1978, Miekka and Ingham investigated precipitation using PEG and found that the higher the polymer concentration, the more efficient the precipitation effect [42]. More recent studies show that this effect in the form of better retention is also observed with SXC [16,25,29]. An increasing concentration of polymer leads to increased osmotic pressure, which favors the association of target with the stationary phase [1,2,16,29,31]. An upper limit of this effect exists due to the concentration-related compression of polymer molecules, which leads to smaller hydrodynamic radii [25]. This is supported by the assumption that steric exclusion for chromatography requires the presence of a dilute polymer solution [16], because in a semi-dilute polymer concentration regime, the depletion attraction is no longer dependent on the polymer size and the strength of the interaction is a decreasing function of the concentration [17].

PEG concentrations from 2% to 22% were tested with a variety of targets for chromatographic application (refereeing to the final applied concentration) [2,28]. It is noticeable that due to the increase of the steric exclusion effect, the impurity removal capacity of the method decreases at higher polymer concentrations [29,43]. Sufficient DNA digestion is therefore crucial, as the removal of impurities is based on the size difference between the target and the impurities and small impurities are expected to be removed more efficiently [16]. These influences arise since the selectivity of the method correlates with the molecular weight of the target, retaining larger molecules more strongly [1]. Large targets precipitate at smaller PEGs and lower final PEG concentrations, while small targets require higher concentrations of larger PEGs [44]. This behavior observed for protein precipitation might apply as well for SXC and gives an indication for the selection of PEG size and concentration. In other words, the hydrodynamic radius of the target determines the optimal polymer concentrations and molecular weight.

As a consequence, different process optima have been presented. For instance, Levanova and Poranen found that for the purification of RNA molecules using CIM monoliths, PEG6000 in a final concentration above 7% is favorable [31]. Purification of lentiviral vectors was optimized to 12.5% PEG4000 on regenerated cellulose membranes [16]. An influenza A virus defective interfering particle was purified at 8% PEG6000 [27]. In general, PEG with a molecular weight of 6000 to 8000 Da was predominantly used [1,2,18,21,22,24,26,27,29,31], although an overall range of molecular weight of 2000 to 1200 Da and even mixtures of different molecular weights were tested [1,16,21,29]. PEG with a higher molecular weight retains the targets more efficiently than low-molecular-weight PEG of the same concentration [1]. However, shorter PEG molecules should be favored, as they offer a larger concentration-dependent optimization window, thus making it easier to find the retention maximum and impurity minimum [22]. It can be said that the yield increases with increas-

ing PEG concentration and molecular weight, whereby the concentration-dependent effect was reported to dominate the effect [25]. As a rule, the chain length of the polymer determines the range of depletion interaction and the polymer concentration its strength [17,45]. Both variables—polymer size and concentration—influence the viscosity of the capture buffer, which increases with increasing PEG size or increasing PEG concentration [46]. Therefore, pressure is a critical aspect to be monitored to avoid exceeding the operating pressure of the chromatographic system or the modules [1,16]. Concentrated PEG stock solutions are prepared, which are diluted to the final concentration through mixing in the chromatography system. Related methods are described in the following section.

3.2.2. Mixing Approaches of PEG with the Feed Solution

When loading the feed solution of the target onto the stationary phase, there are two mixing strategies of the PEG buffer and the feed solution that have been applied. One involves mixing the feed solution with PEG buffer inside the chromatography system, meaning that two solutions are pumped simultaneously into the system via two pumps, and the solution is mixed inline via a mixer, preferably a dynamic mixer to ensure an adequate mixture of the viscous PEG-buffer with an aqueous feed solution. A static mixer is likely to be insufficient to mix the viscous solution. This mixing method is often referred to as inline or internal mixing. External or offline mixing refers to mixing the PEG buffer with the feed solution in a beaker or bottle, e.g., with a magnetic stir bar, and then loading the ready-mixed solution. Different mixing ratios of the feed solution with the PEG buffer are possible to reach the desired PEG working concentration, depending on the stock concentration of the PEG buffer. Often a 1:1 or 1:3 mixing ratio (corresponding to a dilution of $^1/_2$ or $^1/_4$) is applied.

