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## Review

# Downstream Processing: From Egg to Cell Culture-Derived Influenza Virus Particles

The establishment of cell culture-derived vaccine production requires the development of appropriate downstream processes. Until today, many of the downstream methods applied originate from egg-derived production processes. These methods have often been slightly modified in order to account for the new demands. However, efforts are currently underway to optimize these processes focusing, for example, on ion exchange or affinity based membrane adsorption chromatography. This review covers the main aspects relevant for the downstream processing of egg and mammalian cell culture-derived whole influenza viruses.

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## 1 Introduction

Two hundred years ago, the English physician Edward Jenner developed the first human vaccine. Since Jenner's time advances in immunology have led to an enhanced understanding of how the human immune system eliminates invading bacteria and viruses. The principal of vaccination is the induction of a primed state in the vaccinated person or animal, so that, following exposure to a pathogen, a fast secondary immune response is generated. This enables the challenged host to accelerate the elimination of infectious particles and so to protect against clinical disease. There are several types of vaccines. Live vaccines are in general based on attenuated, heterologous and recombinant infectious viruses or live organisms. Inactivated vaccines are inactivated preparations of virus particles or subcellular fractions (split vaccines) and specific proteins (subunit vaccines) of the virus. Vaccines can also be based on synthetic peptides, recombinant vectors and plasmid DNA. Today, a large number of vaccines are used to control the outbreak of diseases like measles, mumps, rubella, polio, hepatitis A & B, yellow fever, rabies, human papillomavirus induced cancer, rotavirus gastroenteritis and influenza. Due to its annual death rate and potential to cause pandemics, influenza remains a major public health concern. Current strategies to control influenza outbreaks are mainly centered on prophylactic vaccinations in conjunction with antiviral medications. In the case of influenza, it has to be distinguished between seasonal and pan-

demical vaccines. Seasonal influenza vaccines are commonly a trivalent dose, containing three different strains of influenza A and B viruses. These strains are subject to annual changes and recommended by the World Health Organization (WHO). Pandemic influenza vaccines contain the respective pandemic virus subtype. The precise prediction of such a subtype has not been possible so far. Therefore, only small numbers of doses are stockpiled and the production of large quantities will be initiated after a pandemic influenza subtype has been identified in humans.

Traditionally, influenza vaccines are produced in embryonated chicken eggs. This process has several drawbacks such as the lack of scalability and the risk of involving allergic reactions induced by egg proteins. Additionally, it is challenging to produce avian pandemic strains in fertilized eggs. These disadvantages have led to the development of mammalian cell culture based production processes. Currently, there are several cell lines used for the production of influenza viruses, for example, Per.C6 [1], Vero cells [2], and MDCK cells [3–6].

The establishment of cell culture-derived vaccine production requires the development of appropriate downstream processes. Until today, many of the downstream methods applied originate from egg-derived production processes. These methods have often been only slightly modified in order to account for the new demands. However, efforts are currently underway to optimize these processes focusing, for example, on ion exchange or affinity based membrane adsorption chromatography.

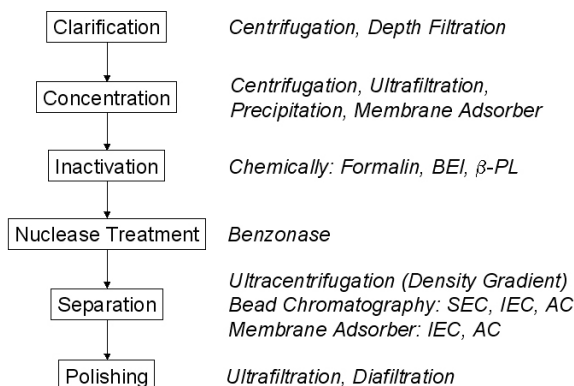
This review article covers the main aspects relevant for the downstream processing of egg and mammalian cell culture-derived whole influenza viruses. The majority of currently available human influenza vaccines are split and subunit vaccines. For these vaccines the membrane protein hemagglutinin or the

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required membrane fractions are generally derived from concentrated and purified virus particles by detergent solubilization. Some alternative vaccines are based on attenuated whole influenza viruses. However, in both cases separation from cell debris and purification of virus particles are the first steps for vaccine production.

Influenza virus particles consist of a host cell membrane derived lipid bilayer envelope in which virus encoded glycoproteins, hemagglutinin (HA), neuraminidase (NA) and the matrix protein M2 are embedded. HA and NA form “spikes” protruding from this membrane. Separation of the influenza virus from the culture medium or allantoic fluid is based either on the size of the virus particles or on the properties of their outer membrane compared to contaminating components of the virus harvest.

Downstream processes for the purification of influenza viruses are generally subdivided as illustrated in Fig. 1. Prior to a first concentration step large contaminants are removed from the cultivation broth or allantoic fluid. Concentration methods are centered on ultracentrifugation [7, 8], ultrafiltration or diafiltration [9, 10], and chromatographic methods based on ion exchange [8, 11, 12] and specific affinity [13, 14]. In the past, it has also been shown that influenza viruses can be successfully concentrated by alcohol [15] and polyethylene glycol [16, 17] precipitation. Concentrates are further processed by density gradient ultracentrifugation [7, 9, 18, 19] or any type of chromatographic method [13, 14, 20]. For the production of egg and mammalian cell-derived inactivated influenza vaccines, these steps are commonly followed by an inactivation step. This is generally done chemically using formalin [2], binary ethyleneimine (BEI) [20, 21] or  $\beta$ -propiolactone ( $\beta$ -PL) [22].



**Figure 1.** Options for unit operations in downstream processing of inactivated whole influenza virus particles.

To comply with regulatory requirements for host cell DNA levels, the majority of production processes include a DNAase treatment. This procedure is frequently combined with chemical inactivation in sequential order. To remove the applied components, DNA fragments and other residual contaminants, affinity or ion exchange chromatography and/or diafiltration are conducted as a final purification step. In the case of split or

subunit vaccines the membranes of the purified virus particles are then detergent solubilized and the target product is isolated. However, these latter unit operations are not the subject of this review.

Influenza vaccine production processes and methods are evaluated by a broad range of assays, including infectivity, antigenicity, HA activity, sterility, innocuity, and residual levels of host cell DNA and protein. Viral infectivity is typically assayed *in vitro* by the tissue culture infective dose (TCID<sub>50</sub>) and *in vivo* by the infectivity rate of mice or chick embryos (LD<sub>50</sub>). Antigenicity is often characterized in guinea-pigs, ferrets or mice. Here, the antigenic potency is assayed after a single injection of the virus preparations by a HA inhibition assay of animal blood samples. The HA activity assay reflects the activity of virus particles to agglutinate erythrocytes and is comparatively robust with respect to changes in starting materials, buffer composition and sample matrix. In addition, it is often possible to obtain closed material balances, and therefore this assay allows quantitative comparisons of individual unit operations. On the other hand, the data obtained may not be directly comparable from strain to strain for the production of whole virus vaccines [23]. Furthermore, the results do not necessarily correlate with the determination of the HA antigen content of the final product as specified by the European Pharmacopoeia, where immunochemical methods are required for HA quantification [24]. Currently, this is mainly done by a single radial immunodiffusion assay (SRID) [23], but ELISA assays and HPLC methods [25] have been established as well. Levels of residual contaminants are generally evaluated by total protein and dsDNA assays.

