Purification of Cell Culture-Derived Human Influenza A Virus by Size-Exclusion and Anion-Exchange Chromatography

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ABSTRACT: A process comprising of size-exclusion chromatography (SEC) and anion-exchange chromatography (AEC) was investigated for downstream processing of cell culture-derived influenza A virus. Human influenza virus A/PR/8/34 (H1N1) was propagated in serum-free medium using MDCK cells as a host. Concentrates of the virus were prepared from clarified and inactivated cell culture supernatants by cross-flow ultrafiltration as described before. SEC on Sepharose 4 FF resulted in average product yields of 85% based on hemagglutination (HA) activity. Productivity was maximized to 0.15 column volumes (cv) of concentrate per hour yielding a reduction in total protein and host cell DNA (hcDNA) to 35 and 34%, respectively. AEC on Sepharose Q XL was used to separate hcDNA from virus at a salt concentration of 0.65 M sodium chloride. Product yields >80% were achieved for loads >160 kHAU/mL of resin. The reduction in hcDNA was 67-fold. Split peak elution and bimodal particle volume distributions suggested aggregation of virions. Co-elution with hcDNA and constant amounts of hcDNA per dose indicated association of virions to hcDNA. An overall product yield of 52% was achieved. Total protein was reduced more than 19-fold; hcDNA more than 500-fold by the process. Estimation of the dose volume from HA activity predicted a protein content at the limit for human vaccines. Reduction of hcDNA was found insufficient (about 500 ng per dose) requiring further optimization of AEC or additional purification steps. All operations were selected to be scalable and independent of the virus strain rendering the process suitable for vaccine production.

Introduction

Influenza disease impacts every year up to 10% of the world’s population, that is, up to 500 millions of people (Gerdil, 2003). Infection is caused by influenza A and B viruses, lipid-enveloped RNA viruses that belong to the Orthomyxoviridae family. Vaccination with attenuated or inactivated viruses or viral components is the principal means of prophylaxis for human but also veterinary use. Traditionally, influenza virus is propagated in fertilized chicken eggs even at large-scale production (Bardiya and Bae, 2005). Some years ago, mammalian cells were proposed as an alternative for virus replication either using MDCK (Tree et al., 2001), Vero (Kistner et al., 1998), or PER.C6 cells (Pau et al., 2001). Similarly, the production of other vaccines in mammalian cells, for example, against rabies (Kumar et al., 2002) or Japanese encephalitis (Sugawara et al., 2002), has been reported. Viral vectors for gene therapy are routinely produced with mammalian packaging cell lines (Konz et al., 2005; Segura et al., 2005; Transfiguracion et al., 2003).

Precipitation methods for the concentration and purification of influenza virus were among the first to be described (Cox et al., 1947). In 1966, the use of gradient density centrifugation with zonal ultracentrifuges was introduced for large-scale preparations (Reimer et al., 1966, 1967). At this time influenza virus was still exclusively propagated in the allantois of fertilized chicken eggs. But even today the use of density gradient centrifugation seems to be common in the production of egg-derived and cell culture-derived influenza vaccines (Bardiya and Bae, 2005).
More recently, the use of cross-flow ultrafiltration has become popular. Ultrafiltration of cell culture-derived influenza virus was demonstrated to result in high concentration factors and product yields if operated under appropriate conditions (Kalbfuss et al., 2006; Nayak et al., 2005).

A first publication on the purification of bacteriophages by size-exclusion chromatography (SEC) on controlled pore glass (CPG) dates back to the 70s (Geschwender et al., 1969). Heyward et al. (1977) used a specifically designed CPG with pores of 729 Å for the purification of influenza virus after precipitation with polyethylene glycol. More recently, the application of SEC has been reported for turkey coronavirus (Loa et al., 2002), pseudotyped vesicular stomatitis virus (Transfiguracion et al., 2003), Moloney murine leukemia-derived retrovirus (Segura et al., 2005), and equine influenza virus (Nayak et al., 2005). Common to all studies was the recovery of virions in the void volume, that is, the pore size of the chromatography media was chosen to exclude the virus.