In the literature, both approaches have been performed with varying outcomes. The use of inline vs. offline mixing throughout the SXC publications is more or less balanced. Some studies performed inline mixing on purpose and stated this an advantageous process setting, but without showing comparative data of offline mixing. In these publications, the application of a dynamic inline mixer was anticipated to prevent precipitation of the target product in the lines of the chromatography system before reaching the column. Inline mixing shortly before the column would then encourage the interaction of the target with the stationary phase, rather than with one another [1,18,31]. On the other hand, offline mixing and loading of the premixed solution via a loop worked well without any process impairments, such as low product recovery or pressure issues, in several studies [22–24,26,28,29,47].

The statement that inline mixing works better is thus in contrast to studies in which offline mixing also worked well and enabled high product recovery. Comparative studies were carried out by Labisch et al. and Eilts et al., and the two approaches were different. The results are interesting because in the first study, it was shown that inline mixing led to a significantly higher product recovery (40% in difference), in this case a lentiviral vector compared with applying an offline-mixed solution that was mixed shortly before loading [16]. The second study compared loading an inline mixed Orf vector solution with an offline mixed solution that was incubated for 12 h. The offline mixed and incubated sample resulted in a 25% decline in product recovery in the elution fraction [25]. Such a long incubation time is very unlikely to be performed in an efficient purification process; however, it demonstrates the same trend. One common observation can be made with these two comparative studies. The target product was not found in any other fraction (flow through or wash fraction).

Aggregation or precipitations effects are often discussed as possible reasons for a preferred inline mixing strategy. Incubation of the target Orf with PEG buffer and time-dependent size monitoring revealed that the aggregate size increased over time for 60 min and an increase in PEG concentration or molecular weight resulted in an increased aggregation size [25]. The size increase over 60 min was for instance approximately from

900 nm to 1500 nm with 6% PEG 6000. Thus, the size roughly doubled, which is not a huge aggregation or precipitation effect.

When the theoretical basis of the method is considered, the preferred inline mixing seems reasonable. The free energy of the system that is present upon the addition of the polymer to the feed solution, as explained in Section 2, can be reduced by the association of the target with the stationary phase or with one another. When no stationary phase is available (during offline mixing), the targets will likely aggregate and an association with the membrane later on is less likely, since the driving force of the molecule association has already been reduced [16,18], or as discussed elsewhere, it could have led to filtration effects of large aggregates on the membrane, making elution difficult [25]. The reversible formation of γ-globulin precipitates on the stationary phase was observed by Wang et al. by electron microscopy [21]. The reversible association with the stationary phase probably also depends on the physicochemical properties of the target. Strong charge attraction or van der Waals forces of very large aggregate complexes could be too strong and make dissolution during elution difficult. This could explain why the mass balance could often not be closed. In summary, both mixing strategies are in general feasible for SXC, but depending on the target, either mixing strategy might not work. Inline mixing also has the advantage that sample conditioning is not required [18] and the time from mixing until reaching the column can be controlled better, eliminating variances that could hamper the success of the purification step [28].

3.2.3. Buffer Systems and Influence of Salt

Besides the PEG molecular weight and concentration, the buffer composition affects the retention behavior, and buffer screening might be necessary. Generally, one would start with a buffer composition known to be well suited for the target in terms of preserving the target's stability. For example, Eilts et al. tested the buffer systems citrate phosphate buffer (CPB), phosphate-buffered saline (PBS), tris(hydroxymethyl)aminomethane (TRIS), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and observed that CPB was less suited than the other buffer systems, as SXC recovery was lower due to product loss during the loading and wash step [25]. In other publications, mainly TRIS [2,16,22–24,26,31,47] or PBS-based buffer systems [18,27,29] were used.

$$\begin{array}{c} SO_4^{2-} < HPO_4^{2-} < C_2H_3O_2^- < Cl^- < Br^- < NO_3^- < ClO_3^- < I^- < ClO_4^- < SCN^- \\ \xleftarrow[Kosmotropic]{} \qquad \xrightarrow{Chaotropic} \\ NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+} \end{array} \quad (1)$$