## 2 Concentration and Partial Purification of Influenza Virus Particles

Virtually, all chromatographic purifications of influenza viruses performed on a production scale use a concentrate of the allantoic fluid or the cultivation broth as the starting material. These concentration steps are classically based on centrifugation, but in recent years this method has been frequently substituted by different types of ultrafiltration and diafiltration.

### 2.1 Virus Concentration and Purification by Centrifugation

In 1936, Elford and Andrewes demonstrated that influenza viruses could be concentrated by high-speed centrifugation [26]. In the early 1950s, similar methods have been described and evaluated for the concentration of egg-derived influenza viruses [27, 28]. Even today, the use of density gradient centrifugation is common in the production of egg-derived and cell culture-derived influenza vaccines [29]. The precise method mainly depends on the applied equipment. In general, the harvested allantoic fluid and the cultivation broth are clarified by low-speed centrifugation and the virus is subsequently sedimented in an ultracentrifugation step. The pelleted virus is re-

suspended and in some methods allowed to swell. To purify the concentrated virus by centrifugation, the virus solution is centrifuged through a sucrose gradient. The precise gradient range varies, but the majority of publications described a gradient ranging from 20–60 % [7, 8], with the target virus at 40–45 % sucrose [7]. Sucrose can be subsequently removed by pelleting of the virus. In the 1970s, Reimer et al. [18, 30, 31] and Gerin and Anderson [32] introduced a zonal ultracentrifugation method to improve the purity of the harvested virus and the efficiency of the method. Here, Reimer et al. [18] demonstrated that this method allowed a tenfold concentration of the virus with respect to the allantoic fluid and an approximately 100-fold purification with respect to the protein content.

## 2.2 Virus Concentration and Partial Purification by Dia-, Ultra-, and Microfiltration

Diafiltration, ultrafiltration, and cross flow microfiltration are frequently used in the biotechnological industry in order to remove or concentrate insoluble larger particles from the suspending medium. Important parameters for the concentration of whole virus particles are permeate flux, transmembrane pressure and pore size. Process efficiency increases with pore size and permeate flux. However, in order to minimize virus losses, a pore size has to be chosen which is significantly smaller than the virus particles. Increased permeate fluxes result in high wall shear rates and since influenza viruses are fragile this could lead to damage of virus particles. Therefore, safety margins have to be empirically defined for the applied process.

First purification studies of influenza viruses from allantoic fluid were described in 1943 [28]. They applied collodion membranes with average pore diameters ranging from 425 to 97 nm. Mice infectivity tests indicated that influenza A (PR8) was retained by an average pore diameter of 172 nm or less and influenza B (Lee) by a diameter of 180 nm. Due to the simplicity of the process, these methods have gained increasing interest over the last 10 years for the concentration of viruses, e.g., adenovirus [33], adeno-associated viruses [34], murine leukemia virus [35], lentivirus [36], parvovirus [37], bromovirus [38] and influenza virus [10, 22].

Nayak et al. [20] described the ultrafiltration of MDCK cell-derived equine influenza virus particles by a plate-type flat sheet polyethersulfone membrane (100 kDa MW cut-off). Prior to concentration, the cell culture broth was clarified via depth filtration (1  $\mu\text{m}$  pore size) and inactivated by BEI. Based on a HA activity assay, they obtained an approximately 20-fold concentration factor in the retentate with a total HA recovery of 95 %. Total protein and host cell DNA reduction was reported to be 88 % and 93 %, respectively.

Wickramasinghe et al. [10] tested different ultrafiltration SartocoSlice 200 cassettes for the concentration of human influenza A/PR/8/34, H1N1. The membranes were made of polyethersulfone with a nominal filtration surface area of 0.018  $\text{m}^2$ . The pore size ranged from 100 kDa to 0.45  $\mu\text{m}$ . The tested virus was produced in MDCK cells maintained in serum-free medium and virus particles were clarified by depth

filtration (1  $\mu\text{m}$  pore size) prior to  $\beta$ -PL inactivation. They identified an optimal pore size of 300 kDa using a wall shear rate of 5700  $\text{s}^{-1}$ . Under these experimental conditions virus losses based on the HA activity assay were negligible and the total protein and host cell DNA reduction was reported to be about 90 % and 93 %, respectively. In the case of membranes with pore sizes close to the virus particles (0.1  $\mu\text{m}$ ) they observed an internal fouling, indicating the importance of careful selection and optimization of membrane pore size and operating conditions.

Kalbfuss et al. [22] tested three different polysulfone hollow-fiber modules with cut-offs of 750 kDa, 0.1  $\mu\text{m}$  and 0.45  $\mu\text{m}$ . Applied virus samples were produced under similar conditions (MDCK, serum-free) to those described for the experiments conducted by Wickramasinghe et al. [10]. However, clarification was slightly different. Primary clarification was done by depth filtration (0.65  $\mu\text{m}$ ) prior to  $\beta$ -PL inactivation. After inactivation precipitates were removed by a microfiltration step (0.45  $\mu\text{m}$ ). The 0.45  $\mu\text{m}$  hollow fiber membrane did not retain the virus. The 0.1  $\mu\text{m}$  and the 750 kDa hollow fiber membrane, however, retained the virus with recoveries in the retentate of 54 % and 100 %, respectively. The total protein and host cell DNA reduction for the 0.1  $\mu\text{m}$  hollow fiber membrane was given with 91 % and 84 % and for the 750 kDa hollow fiber membrane with 88 % and 61 %, respectively.