Whereas SEC seems to be efficient at the removal of small solutes and proteins, separation from host cell DNA (hcDNA) often remains difficult. This may be due to the size of genomic hcDNA fragments (see discussion) but also due to association of virions with hcDNA (Konz et al., 2005). The very low hcDNA burden admitted in products for pharmaceutical use typically requires the incorporation of dedicated purification steps. One common approach seems to be the digestion of hcDNA by nuclease-treatment (Konz et al., 2005; Transfiguracion et al., 2003). In other studies hcDNA was precipitated selectively from preparations of poliovirus using protamine sulphate or from adenovirus using cationic detergents (Amosenko et al., 1991; Goerke et al., 2005). Kumar et al. (2002) reported the clearance of hcDNA from cell culture-derived rabies vaccine using a second density gradient centrifugation step. Knudsen et al. (2001) evaluated the removal of hcDNA by adsorption to anion-exchange membranes operated in flowthrough mode for the process-scale purification of antibodies. Interestingly, ion-exchange chromatography was also mentioned in the context of a commercial influenza vaccine (Brands et al., 1999); however, no details on the downstream process were given.

In this study, the suitability of SEC followed by anion-exchange chromatography (AEC) was investigated for the preparation of human influenza vaccines. A diafiltration step after AEC required for desalting and final concentration was assumed but not realized (Fig. 1). Concentrates of human influenza virus A/PR/8/34 (H1N1) were prepared by cross-flow ultrafiltration as described before (Kalbfuss et al., 2006). Using these concentrates different SEC media (Sepharose CL-2B, Sepharose 4 FF, Sepharose 6 FF, and Superdex 200 p.g.) were tested for their ability to separate virus from protein and hcDNA. Separation was investigated in closer detail for Sepharose 4 FF as a function of column load. Four independent batches were processed under conditions derived from this loading study. The selectivity of two AEC media (Sepharose Q FF and Sepharose Q XL) between virus and hcDNA was compared. Sepharose Q XL was used to adsorb hcDNA from SEC-purified virus in negative mode (i.e., virus in flowthrough) after identification of the optimal salt concentration in batch adsorption experiments. Product yields on the basis of hemagglutination (HA) activity are reported for both chromatography steps. The removal of protein and hcDNA are reported wherever appropriate. Concentrates and product fractions of chromatography operations of two batches were analyzed by SDS–PAGE and agarose gel electrophoresis. Particle volume distributions were determined by dynamic light scattering analysis.

**Materials and Methods**

**Preparation of Virus Concentrates**

Virus concentrates were prepared as described before (Kalbfuss et al., 2006). Human influenza virus A/PR/8/34 (H1N1) from NIBSC (United Kingdom, Code 99-716) was propagated in roller bottles with serum-free medium (Ex-Cell MDCK, JRH Biosciences, United Kingdom, Cat.
No. 14580) using MDCK cells as a host (ECACC, United Kingdom, Code 841211903). Cell culture supernatants were clarified by a combination of depth and membrane filters (0.65 μm polypropylene mesh and 0.45 μm polyethersulfone membrane, GE Infrastructure, United States). The virus was inactivated chemically with β-propiolactone (3 mM pH 7.5, incubated at 37°C for 24 h). Inactivated supernatants were concentrated about 20-fold by cross-flow ultrafiltration (polysulfone hollow-fiber, 750-kDa cut-off, GE Healthcare, Sweden). Concentrates were either frozen at −70°C and later used in scouting experiments or processed immediately.

Hemagglutinin Stability

Stability of HA activity was investigated by titrating inactivated supernatant to different pH using 1 M sodium hydroxide or hydrochloric acid while stirring in a beaker (pH was measured with an electrode and recorded for each reaction). Titrated supernatants were incubated for 2.5, 4.5, or 23 h at room temperature and neutralized immediately afterwards. Neutralized supernatants were analyzed for HA activity.