Salts decrease the retention of the target on the stationary phase. The retention decreases as the salt concentration is increased and according to the Hofmeister ranking [1], (Equation (1)) [48]. This effect occurs because salts compact PEG [49]. A compacted PEG molecule, considered to be a penetrable hard sphere, generates a thinner depletion zone since the steric exclusion effect is less pronounced as the thickness of the depletion zone is in dilute polymer solutions half the diameter of the penetrable hard sphere [17]. This leads to a reduced depletion attraction that can be compensated by increasing the PEG concentration [2]. According to the Hofmeister series, more kosmotropic salts decrease the retention more strongly, and thus a higher amount of NaCl was necessary to achieve the same reduction in retention of IgM compared with $(NH_4)_2SO_4$ [1]. On the other hand, another study could not reproduce this data with Orf and achieved an increased yield with Na_2SO_4 compared with NaCl, while the best recovery was obtained with $MgCl_2$ [25]. Almost all other SXC studies used NaCl as a salt additive with a concentration ranging between 150 and 200 mM [2,16,22–24,26,47]. Another interesting aspect is that a salt concentration in the loading buffer of 200 mM NaCl for Orf [25] and 400 mM for nucleic acids [31] increased product recovery compared with lower NaCl concentrations in the loading buffer, which can be explained by a reduced charge repulsion of the targets allowing for improved capture of the target on the stationary phase. A certain conductivity in the

loading buffer is therefore beneficial, yet the stability of the target with respect to salts must be considered to avoid degradation of the product.

The pH of SXC buffers was in general between 6 and 8 [1,2,21,31], with most of the protocols purifying virus targets applying pH 7.4 [16,22,24]. Surprisingly, no virus retention was achieved when performing SXC with a hepatitis C virus (HCV) feed solution at pH 7.4. Moreover, at pH 8, 10, and 11, huge virus breakthroughs were observed and only a narrow pH optimum at pH 9 $\pm$ 0.5 was identified. The difference in pH optimum can be explained by different isoelectric points (pI) of the targets [26]. The pI of HCV is expected to be alkaline, whereas the pI of most other viruses is acidic. For protein precipitation studies with PEG, a pH near the pI was reported to require a lower PEG concentration [50]. The same effect was reported for IgM and bacteriophages during SXC: the retention increased near the pI and a lower PEG concentration was required [1]. The reason is that the target is minimally self-repellent at its pI and favors self-association through steric exclusion by PEG [2]. Thus, the depletion interaction of the targets with one another or with the stationary phase can be changed at different pH due to resulting attraction or repulsion depending on the charges of the target and the stationary phase. In this way, retention of the targets can be promoted or weakened. As typically uncharged stationary phases are used, the described effect would apply between targets, as the depletion interaction can also apply between several particles that form multilayers, as discussed in more detail in Section 3.3. Hence, a pH that is not too far from the pI of the target and at the same time preserves its stability should be selected.

3.3. Scalability

SXC has been applied for purification for a variety of biomolecules, as described in Section 4. To date, SXC has not been applied in large-scale productions of biopharmaceuticals. The transfer from a small-scale laboratory method to a large-scale purification method involves some hurdles. First, as mentioned in Section 3.1.2., there is a limited availability of commercially available stationary phases through all process scales, which is currently limited to monoliths. A scalable membrane module portfolio could further promote the development of downstream processes based on this method, as the customer could purchase modules for larger scales after successful implementation and testing at a small scale and once the target product receives market approval and is produced in large batches. Second, the skepticism towards new methods leads to the preferred application of established methods accepted by the regulatory authorities. The first scale-up approach of SXC was reported by Lothert for the purification of 200 L Orf with monoliths, but recoveries of SXC runs showed great variations with limited reproducibility [51]. The scale-up difficulties in this case likely stem from a rapid transfer of a small-scale optimized protocol to a very large scale without knowing the critical process parameters for scaling up and whether SXC scales just like other chromatography methods.

A holistic study on SXC scaling up was recently published by our team [43] using membrane modules with regenerated stabilized cellulose membranes to purify lentiviral vectors. Two key aspects were demonstrated. First, it was observed that the stained target was mainly captured on the first membrane layer and likely formed multiple layers of captured target on the first layer (Figure 4). Hence, no capture in the depth of the stationary phase occurred, which is distinct from classical chromatography methods.