In summary, ultra-, micro- or cross flow filtration represent a powerful tool for the large-scale concentration of complex biomolecules like viruses and viral vectors. However, careful selection of membrane cut-off and operating conditions are essential in order to maximize the benefits of these operations for the concentration of viral products. In the presented studies, the exclusion limits identified for influenza viruses varied for mammalian cell-derived virus particles from 100 to 750 kDa. The exclusion limit for egg-derived virus particles was approximately 180 nm. The diameter of influenza viruses ranges from 80 to 120 nm [39, 40]. However, influenza viruses have a tendency to aggregate and the size distribution of virus aggregates from MDCK cell-derived virus particles ranges from 60 to 600 nm [41] or even 1  $\mu\text{m}$  [22]. The degree of aggregation seems to depend on the production process. Accounting for the varying starting materials and the fact that the rating procedure from the pores can differ between suppliers, some variation in the exclusion limits for influenza viruses and elimination rates of contaminants are expected. Process efficiency increases with larger pore sizes and allows a higher degree of protein and host cell nucleic acid elimination. Larger pore sizes and a reduced permeate flux also reduce the wall shear rate to which fragile virus particles are subjected during the concentration step. Hence, the optimal exclusion limit and operating conditions for every process still have to be identified empirically based on the product recovery, the concentration factor achieved and the elimination of contaminants. Additionally, if the target products are whole virus particles, viral fragments can be eliminated at this step. On the other hand, if split vaccines or subunit vaccines are produced, the pore size can be chosen to include viral fragments in the product fraction in order to increase the overall yield.

### 2.3 Virus Concentration by Precipitation

Currently, precipitation is not applied in any large-scale production process reported for influenza vaccines. In the past, precipitation with ethyl and methyl alcohol [15] and polyethylene glycol [16, 42, 43] was described for egg-derived influenza viruses. Due to the simplicity of this unit operation and its potential in large-scale operations (10,000–20,000 L working volume), there seems to be an awakening interest in the precipitation of contaminants like host cell DNA and proteins as a primary purification step. However, an economically efficient precipitation agent for contaminants or the target virus has still to be identified.

## 3 Purification or Capturing of Influenza Viruses by Liquid Chromatography

### 3.1 Virus Isolation by Size Exclusion Chromatography

Size exclusion chromatography (SEC) separates molecules based on their hydrodynamic volume. The size of influenza viruses and their tendency to form aggregates has been discussed in the previous section. The molecular weight of influenza virions typically exceeds  $1 \cdot 10^5$  kDa. Conversely, the molecular weight of most soluble biopolymers is not larger than a few hundred kDa. Hence, a size-based fractionation offers a promising approach to the separation of virus particles from contaminating host cell proteins and nucleic acids as demonstrated for the turkey coronavirus [44], the vesicular stomatitis virus [45] and the influenza virus [20, 42, 46]. Due to the size of virus particles, they are not able to penetrate the pores of most chromatography media and hence elute at the void volume of the columns used.

Heyward et al. [42] applied controlled pore glass (CPG) with a pore size of 729 Å for the purification of an egg-derived influenza virus (A/Ann Arbor/60) after polyethylene glycol precipitation. As expected from the size of the influenza virus particles, virions were eluted in the void volume and the contaminating proteins well separated from the viral fraction, leading to a typical double peak in the UV spectrum. The overall recovery of the CPG-SEC was above 80% of the loaded virus infectivity and the purity of the virus preparation was comparable to virus preparations purified by conventional density gradient procedures as judged by SDS-PAGE.

More recent applications of SEC have been reported for the purification of equine and human influenza virus particles [20, 46]. Nayak et al. [20] separated BEI inactivated MDCK cell-derived equine influenza virus particles (A/Newmarket/1/93) from concentrated cultivation broth by Sepharose CL-2B chromatography. According to the manufacturer's datasheet, the fractionation range of Sepharose CL-2B is 70–40,000 kDa, hence whole virions or aggregates of virions are eluted at the void volume. Small solutes are retained from the SEC medium, leading to a double peak in the UV trace as shown by Nayak et al. Based on HA activity, viral recovery in the void volume of the SEC process of the concentrate was approximately 38%. The remaining HA activity was distributed over later fractions,

which most likely account for deteriorated virions and small membrane fragments containing HA molecules. Therefore, it can be concluded that SEC allows the fractionation of whole virions from damaged viral or cellular membrane particles. Due to the budding process of influenza viruses, the membrane structure of these particles is identical to those of whole virus particles. Typically, only the virus fraction at the void volume is collected for further processing. Based on the concentrated starting material, Nayak et al. achieved a total protein and host cell DNA reduction in this fraction of about 66% and 82%, respectively. By combining the ultrafiltration step with SEC the overall virus recovery was 36% with a protein and DNA reduction of 96% and 99%, respectively.

Kalbfuss et al. [22] compared four different SEC media: Sepharose CL-2B, Sepharose 4FF, Sepharose 6FF, and Superdex 200 prep grade (pg) with an exclusion limit of 30 nm, 30 MDa, 4 MDa, and 500 kDa. Consequently, intact virus particles could not penetrate the pores of these chromatography media. As a model, the authors used  $\beta$ -PL inactivated MDCK cell culture-derived (serum-free medium) human influenza virus particles (A/PR/8/34) from concentrated cultivation broths. Elution from all tested chromatography media led, as in previous studies, to the characteristic double peak pattern in the UV trace with the first peak at the void volume representing the viral fraction. The second peak resulted from the elution of small solutes like proteins, amino acids, nucleotides, etc. [46]. In addition, Kalbfuss et al. were able to demonstrate that host cell DNA is larger than contaminating proteins and as a consequence elutes earlier than small contaminants. In all tested chromatography media, host cell DNA was co-eluting with the virus fraction and the quantity of co-eluting DNA was steadily increasing from Sepharose CL-2B, Sepharose 4 FF, Sepharose 6 FF, to Superdex 200 pg. On the other hand, the viral fraction was heavily tailing in the case of Sepharose CL-2B, clearly suggesting that damaged virus or cellular membrane particles were able to enter a fraction of the pores as already described by Nayak et al. [20]. Therefore, the authors selected Sepharose 4 FF for their further studies and achieved an overall virus product yield of 85% based on the SEC load. Total protein and host cell DNA reductions were 65% and 66%, respectively.

The overall yields of the presented studies vary tremendously. The recovery achieved with CPG, Sepharose CL-2B and Sepharose 4FF was 80% [42], 38% [20] and 85% [46]. However, it has to be considered that the starting material in terms of (1) influenza virus strain, (2) production process (egg versus MDCK cell-derived; time of harvest), (3) clarification, and (4) concentration (precipitation and ultrafiltration) differed in all studies. In particular, the clarification and concentration step can result in virus particle damage and therefore the accumulation of viral breakdown products, which cannot be differentiated from whole virions by the majority of analytical assays. This also applies for small cell debris remaining in the culture broth after clarification. These particles are separated from whole virus particles by SEC, leading to an apparent low recovery in the product fraction. Additional points to consider in this context are degradation of virus particles by unspecific protease activity towards the end of upstream processing due to cell lysis and storage conditions after harvesting,

inactivation and concentration. Aside from these considerations the overall yields of the CPG and Sepharose 4 FF studies are comparable. Considering the yields of the SEC purified (Sepharose CL-4B) vesicular stomatitis virus (53 %) [45] and moloney murine leukemia virus (70 %) [47], the extremely low yield (38 %) from Nayak et al. might not be representative for the method used. One possible explanation for the low yields is obviously the tailing of the void volume peak applying Sepharose CL-2B as described by Nayak et al. [20] and Kalbfuss et al. [46]. Hence, purity of the virus particles has to be balanced with the yield in particular in the case of Sepharose CL-2B.