Testing the stability with respect to salt, 200 μL of phosphate buffer (10 mM, pH 7.3) containing different concentrations of sodium chloride (NaCl) were mixed with 100 μL of SEC-purified virus (150 mM NaCl, pH 7.3) in microtiter plates. Plates were incubated for 2 h at room temperature. After incubation, 100 μL of each reaction were transferred into a sample tube and diluted 1:10 in appropriate phosphate buffer to reduce the concentration of NaCl to 150 mM. Diluted reactions were analyzed for HA activity.

Batch Adsorption

Batch adsorption was conducted in microtiter plates. Unused Sepharose Q XL resin (GE Healthcare Bio-Sciences, Sweden) was washed with water three times and adjusted to an approximate volume fraction of 62.5% in a graduated cylinder. Eighty microliters of resin slurry (approximately 50 μL of packed volume) were mixed with 120 μL of phosphate buffer (10 mM, pH 7.3) containing different concentrations of NaCl. In the next step, 100 μL of SEC-purified virus (dissolved in 150 mM NaCl, 20 mM phosphate buffer, pH 7.3) were added to each well resulting in a final volume of 300 μL. Plates were incubated in a Thermomixer Comfort (Eppendorf, Germany) for 2 h at room temperature shaking with 1,000 rpm. After incubation, 100 μL of each reaction were transferred into a sample tube and diluted 1:10 in appropriate phosphate buffer to reduce the concentration of NaCl to 150 mM. Diluted samples were analyzed for HA activity and hDNA content.

Chromatography

An ÄKTaexplorer 100 was used for column chromatography. SEC media (Sepharose CL-2B, Sepharose 4 FF, Sepharose 6 FF, and Superdex 200 p.g.) were packed into XK 16/40 or Tricorn 10/30 columns. AEC media were packed into Tricorn 10/15 columns. First experiments with ion-exchangers were conducted with HiTrap screening columns (all from GE Healthcare Bio-Sciences, Sweden). Void volumes were determined with BSA-coated latex beads of 100-nm diameter (Postnova Analytics, Landsberg, Germany) or estimated from the virus-containing peak. The packed bed of each column was tested regularly by determination of the asymmetry and HETP with acetone (2% v/v dissolved in eluent). Specifications of columns are summarized in Table I. Columns were sanitized with 0.5 M sodium hydroxide for at least 1 h before and after experiments. Columns were stored in 20% (v/v) ethanol at room temperature. The feed material (i.e., virus concentrates or pooled SEC eluate fractions) was injected using a 50 or 150 mL superloop (GE Healthcare Bio-Sciences, Sweden). Eluates were either fractionated by a Frac-950 fraction collector (GE Healthcare Bio-Sciences, Sweden) or using the outlet valve of the chromatography system.

Table I. Specifications of chromatography columns.

| #  | Medium          | Exclusion limita | Hb (cm⁻¹) | Voc (mL) | ε² | HETPe (μm) | Asym, f |
|----|-----------------|-----------------|-----------|---------|----|------------|---------|
| 1  | Sepharose CL-2B | 30 nm           | 31.5      | 63.3    | 0.38 | 208        | 1.09    |
| 2  | Sepharose 4 FF  | 30 MDa          | 23        | 46.2    | 0.36 | 405        | 1.16    |
| 3  | Sepharose 6 FF  | 4 MDa           | 25        | 50.3    | 0.34 | 256        | 1.08    |
| 4  | Superdex 200 p.g.| 500 kDa        | 23        | 46.2    | 0.41 | 103        | 1.88    |
| 5  | Sepharose 4 FF  | 30 MDa          | 30        | 23.6    | 0.36 | 271        | 1.07    |
| 6  | Sepharose 4 FF  | 30 MDa          | 31        | 62.3    | 0.35 | 193        | 1.05    |
| 7  | Sepharose Q FF  | —               | 15.7      | 3.1     | —   | 760        | 1.22    |
| 8  | Sepharose Q XL  | —               | 15.7      | 3.1     | —   | 956        | 1.04    |