Therefore, increasing the membrane volume without increasing the membrane surface area of the first layer would not result in increased capacity of the module. The second key aspect describes the need to scale the flow rate with respect to the incident flow area of the first membrane layer, which is in contrast to scaling the flow rate in relation to the membrane volume, as done for other membrane chromatography methods. Over a range of four module sizes with an overall scaling factor of 98, product recovery was reproducibly high when a minimal flow rate of 1.4 $mL \cdot min^{-1} \cdot cm^{-2}$ was applied [43]. It should be noted that pressure was not an issue at any state of the large-scale runs, in contrast to when a small-scale liquid chromatography system was used. This study showed

that scaling up with reproducible results is possible through a mechanistic understanding of the method and the identification of critical process parameters. This gives hope that SXC will be perceived as an industrially interesting process in the future, as scalability has been demonstrated.

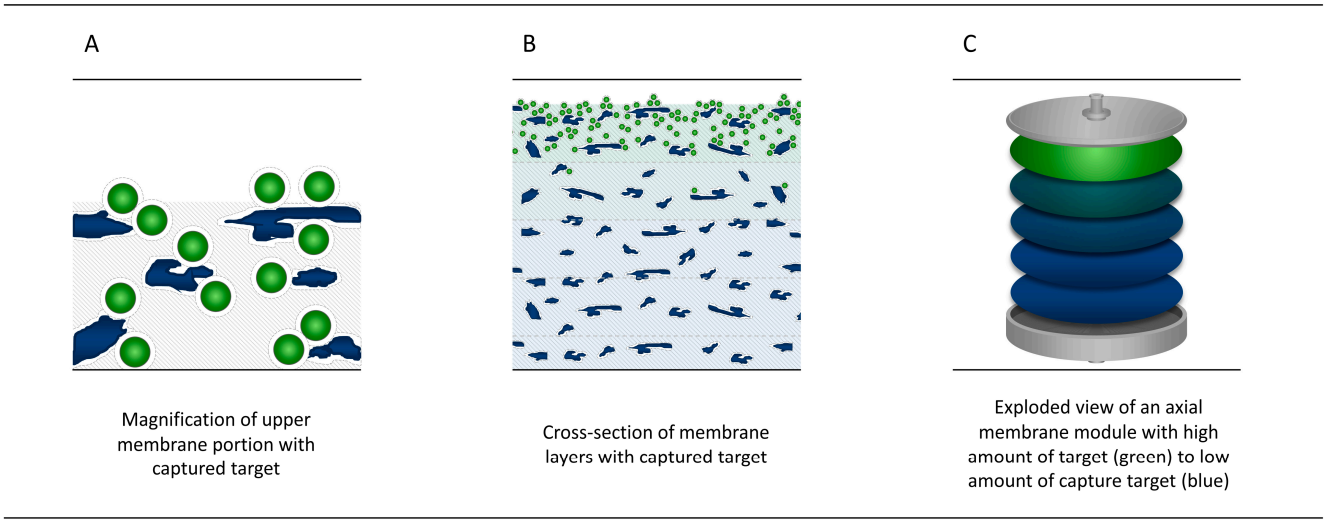

Figure 4. Capture of the target on the upper membrane layers. (**A**) Magnification of upper membrane portion, as shown in Figure 2. (**B**) Cross section of membrane layers with captured targets. More of the target was found on the first membrane layer. (**C**) Visualization of the captured target on the membrane. A decreasing amount of the target from the upper to bottom membrane layer was observed (high target amount = green, low target amount = blue).

4. Applications of SXC

SXC has been used as a purification technique for a variety of molecules. The targets that were purified are listed in Table 2, including process performance.

Table 2. Characteristics of targets that have been purified by SXC with respective process performance. Abbreviations: HCP—host cell proteins, HCD—host cell DNA, n.a.–not applicable.

Target	Size	Shape	Maximum Recovery	Impurity Removal	References
Bacteriophage M13KO7	916 × 7.2 nm	rod	>90%	>99% HCP, 93% DNA	[1]
IgM	25–35 nm diameter [4]	radial pentamer	≤90%	95% HCP	[1]
IgG	12 nm diameter [5]	Y-shaped	87%	99% HCP	[2]
γ-globulin	12–35 nm diameter	various	n.a.	n.a.	[21]
Influenza A virus	80–120 nm diameter	spherical, sometimes filamentous	>95%	>99% HCD, 92% HCP	[22]
ssRNA dsRNA	700–6374 nt and 500–6374 bp length	various	>90% retained on the column	n.a.	[31]
Baculovirus [1]	200–300 nm × 30–60 nm	rod	91%	>99% total protein, 85% total DNA	[29]
Parapoxvirus ovis (Orf)	220–300 nm × 140–200 nm	rod	67% to >90%	>98% total protein, >60% total DNA	[23–25,47]
Hepatitis C virus	30–80 nm diameter	spherical	>97%	>99% total protein, 84% total DNA	[26]
OP7 [2]	80–90 nm diameter	spherical	n.a.	89% total protein	[27]
Adeno-associated virus (AAV)	20 nm diameter	near spherical	>95%	≥80% total protein, ≥94% total DNA	[18]
Lentiviral vector	120 nm diameter	spherical	≥86%	80% total protein and total DNA	[16]
Latex particles [3]	190 nm diameter	spherical	≤21% for CPS, ≤7% for CPH	n.a.	[28]