In summary, SEC allows the separation of whole virions from virus or cellular membrane particles. Influenza viruses are enveloped and bud from the plasma membrane. Hence, the membrane composition of whole virions is identical to that of virus or cellular debris, making their separation challenging by other chromatographic methods like affinity chromatography. In an optimized process, yields of 80 % and more can be achieved. However, in contrast to veterinary vaccines [48], product purity after SEC fractionation is usually not sufficient for human vaccine production where the level of contaminating proteins and host cell DNA needs to be further reduced by additional unit operations in order to comply with regulatory requirements. In addition, using SEC unit operations might not be economically feasible for large-scale production processes due to the restrictions in column size, stability of gel matrices and limitations in loading volumes.

### 3.2 Virus Purification by Ion Exchange Chromatography

Ion exchange chromatography (IEC) relies on charge to charge interactions between the viral envelope and the charges immobilized on the resin. Target molecules are displaced from chromatography media by increased ionic strength. Alternatively, the adsorbed targets can be eluted by a change in pH leading, in the case of influenza viruses, to an unfavorable overall charge of the viral envelope or the matrix at which the target does not bind. However, the required change in pH or ionic strength for a complete elution can affect the product activity or the structural stability of virions. Influenza viruses are in general sensitive to pH changes [49], in particular in acidic conditions, but comparatively stable in the presence of high salt concentrations [46, 50]. Hence, one of the most important points to consider for this type of chromatography is product stability at the running conditions used. IEC can be subdivided into cation (CEC) and anion exchange chromatography (AEC). Both methods can be applied in a positive mode, i.e., virus particles adsorb to the chromatography medium, and in a negative mode, i.e., contaminants adsorb to the matrix and the virions do not bind. Different variations of CEC and AEC have been applied for the purification of influenza viruses from allantoic fluid and cell culture broth [8, 46, 49, 51–53]. However, due to an acidic pI (5.0) of influenza virions ( $A_2$ /Singapore/57) [54] and possible losses of HA activity at lower pH, anionic exchanger resins are commonly favored.

Neurath et al. [12] demonstrated in 1967 the applicability of cation exchangers for the separation of influenza viruses from allantoic fluid. Interestingly, it could be demonstrated that not only the type of ion exchanger but also the ionic form has an influence on the adsorption behavior of the virus to the chromatography medium. The binding of different egg-derived influenza virus types ( $A_2$ /Japan/170/62,  $A_2$ /Taiwan, A/PR8,  $A_1$ /Ann Arbor/1/57, and B/Maryland/1/59) was compared to a sodium and calcium form of the cation exchanger CG-50, type 1. Influenza A virus subtypes did not bind to the sodium form but adsorbed to the calcium form of the resin and could, therefore, be separated from a large amount of contaminating proteins of the allantoic fluid [12]. However, the B strain virus failed to adsorb to either form of the resin. Hence, this method cannot be used for a general vaccine production process. Neurath et al. also demonstrated product stability via a potency test in mice and no differences were observed between crude and purified viruses. Unfortunately, HA activity assays or other common assays to allow a quantitative evaluation of the method were not carried out.

Matheka and Armbruster [49] compared anion and cation exchange resins for the purification of egg-derived virus particles (influenza A/PR/8). As expected from the pI of influenza virions, anion exchangers were shown to be more suitable to adsorb influenza viruses at pH conditions close to neutral. Furthermore, Matheka and Armbruster tested the effect of bead size as well as basicity of anion exchangers. As anticipated, bead size was not relevant for the separation of influenza virions from contaminating proteins. On the other hand, it is known that bead and pore size of beads contribute to the overall capacity of the applied resin in particular for the purification of large particles. However, this was not described by the authors. The tested basicity affected the adsorption behavior of the virus particles. The higher the basicity, the greater the ionic strength required to displace the viruses. In a follow-up publication [53] Matheka and Armbruster demonstrated the fractionation of influenza viruses by eluting adsorbed virions from an anion exchange resin (Amberlite IRA 400) with an increasing NaCl concentration in the elution buffer (citrate buffer pH 6.2–6.4). Comparing three egg-derived influenza virus types (A/PR/8, A/FM/1, B/Lee) the authors observed clear differences in the adsorption characteristics of the strains. This is in agreement with other studies [8], hence close attention should be paid to the viral strain applying ion exchange chromatography processes. One additional interesting finding was the variation of virus particle diameters (40–200 nm) during elution. Larger virus particles (100–200 nm) and virus aggregates were eluted in fractions with low ionic strength and small virions (40–60 nm) at high salt concentrations. This is in accordance with later observations [8, 55]. Hence, larger virions seem to bind less tightly to the chromatographic media than smaller ones. This is most likely due to the spherical shape of the virus particles as the contact area of the chromatographic support does not increase proportionally to the size of virions. Therefore, larger virions are eluted at lower ionic strength.

Iordan et al. [8] described a downstream process scheme comprising a weak (DEAE-Sephadex) anion exchanger, a concentration step via ultrafiltration (100 kDa cut-off) and a de-

salting step via SEC (Sephacrose 6B). They used two egg-derived influenza strains as a model: A/PR/8/34 and A/Krasnodar/101/59. For both strains they achieved an overall recovery of approximately 60% with a volumetric concentration factor of about 56. Slight differences could be observed in the protein removal rate. The total protein reduction varied between 90% and 97% for A/Krasnodar/101/59 and A/PR/8/34, respectively. These differences were most likely due to a shift in ionic strength required for desorption of the virus particles. The influenza virus strain A/PR/8/34 required a higher NaCl (0.8 M) concentration than A/Krasnodar/101/59 (0.5 M) in order to elute from DEAE-Sephadex. This fact was utilized in the washing step. A/Krasnodar/101/59 was washed with 0.15 M NaCl and A/PR/8/34 with a combination of 0.15 and 0.5 M NaCl resulting, in the case of A/PR/8/34, in high product purity. Total protein removal was mainly due to the AEC unit operation. The following concentration and desalting steps accounted only marginally to the overall protein reduction. However, HA activity losses were mainly due to these two unit operations. Only small amounts of virus particles were lost during AEC. The virus particles lost during column loading and washing might have been larger viral aggregates, binding less tightly to ion exchange resins according to the literature [55]. The differences in adsorption characteristics of the two strains could be due to the variation of the overall surface charge or the aggregation behavior of the virus particles. They could also be caused by the differences in adsorption characteristics of the viral strains to proteins from the allantoic fluid or cell culture media. These differences could be reduced by the use of protein-free media during the upstream processing in the case of cell culture-derived virus particles. Nevertheless, until today, elution conditions for an AEC have to be determined empirically for every strain of influenza virus and AEC process.