aHydrodynamic diameter or molecular weight of globular protein excluded from the medium.
bBed height.
cVolume.
dPorosity of column packing.
eHeight of one theoretical plate.
fAsymmetry of the elution peak for acetone.
SEC runs were conducted at a constant flow rate of 60 cm/h using phosphate buffer (20 mM, pH 7.3) containing up to 0.65 M NaCl as eluent. Complete batches were processed automatically by multiple injection-elution cycles. The product fractions from each run were pooled and loaded onto an AEC column. Presentation over $K_v = (elution volume - void volume)/(column volume - void volume)$ instead of elution volume or retention time was preferred in scouting experiments for better comparability between chromatography media. Area normalized concentrations (i.e., density distributions) instead of absolute concentrations were used for plotting HA activity, DNA, and total protein such that areas below the curves can be compared between different analytes.

AEC runs were operated at a constant flow rate of 200 cm/h. The same eluent as for SEC was used to equilibrate columns and wash out unbound feed material. Virus was collected in the flowthrough. Adsorbed hcDNA and other impurities were desorbed by a salt gradient up to 1.5 M NaCl. Columns were cleaned afterwards by a mixture of 0.1 M hydrochloric acid and 2 M sodium chloride followed by a mixture of 1 M sodium chloride and 1 M sodium hydroxide (5 cv/23 min contact time and 7 cv/32 min contact time, respectively).

Analytical Methods

Analytical methods were performed as previously described (Kalbfuss et al., 2006; Wickramasinghe et al., 2005). Briefly, total protein was quantitated colorimetrically using the Coomassie dye-binding assay. hcDNA was quantitated by the fluorescence enhancement of PicoGreen upon binding to double-stranded DNA. Product fractions of both chromatographic purification steps were analyzed by SDS–PAGE and agarose gel electrophoresis. Particle volume distributions were obtained by dynamic light scattering analysis. A refractive index of 1.340 and a dynamic viscosity of 1.075 mPa·s were determined for the SEC eluent (650 mM NaCl + 20 mM phosphate buffer, pH 7.3).

HA activity served as a measure for the virus content. An improved assay format involving measurement of light extinction at 700 nm and non-linear regression gave rise to an analytical error <15% (confidence interval, $\alpha = 0.05$). HA activity was measured immediately after experiments since freezing in phosphate buffer led to significant losses in activity.

Due to unavailability of an A/PR/8/34 (H1N1) reference antigen for calibration, ELISA or SRID measurements could not be established. The concentration of hemagglutinin protein therefore had to be estimated from HA activity. The content was calculated under the assumption of one virion bound per erythrocyte at the agglutination-endpoint (Donald and Isaacs, 1954). According to the composition of virions reported by Knipe and Howley (2001) the total protein mass of influenza virions sums up to $2.66 \times 10^8$ g/mol. The fraction of hemagglutinin protein was determined to be 36.7% for egg-derived A/PR/8/34 (H1N1) by Oxford et al. (1981). This is in good agreement with the theoretically derived 35% based on calculations with values from Knipe and Howley (data not shown). Since the concentration of erythrocytes used for the assay was adjusted to $2 \times 10^8$ mL$^{-1}$, the virus particle concentration can be estimated according to $N_v = a_{\text{HA}}/(\text{HAU} (0.1 \text{ mL})^{-1}) \times 2 \times 10^8$ mL$^{-1}$ where $a_{\text{HA}}$ is the specific (i.e., per volume) HA activity. Using Avogadros number for conversion, the total protein mass of virions and the mass fraction of hemagglutinin, the mass concentration of hemagglutinin can be estimated by $c_{\text{HA}} = N_v/6.023 \times 10^{23} \times 2.66 \times 10^8$ g mol$^{-1} \times 0.367$. A specific HA activity of 1 HAU/100 μL thus corresponds to a mass concentration of 3.24 ng/mL.