[1] *Autographa californica* multicapsid nucleopolyhedrovirus, vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped. [2] Influenza A virus defective interfering particle [3] Hydroxylated polystyrene particles (CPH) and sulfated polystyrene particles (CPS) [4] Molecule size derived from [52]. [5] Molecule size derived from [53].

When analyzing the targets that were purified with SXC with the respective publishing date, it becomes remarkably clear that the method was first mainly established for the purification of γ-globulins (including IgM and IgG) and bacteriophages from 2012 to 2014. From 2017 onward, the targets purified by SXC were mainly viruses, except for one report on RNA molecule purification [31] and one report on latex particle purification [28]. The viruses or viral vectors purified were mainly large, enveloped viruses. A wide variety of target sizes as well as molecule shapes were successfully purified, offering a platform technology characteristic of SXC.

The overall process performance for all targets was, except for the latex particles, very good, yielding recovery of 85% or higher under optimized conditions. Here, it must be noted that the use of latex particles was intended to serve as a model for biological nanoparticles of comparable size, as this would enable faster process development. However, the latex particles remained largely in the stationary phase during elution and were therefore not suited as a model [28]. Impurity removal is another important aspect of the purification method and demonstrates the selectivity to retain mainly the target. By this, 80% or more of the contaminating proteins were removed, often reaching the detection limit of the analytical protein assays. The removal of DNA was 60% or higher (Table 2).

Large, fragile molecules, such as enveloped viral vectors, are an interesting target species for SXC, as conventional chromatography techniques often reduce virus infectivity due to harsh buffer conditions [54,55]. For example, elution with high salt concentrations or by changing the pH, which is typically performed during elution of anion exchange chromatography (AEX), was reported to reduce the infectivity of lentiviral vectors [56,57] and measles virus [58,59]. An immediate posttreatment step (buffer exchange or dilution to reduce conductivity) is therefore necessary [60,61]. Since SXC is a gentle chromatography method that does not rely on chemical interaction with the stationary phase and thus does not require any harsh change in buffer composition (pH or salt), it was shown to be well suited to purify enveloped viruses, which tend to be very fragile, and might offer a promising alternative purification method for these modalities [16]. Additionally, as the separation process of SXC is size-dependent and not based on electrostatic interactions, protocols are easier to be transferred for other targets, such as viruses, which are highly diverse. This explains why that in the last few years SXC was predominantly applied to purify viruses, which is in general a new field compared to antibody bioprocessing, and there are many hurdles still to be overcome for efficient and successful virus bioprocessing with no one-fits-all solution due to the heterogeneity of different virus types [62,63]. Other interesting target species are extracellular vesicles and virus-like particles.

For antibodies, which were used as a target species in the very first SXC publications [1,2,21], other chromatography techniques such as protein A affinity chromatography and ion exchange chromatography are well established [64,65], and hence present no need to establish SXC to purify antibodies. We therefore assume that no further research on γ-globulin purification by SXC was published since then.

Recently, a study was published using SXC as a sample preparation technique for physicochemical characterization (including size distribution, electrophoretic mobility, and visual appearance by electron microscopy) of Orf viral vectors. It was reported that sample preparation with SXC was superior to commonly performed ultracentrifugation methods, which are time-consuming and costly [47]. Hence, SXC might offer the opportunity to prepare a desired target of any size and shape for subsequent characterization methods such as electron microscopy, which requires purified samples.