Jordan et al. [8] compared AEC in combination with concentration and SEC unit operations with a downstream process based on sucrose gradient (20–60%) centrifugation. Here, the overall yields for both influenza strains were significantly reduced (40–50%) and the degree of viral aggregation was higher in the case of the gradient centrifugation method. There were no considerable differences in the degree of protein reduction for both methods. However, the volumetric concentration factor of the centrifugation method was two to three times higher compared to the AEC based process. Of course, this was due to the ultrafiltration step following AEC, which can be optimized by reducing the retentate volume by increasing operating time and by reducing the dead volume of the applied ultrafiltration equipment.

All the IEC studies described so far are centered on the purification of egg-derived influenza virus particles. However, although these results cannot be directly transferred to the downstream processing of cell culture-derived virions, they offer a good starting point for the design of corresponding unit operations.

Kalbfuss et al. [46] investigated the suitability of AEC after SEC in order to remove residual host cell DNA in positive and negative mode. They tested two different Sepharose Q based ion exchange matrices (Sepharose Q FF and Sepharose Q XL) for the separation of DNA from a MDCK cell-derived human

influenza virus (A/PR8/34), which was  $\beta$ -PL inactivated. NaCl in phosphate buffer (pH: 7.3) was used as a displacer for the positive mode AEC application. Both media displayed different selectivity with respect to the virus particles and the host cell DNA. Sepharose Q FF did not separate the virus from the host cell DNA, while partial separation was achieved with Sepharose Q XL. The use of Sepharose Q XL led to a split peak elution of the desorbed virus, while one of the eluted peaks co-eluted with the host cell DNA. To apply AEC in negative mode, the loading buffer was conditioned with 0.65 M NaCl, resulting in a flow through the virus particles with recoveries of  $\geq 80\%$  based on the load. However, the overall yield depended on the amount of loaded virus and reduced yields were observed at low loads, most likely due to residual binding capacities of the stationary phase. Naturally, loads above or close to the column capacity also resulted in reduced yields.

As an alternative to bead chromatography, membrane adsorbers are increasingly used for the purification of large particles, i.e., viruses. Kalbfuss et al. [11] tested anion exchange membrane adsorbers (AEX MA) for the direct capturing of clarified (0.45  $\mu\text{m}$ ),  $\beta$ -PL inactivated human (A/PR/8/34; H1N1) and equine (A/Newmarket/1/93; H3N8) influenza virus particles. For their studies, they used Sartobind Q and D MA 75 membrane adsorbers comparing different production systems as well as virus harvest from cultivations in serum-free and serum-containing media. Sartobind membranes are based on stabilized reinforced cellulose with pore sizes of  $> 3 \mu\text{m}$ . The functional group of Sartobind D and Sartobind Q is diethylamine (weak AEX) and quaternary ammonium (strong AEX), respectively. The results obtained indicated that both AEX MA were suitable to capture influenza A viruses. In both cases viral displacement could not be achieved by a shift in pH. However, elution with NaCl resulted in an almost complete elution from Sartobind Q. Compared to Sartobind Q, the displacement from Sartobind D resulted in significantly lower recoveries and a delayed onset of elution, i.e., the elution occurred at higher salt concentrations. Hence, virus particles interacted stronger with Sartobind D than with the Sartobind Q membrane, probably due to its charge density (5–6  $\mu\text{mol cm}^{-2}$ ) being higher than that of Sartobind Q (3–4  $\mu\text{mol cm}^{-2}$ ). It is likely that the higher charge density of Sartobind D will not only lead to higher capacity as it is the case for small colloids, but also result in an increase in the number of interaction sites and therefore binding strength of virions to the stationary phase. The authors reported overall recoveries of the Sartobind Q and Sartobind D membrane adsorbers to be 86% and 38%, respectively. The high losses with Sartobind D MA are in contrast to the findings of Jordan et al. [8] who applied bead-based weak anion exchangers (DEAE-Sephadex) with a 88% recovery of the adsorbed virus particles (egg-derived, A/PR/8/34) after elution with 0.8 M NaCl in Tris-HCl buffer, pH 7.4. Moreover, Jordan et al. did not observe a strong tailing during the elution process [8]. On the other hand, desorption of the virus particles from both membrane adsorbers was noticed over a wide range of salt concentrations (0.3–1.5 M), which is in agreement with observations of Kalbfuss et al. (MDCK cell-derived A/PR/8/34, Sepharose Q) and Matheka and Armbruster (egg-derived A/PR/8, A/FM/1, B/Lee, Amberlite IRA 400) [46, 53].

Similar to bead-based separations, the observed tailing can be explained by the variation in size of the virus particles due to aggregation, the complexity of the budding process and breakdown products of virus particles [56]. The process with Sartobind Q allowed a significant reduction in total protein content (~77%). However, due to the broad elution of the virus particles it was not possible to separate the virus from contaminating host cell DNA [11].

Comparisons of purifications of virus harvest produced in serum-free and serum-containing media indicated higher capacity with the latter medium. This can be explained by a shift in the adsorption equilibrium, which could be due to differences in the ionic strength or adsorption competitors in the respective medium. On the other hand, neither the virus type (human H1N1 and equine H3N8) nor the cultivation system (roller bottles and stirred tank) showed a significant effect on membrane capacity [11]. The drawback shown in this study is the low concentration factor of approximately seven. This is most likely caused by the tailing of the elution peaks or a slow desorption kinetics of the virus particles. A reduction in flow rate or even an incubation of the virus in desorption buffer would overcome these problems but also reduce process productivity. However, the productivity of  $67 \text{ L m}^{-2} \text{ h}^{-1}$  [11] of Sartobind Q MA was remarkably high compared to other chromatographic media or even cross flow ultrafiltration operations.

An interesting observation with respect to the use of AEX MA was reported by Goyal et al. [57]. They observed that the adsorption of egg-derived PR8 influenza virus on AEX MA (Zeta Plus filters series S) was dependent on the pH of the feed. Maximum capacity was observed at pH 6.0 with a strong decline towards acidic pH (50% of maximum capacity at pH 5.0) and a less pronounced decline towards alkaline pH. When applying immediate neutralization after elution full recovery was achieved based on HA activity with different basic elution buffers.