Results

Size-Exclusion Chromatography

SEC was operated in group separation mode (separation of void fraction from retarded molecules). In scouting experiments, columns 1–4 (packed with Sepharose CL-2B, Sepharose 4 FF, Sepharose 6 FF, and Superdex 200 p.g.) were loaded with 10% of their column volume (cv). Virus concentrate (produced by cross-flow ultrafiltration) from the same batch was used. UV absorbance at 280 nm was recorded online. Eluates were fractionated coarsely and analyzed offline for HA activity, total protein, and hcDNA (Fig. 2). The UV trace of all runs showed two distinct peaks corresponding to the $K_v$ of the void volume and small solutes (data not shown). Between the peaks the UV trace did not reach the baseline. According to offline-measured HA activity partial retention of the virus population occurred with Sepharose CL-2B (largest pore size). For all other media, the virus eluted exclusively in the void volume. Protein and hcDNA eluted between the two peaks detected by the UV monitor. A shift was observed from larger to smaller $K_v$ with decreasing pore size of the chromatography medium. In the case of Superdex 200 p.g. (smallest pore size) almost no separation between the virus and protein or hcDNA occurred.

Viral and host cell protein were not distinguishable by the protein assay used. The correlation of HA activity and total protein in the first fractions of runs with Sepharose CL-2B and Sepharose 4 FF, however, suggested that the assay was mainly detecting viral protein in these fractions. According to this correlation, best separation of the virus from impurities was achieved with Sepharose CL-2B. Nevertheless, Sepharose 4 FF was chosen for preparative runs since elution of the virus occurred exclusively in the void volume. Elution in the void volume leads to narrower peaks (absence of mass transfer, no spread due to the size distribution of the virus population), and hence less dilution of the product. In addition, Sepharose 4 FF is more rigid than Sepharose CL-2B and therefore better to handle (easier column packing, higher flow rates).
In the next step, the column load (injection volume) was optimized for a good compromise between productivity and purity of the product. Column 5 was loaded with 0.07, 0.14, and 0.28 cv using virus concentrate from the same batch. Again, UV absorbance was recorded at 280 nm and fractionated eluates were analyzed offline (Fig. 3). Only the total protein concentration was considered for optimization since hCDNA can be removed more efficiently in the anion-exchange step. Similar to the scouting experiment with Sepharose 4 FF, HA activity seemed to correlate with total protein within the first fractions. Due to the higher resolution (smaller fraction size), however, the correlation was more evident. According to the profiles no baseline separation between viral protein and host cell protein could be achieved (even at the lowest load). Separation became worse with increasing volume load due to resulting peak broadening.

Based on the results fractionation limits for viral preparations were defined such that approximately 95% of eluted HA activity is recovered in the product fraction (Table II). The starting fractionation limit was set to 0.3 cv independent of the load. The ending fractionation limit was calculated according to the load adding 0.14 cv for axial dispersion. Pools of the collected fractions assigned to the product fraction contained 93% of eluted HA activity on average. The amount of eluted total protein in the product fraction increased from 35% for a load of 0.07 cv to 48% for a load of 0.28 cv. Productivity increased from 0.08 to 0.27 cv of feed per hour while dilution of the product decreased from 0.32 to 0.64 (a yield of 95% was assumed for calculation). Depending on process requirements, productivity needs to be traded off versus purity. The optimum clearly depends on product specifications. In the case of this study an arbitrary choice was made selecting a moderate load of 0.15 cv (corresponding to a productivity of 0.15 cv of feed per hour).

In order to test the maximum number of injections before regeneration of SEC columns becomes necessary, column 5 was loaded 13 times with 0.15 cv of virus concentrate. Virus was collected from 0.30 to 0.58 cv elution volume. Individual runs were assigned to five groups and the pooled product fraction of each group was analyzed for HA activity. An overlay of all UV traces is given in Figure 4. The traces were almost indistinguishable. The specific HA activity of the pools remained constant within the precision of the assay. The unit operation was therefore considered non-critical with respect to column cleaning. With respect to process hygiene, however, intermediate sanitization may be advisable in particular if the feed cannot be guaranteed to be sterile.