Altogether, SXC can be applied to a variety of targets. The clear advantages that we would like to emphasize here are the platform technology characteristics of the SXC method. Targets of different sizes, shapes and surface characteristics can be purified with this method, since the separation principle is not based on chemical interaction. This is especially advantageous for sensitive targets, such as enveloped viruses, VLPs or exosomes, as mild elution is possible. It must be noted that the implementation of SXC in the industry is still in its infancy. All published data were performed on a small research scale, with

only one case reported for a large-scale application for the purification of an Orf vaccine, which was not successful [51]. For a more detailed discussion on scalability, see Section 3.3.

SXC can be implemented at various stages in the DSP process. In previous experiments with viral vectors, SXC was performed after clarification. In contrast to other chromatography methods, such as AEX, where the feed material has to be adjusted to a certain conductivity [66,67], for SXC the clarified material can be loaded directly without pretreatment. A tangential-flow filtration (TFF) step before SXC is also conceivable if the product has to be concentrated beforehand. Process steps after SXC can look different. A subsequent polishing step with sulfated cellulose membrane adsorbers (SCMA) [26], Capto™ Core 700 [23,24], hydrophobic interaction chromatography (HIC), and AEX [23] was evaluated. The combination of two chromatography steps could be sufficient for regulatory release, depending on the required dose. In general, however, it can be assumed that after SXC, the remaining PEG still has to be removed and the product concentrated and formulated. An ultrafiltration and diafiltration step would be particularly suitable for PEG removal, in which this could be potentially accomplished [16]. It is expected that due to the pronounced size difference of PEG and the target (i.e., large entities such as viral vectors) the residual PEG could be removed in a subsequent ultrafiltration step [16]. However, this has not been analyzed to date and verification of whether acceptable residual PEG levels can be reached is pending. Other methods to remove PEG might be considered. PEG concentrations in the eluate were reported to be as high as between 7 $g \cdot L^{-1}$ [16] and 1% (w/v) as estimated in [47]. This is one of the main obstacles that often discourages people from using it. A final measurement of residual PEG would likely need to be implemented before the final release of drugs purified with SXC due to immunogenicity concerns of PEG [68,69], which have been gaining more attention in recent years after many years of using PEG in pharmaceutical applications. PEG, which is a component of the Comirnaty® vaccine formulation, triggered anaphylaxis in some patients due to an allergic reaction. Nevertheless, it should be noted that PEG allergies are observed very rarely, and therefore the vaccination was considered safe and the presence of PEG was not an exclusion criterion for market approval [70].

5. Conclusions and Future Perspectives

SXC is currently a rather little-known chromatography method whose principles are based on well-known precipitation studies from which a lot of mechanistic understanding can be derived. Nevertheless, some SXC studies from the first reference 11 years ago until today were needed to further extend the mechanistic understanding of the method. It has been shown that SXC can serve as a platform technology that can be used to purify biomolecules of various shapes and sizes. The capture of the target is based on depletion interaction and not on direct binding, such as covalent, electrostatic, and hydrophilic/hydrophobic interactions, making it easily adaptable for new targets. The interplay of target size and buffer composition as well as stationary phase are crucial for optimization in this regard. A hydrophilic porous stationary phase, preferentially membranes or monoliths, serves as a stationary phase, and the association of the target with the stationary phase is achieved by mixing the feed solution with a PEG-containing buffer. The mixing strategy of the two solutions, as well as the PEG buffer composition in terms of PEG size and concentration, salt type and concentration, and pH, are of major importance, and one or more of these factors might require optimization according to the target properties.

In contrast to classical chromatographic methods, the product is captured on the upper membrane layers and not in the depth of the stationary phase. This results in new requirements for scaling SXC. The few data on how to scale SXC to date are promising and indicate that the method can potentially be implemented in large processes when scaling the flow rate related to the membrane area of the first membrane layer of membrane chromatography modules. Specifically for large, fragile, and heterogeneous targets such as

viruses, SXC offers gentle purification that allows for preserving the infectivity, in other words, the functionality, of these molecules.

Avoiding overpressure and removing residual PEG are among the challenges of a process implementation to be mentioned here, but they appear to be capable of being solved. To avoid overpressure, the use of online analytics such as multiangle light scattering might be restricted, depending on the other factors that contribute to the pressure, such as the liquid chromatography system design, the PEG buffer composition, and applied flow rate.

On the road to large-scale SXC use in pharmaceutical production, there is certainly hurdles to overcome, but overall it can be concluded that SXC is a promising and interesting method for all those biomolecules for which existing purification methods are strongly limited.

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