In summary, IEC is an excellent tool for the capturing and purification of egg and cell culture-derived influenza virus particles. AEC is generally more suitable for the adsorption of virus particles. However, nucleic acids also bind to the AEC resins, making an efficient separation of virus particles from host cell DNA very challenging. The reduction in the level of host cell DNA and contaminating proteins by AEC strongly depends on the influenza virus strain. The capacity of AEC media is strongly affected by the type of contaminating proteins and the amount of nucleic acids. Resin capacity can be improved by optimization of the loading and washing conditions (pH, ionic strength) in order to reduce the adsorption of contaminating components. However, the pH sensitivity of the virus particles limits the options for process design. An interesting effect, which should be mentioned here, is the possibility to separate larger virus aggregates from smaller virus particles by AEC. This could potentially allow the separation of viral membrane fragments from whole virus particles, which could be relevant for the production of live vaccines as viral membrane fractions can often not be eliminated by filtration procedures.

### 3.3 Influenza Virus Purification by Affinity Chromatography

Affinity chromatography (AC) allows the purification of biomolecules on the basis of their individual chemical structure or their biological function. Target molecules are separated based on a reversible interaction with a specific ligand immobilized on the chromatography medium. AC offers an excellent specificity which, combined with high capacity chromatography media like membrane adsorbers, represents a powerful unit operation to capture biomolecules. Moreover, with respect to other chromatographic methods, AC offers an unmatched simplicity of operation.

In the case of influenza virus particles the ligand targets are commonly HA and NA. HA is the major influenza virus surface glycoprotein which mediates via a sialic acid binding pocket the attachment of the virus to host cells. Hence, potential ligands for AC of influenza viruses are antibodies specific to HA or NA, sialic acid or sialic acid derivatives and lectins, which interfere with the glycan residues of the glycoproteins HA and NA. Alternative ligands would be peptides and other small molecules specific to HA or NA.

The use of classical immunoaffinity chromatography has been described for whole influenza virus particles [58] and detergent solubilized viral HA and NA [59]. Sweet et al. [58] described the purification of egg-derived whole influenza viruses (A/Moscow/1019/65, A/England/344/68, PR/8-A/Hong Kong/68) by polyclonal rabbit antibodies. Elution of the virus particles was done with an alkaline buffer in a pH ranging from 11.3 to 12.5. However, the infectivity of the eluted fraction was only 50% of the loaded material. Most likely, this is due to the high pH which has been shown to reduce viral infectivity and HA activity [46]. Unfortunately, the pH required for the elution of the target product depends only on the applied antibodies. The application of polyclonal antibodies allows a broader specificity to different subtypes of influenza viruses, but binding kinetics and strength might be suboptimal for an immuno AC. Gerentes et al. [59] applied specific monoclonal antibodies for the purification of detergent solubilized HA and NA from egg-derived influenza viruses (A/Beijing/32/92, A/PR/8/34, RESVIR-8 reassortants of influenza A virus with glycoproteins from A/Johannisburg/33/94 and A/PR/8/34) and were able to elute HA molecules at pH 6 with no loss in HA activity. NA molecules had to be released at an acidic pH (pH < 5) resulting in activity losses. Another concern with immuno AC is the limited number of process cycles and the fact that most biological ligands can usually not withstand harsh cleaning and sanitization conditions. Sweet et al. [58] observed a limited operating lifetime (ten cycles) of the columns, which is a significant economic disadvantage for a production process. On the other hand, this is a common problem with the majority of biological affinity media.

HA is a glycoprotein containing several *N*-linked glycosylation sites. These glycans can be targeted as affinity ligands by specific lectins. Lectins are a class of carbohydrate-specific proteins or glycoproteins which mainly bind to monosaccharides or short oligosaccharide sequences at the nonreducing termini

of a glycan. Hence, specific characteristics of these glycans compared to contaminating molecules can be utilized for the purification of whole influenza viruses [13, 41], viral subunits [60, 61] and recombinant HA [62].

A lectin screen by Opitz et al. [13] showed that MDCK cell-derived whole influenza viruses can be efficiently isolated from the cultivation broth by AC based on the lectin *Euonymus europaeus*. This lectin binds to oligosaccharides containing galactosyl ( $\alpha$  1-3) galactose residues and the target molecules can be displaced by lactose and, if necessary, by lactose containing 2 M NaCl. For their studies, Opitz et al. [13] used a human influenza virus (A/PR/8/34) propagated in MDCK cells which were maintained in serum-containing medium. The overall recoveries of the virus particles heavily depended on the applied chromatography matrix. A maximum recovery of approximately 94% of the loaded virus was achieved with a cellulose membrane. In this particular case, the total protein and host cell DNA reduction based on the starting material amounted to 69% and 99%, respectively. Further experiments showed that this method is applicable for a range of different human influenza A virus strains as well as for influenza B (unpublished data). Hence, this method reflects an efficient capture step for MDCK cell-derived influenza viruses. However, it has to be considered that the glycosylation pattern of the HA glycans strongly depends on the host cell as frequently described in the literature for recombinant proteins and other biotechnological products [63]. Suitable lectins will have to be identified for viruses produced in different host cells, under different cultivation conditions and in different production systems like fertilized eggs.

A general concern associated with AC is the leaching of bioactive compounds. This accounts in particular for lectins having a potential to be toxic. Therefore, the FDA insists that any biological affinity ligand used in the manufacturing process of a biological product meets the same application requirements as the end product. One possible solution to overcome the disadvantages of biological ligands mentioned above is the application of small molecules. Immobilized sialic acid derivatives and sialyllactose have been shown to bind different strains of influenza viruses [64–66]. AC based on sialic acid derivatives ( $\alpha$  and  $\beta$ -ketosides of sialic acid derivatives) has been described for the purification of whole influenza viruses (A/Dunedin/4/73, A/Victoria/3/75, B/Hongkong/8/73) from allantoic fluid [67]. Here, the displacement of virus particles from an affinity Sepharose matrix was done by benzyl  $\alpha$ -ketoside of *N*-acetylneuraminic acid. However, the release kinetics proved to be difficult from the tested sialic acid derivatives, resulting in low total viral recoveries based on a HA activity assay. In order to apply such molecules to AC, a diverse library of sialic acid derivatives would have to be screened to identify ligands with favorable binding and release kinetics. Dissociation constants of  $10^{-4}$ – $10^{-10}$  M are usually considered a good working range [68].

Another alternative is pseudo AC based on heparin and sulfated cellulose. The latter is, in some literature, also referred to as IEC matrix. Heparin is a member of the glycosaminoglycan family of polysaccharides. It is a linear carbohydrate consisting of uronic acid-(1-4)-D-glucosamine repeating disaccharide subunits [69]. Heparin and heparan sulfate have been demon-

strated to interact with several viruses, including herpes viruses [70, 71], dengue virus [72, 73], adeno-associated virus [74], human immunodeficiency virus type 1 [75], moloney murine leukemia derived retroviruses [47] and influenza virus [41]. The interaction of sulfated cellulose with the HA of influenza viruses is currently not fully understood. However, Cellufine® Sulfate is frequently applied for the purification of egg and cell culture-derived influenza viruses [14, 41] and recombinant HA [62]. Opitz et al. [41] compared the capturing of MDCK cell-derived human influenza virus (A/PR/8/34) by Cellufine® Sulfate and heparin column chromatography. Here, based on a HA activity assay, viral recoveries from Cellufine® Sulfate and heparin were 70% and 32%, respectively. Unfortunately, no details were provided on the degree of contaminant removal. Neither does the study of Palache et al. [14]. Peterka et al. [76] reported that the application of Cellufine® Sulfate column chromatography led to low viral yields ranging from 21% and 26.5% based on the HA activity assay and TCID<sub>50</sub> assay, respectively. On the other hand, Kost [77] suggested Cellufine® Sulfate column chromatography for the determination of the virus particle or viral antigen concentrations in process samples. Here, it has to be pointed out that the loading conditions are crucial for a successful application of Cellufine® Sulfate. Depending on the virus strain and the source of the virus particles, the salt concentration required for an efficient adsorption of virus particles often has to be significantly lower than in phosphate buffer saline (unpublished data). The major drawback to the application of Cellufine® Sulfate beads for the purification of influenza viruses is the low productivity of the column chromatography. This can potentially be improved by using specifically sulfated reinforced cellulose membrane adsorbers.

Another option would be the purification of influenza viruses by small peptide ligands. Peptide AC has already been successfully used for the purification of recombinant proteins [78–80]. A prerequisite to the purification of influenza viruses is the identification of peptides with a broad specificity for different influenza types and strains from a peptide library and the immobilization on a chromatographic support.

One important consideration for AC is the immobilization of ligands on the chromatographic support. Various types of pre-activated chromatography media are commercially available. These resins are functionalized with chemically reactive amine, aldehyde or epoxy groups. However, it has to be verified that the ligands to be immobilized are not only functional but also accessible to the target. In particular for large particles like viruses accessibility can be crucial. To account for spherical hindrance of larger particles, small ligands may have to be immobilized via spacers on the chromatographic support. Also, the pore size of the chromatography media has to be considered, in particular for high value ligands. Most chromatography media exclude influenza viruses due to their pore size distribution, which leads to low binding capacities despite a high number of immobilized ligands. Hence, large pore size media as, for example, controlled pore glass or membrane adsorbers or nonporous media should be used to improve process economy.

In summary, AC represents an excellent tool for the capturing and purification of influenza viruses. Overall yields and



productivity of AC unit operations mainly depend on the selected ligand and its binding and release kinetics. In combination with high capacity chromatographic supports AC allows to achieve good concentration factors at the primary capture step. Furthermore, the level of contaminating proteins and host cell nucleic acids can be significantly reduced. Both filtration and centrifugation techniques allow higher volumetric concentration factors, but these methods lack specificity.

The simplicity of AC unit operations and their high specificity is unmatched by any other chromatographic method. However, the level of purity achieved solely depends on the specificity of the ligand used. Major drawbacks to using AC are the high price of ligands, the potential leaching of bioactive compounds and limitations with respect to cleaning and sanitization. Using small molecules like heparin or sulfated glucose molecules (e.g., Cellufine® Sulfate) can help to overcome these disadvantages but also lack high specificity. Nevertheless, Cellufine® Sulfate is currently the only pseudo AC medium which is commercially used for the production of influenza vaccines.

#### 4 Use of Chromatography Beads, Monoliths, and Membrane Adsorbers

Several different chromatography media have been tested for the purification of influenza viruses. Opitz et al. [41] compared numerous column chromatography media with a membrane adsorber for the capturing of influenza viruses. Here, the authors indicated that the capacity of membrane adsorbers was superior to the tested bead-based chromatography material. Additionally, with some of the soft gels, they observed unspecific losses of virus particles, which was not the case with a solid support polymer matrix and the membrane adsorber. However, it should be pointed out that unspecific loss of target material has been observed for proteins [81] and influenza viruses [11], depending on the type of membrane adsorber and the application.

Most commonly applied chromatography media for influenza purifications are porous beads. The main disadvantage here is the limitation in mass transfer kinetics. Transport of solutes into the porous lumen of beads occurs exclusively by diffusion. However, effective diffusivity is low for larger particles, i.e., virions with a diameter close to the pore size. One possibility to overcome this limitation is the application of nonporous beads or large porous membranes and monoliths. The disadvantage of nonporous beads is their low capacity and productivity. Monoliths and porous membranes allow overcoming these disadvantages. Different types of monolithic columns have been used successfully for the purification of tomato mosaic virus [82], moloney murine leukaemia virus [83] and influenza virus [64, 76]. In the case of influenza virus particles purification was based on an anion exchange monolithic column followed by SEC and a concentration step [76] and on pseudo-affinity monolithic columns [64]. Membrane adsorbers were used for the purification of virus particles, for example, aedes aegypti densovirus [84], densovirus [85] and influenza virus [11, 41]. The main advantages of membrane adsorbers compared to bead-based column chro-

matography are the significantly enhanced mass transfer kinetics due to the convective flow through the porous lumen, the higher volumetric concentration factor and the reduced pressure drop during operation and therefore an increased productivity. However, it has to be considered that productivity is also a function of the binding kinetics of the target molecule to the immobilized ligand. Hence, in the case of affinity membrane adsorbers the operational flow rate is mainly limited by the binding and release kinetics of the immobilized ligands and not by the backpressure of the applied chromatography material. On the other hand, the influence of the binding kinetics of the virus to the membrane is not crucial for ion exchange membrane adsorbers. In the majority of applications this leads to an increased productivity but lacks the specificity of an affinity-based purification step. In addition, membrane adsorbers are comparatively easy to scale-up and to validate. Furthermore, due to their low production cost it is economically feasible to integrate these unit operations into single-use production processes. This is a clear advantage over currently available monolithic columns.

#### 5 Downstream Processes of Cell Culture-Derived Influenza Virus Particles

Important requirements on content and purity for whole virion inactivated cell culture-derived human influenza vaccines according to the European Pharmacopoeia 6.0 are: (1) 15 µg of HA are required per strain and dose. (2) Total protein cannot be more than six times the total HA content of the vaccine as determined in the assay, but in any case not more than 100 µg of protein per virus strain and human dose. (3) Bacterial endotoxins have to be less than 25 IU per human dose. (4) Residual host cell DNA, if a continuous cell line is used for virus propagation, has to be limited to 10 ng per single human dose [24].

Naturally, only a combination of different downstream processing methods will allow compliance with these requirements. Unfortunately, very few complete downstream processes have been described for the preparation of cell culture-derived whole influenza vaccines in the literature. Moreover, publications from vaccine producers do not disclose details, making a precise evaluation of the respective methods nearly impossible. The influenza vaccine Influvac [3] is purified and processed from MDCK cell supernatant by a multistep downstream process comprising the following unit operations: (1) capturing of the virus particles by IEC (Cellufine® Sulfate [14]), (2) prefiltration, (3) concentration/buffer exchange by ultra- and diafiltration, (4) nuclease treatment, and (5) a viral inactivation step by formaldehyde.

A downstream process of Vero cell culture-derived influenza vaccines is based on centrifugational steps [9]. The scheme includes: (1) clarification by low speed centrifugation, (2) concentration by ultrafiltration (300 kDa cut-off), (3) purification on a 20–60% continuous sucrose gradient, (4) inactivation by formalin, and (5) diafiltration to remove formalin. An alternative to this scheme is described by Kistner et al. [2]. Here, the slow speed centrifugational clarification is followed by an early

inactivation step (formalin) and a sequential benzonase treatment to destroy Vero cell DNA. The inactivated material is then concentrated (ultrafiltration, 200 kDa cut-off) and further clarified by protamine sulfate precipitation before purification by continuous flow zonal centrifugation over a 0–50 % sucrose gradient. Finally, the virus-containing fraction is concentrated by diafiltration and sterile filtered. Unfortunately, in all three cases, no details are provided concerning yields or contaminant elimination after the individual unit operations. However, in the last example, the residual protein content after the entire process was less than 250 µg and the level of host cell DNA less than 100 pg per dose. This reflects compliance with regulatory requirements at the time of this study.

Methods for the purification of influenza and other viruses based on centrifugation are cumbersome and technically demanding for large-scale productions of virus particles for vaccines of the requested purity. For an economic process, investment cost for the required equipment has to be balanced with operating costs of filtration and chromatography-based processes.

A generic scheme for the downstream process of MDCK cell-derived human influenza virus from Kalbfuss et al. [46] based on (1) clarification by depth filtration (0.45 µm), (2) β-PL inactivation, (3) SEC, and (4) AEC resulted in an overall virus yield of 53 %. Total protein and host cell DNA removal based on the starting material was 96.5 % and 99.8 %, respectively. The applied SEC, as judged by the authors, was efficient for the separation of host cell proteins from virions. However, even after AEC, host cell DNA reduction was not sufficient with respect to the requirements of the European Pharmacopeia. The presented scheme would have to be improved by further optimizing the AEC or introducing an additional purification step – alternatively a benzonase treatment step for further reduction in host cell DNA level. The clear advantage of this scheme is its full scalability and its virtual independence of influenza virus strains.

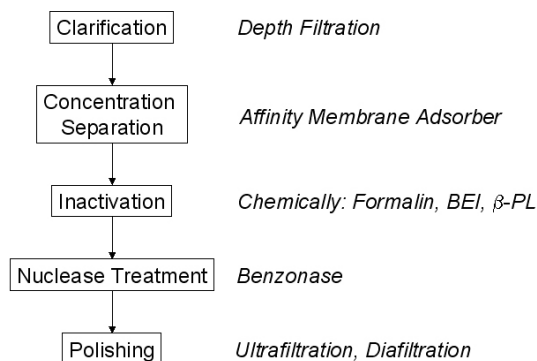
Considering the removal rate of contaminants, in particular host cell DNA, the affinity capture step suggested by Opitz et al. [41] is worth mentioning. They developed a method to capture MDCK cell-derived influenza viruses by lectin AC (EEL polymer and EEL reinforced cellulose membrane), which resulted in a host cell DNA contamination per monovalent bulk of approximately 6 ng, amounting to roughly 20 ng host cell DNA per trivalent dose. Even though the content of host cell DNA after this capture step was twice as much as the required limit, it had been achieved in a single step. Unfortunately, lectin-ligands are not an optimal choice for a production process due to their potential toxicity and the high cost of the ligands. However, alternative ligands with similar or even higher specificity are an interesting tool for a capture step in a production process of influenza or other viral vaccines. With regard to subunit or split vaccines, the remaining host cell DNA could be removed during the follow-up processes. On the other hand, a benzonase treatment might be advisable in order to ensure the absence of larger DNA fragments in the vaccine. This would also be beneficial in case host cell DNA is associated with virus particles and therefore difficult to remove by conventional techniques, as suggested for adenovirus by Konz et al. [86].

Currently, there are three principal types of influenza vaccines in use: (1) whole virus vaccines, (2) split vaccines, and (3) subunit vaccines. Subunit vaccines are based on highly purified HA and NA membrane glycoproteins while split vaccines consist of viral membrane fractions containing these antigens. Most of the unit operations described in this review focused on the purification of whole virus particles. In the case of split and subunit vaccines, the membrane fractions or the viral glycoproteins HA and NA are derived by a detergent solubilization step of separated virus particles. Hence, most methods discussed for the isolation and purification of intact influenza virus particles are important for the production of both whole virus vaccines and split and subunit vaccines.

## 6 Summary and Conclusion

This review describes a wide variety of downstream processing methods for the purification of influenza virus particles derived from eggs and cell culture systems. As a result of the different vaccine production systems and in particular due to significant differences in the analytical evaluation methods, a direct comparison of individual unit operations is almost impossible. For a comprehensive evaluation of cost-effectiveness and performance of a complete downstream process for influenza virus purification, the quantity of HA and the residual level of contaminants is relevant. However, the majority of unit operations described in this review have been evaluated by a variety of assays including infectivity and HA activity. Hence, a direct, quantitative comparison of the described downstream processes for influenza virus vaccine production is hardly possible.

Evaluating the described methods and options for the selection of chromatography materials, a visionary scheme for a generic economic influenza vaccine production process for whole inactivated influenza vaccines is shown in Fig. 2. It includes clarification to remove larger particles from the cultivation broth via depth filtration followed by an in-line capturing step of virus particles by an affinity membrane adsorber. This membrane adsorber should have high specificity for the binding of virions, a large pore size to achieve flow characteristics



**Figure 2.** Hypothetical scheme for a generic downstream process for purifying inactivated whole influenza virus particles, based on an affinity chromatography capturing step.

for high productivity, and an optimal ligand density for high capacity. The next step would be the chemical inactivation of virus particles followed by a nucleic acid digestion by a benzonase treatment. Finally, a diafiltration/ultrafiltration step could be included to further remove residual compounds and to concentrate the final harvest for blending. However, highly specific but strain independent ligands for an optimal capturing process have yet to be identified and, in case anion exchange chromatography is applied for capturing, additional purification steps have to be added.

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