For viral preparations, column 6 was loaded multiple times with 0.15 cv of concentrate. Only two fractions were collected: the product and the waste fraction. The product fraction was collected from 0.30 to 0.58 cv elution volume,
the waste fraction from 0.58 to 1.91 cv. Typically, three to five injections were necessary to process one batch. Specific HA activities, protein and hcdNA concentrations from four independent preparations were summarized in Table III. The product yield (based on HA activity) was 85% on average (corresponding to 95% of eluted HA activity). Total recoveries were slightly higher with 89% on average. About 10% of loaded HA activity was lost. The amount of total protein and hcdNA in the product fraction was reduced to 35 and 34% of the loaded amount, respectively. It needs to be mentioned, however, that the material balances for protein and hcdNA were typically not closed. On average, total recoveries for protein and hcdNA were 69 and 136%, respectively (see discussion).

Anion-Exchange Chromatography

Two experiments were conducted to investigate the stability of HA activity. In order to find a pH working range for AEC, inactivated cell culture supernatant was titrated to various pH values and incubated at room temperature for different periods of time. After neutralization, HA activity was analyzed and compared (Fig. 5). The results revealed that HA activity was only stable within a narrow range between pH 7 and 8. At higher or lower pH degradation occurred. Under alkaline conditions the rate of degradation increased with increasing pH. Under acidic conditions there was a maximum close to pH 5. Below this maximum the rate of degradation reduced again. In contrast, ionic strength

| Load (cv) | Prod. fractionb | Yieldsc |
|-----------|-----------------|---------|
| Start (cv) | Stop (cv) | HA % (eluate) | Protein % (eluate) | Run timed (h) | Prod.e (cv/h) | Dilutionf |
| 0.07 | 0.3 | 0.51 | 87 | 35 | 0.92 | 0.08 | 0.32 |
| 0.14 | 0.3 | 0.58 | 96 | 44 | 0.95 | 0.15 | 0.48 |
| 0.28 | 0.3 | 0.72 | 96 | 48 | 1.02 | 0.27 | 0.64 |

anormalized feed volume loaded onto the column.
bNormalized fractionation limits of the product fraction.
cPercentage yield of eluting HA activity and total protein.
dDuration of one injection-elution cycle.
eNormalized feed volume processed per hour.
fDilution of the product assuming a yield of 95%.

Figure 4. Overlay of UV traces (measured at 280 nm) from 13 subsequent injections on column packed with Sepharose 4 FF. Column was loaded with 15% of its volume using virus concentrate from the same batch. No change in the separation behavior was observed.

Table II. Yield, productivity, and dilution in size-exclusion chromatography on Sepharose 4 FF as a function of the column load.

| Load (cv) | Prod. fraction | Yields |
|-----------|----------------|---------|
| Start (cv) | Stop (cv) | HA % (eluate) | Protein % (eluate) | Run time (h) | Prod. (cv/h) | Dilution |
| 0.07 | 0.3 | 0.51 | 87 | 35 | 0.92 | 0.08 | 0.32 |
| 0.14 | 0.3 | 0.58 | 96 | 44 | 0.95 | 0.15 | 0.48 |
| 0.28 | 0.3 | 0.72 | 96 | 48 | 1.02 | 0.27 | 0.64 |

Table III. Material balances of preparative size-exclusion chromatography on Sepharose 4 FF.

| Lot | Feed | Product eluate fraction |
|-----|------|-------------------------|
|     | V (mL) | $\dot{q}_{\text{HA}}$ (kHAU/100 µL) | $\dot{q}_{\text{prot}}$ (µg/mL) | $\dot{q}_{\text{DNA}}$ (µg/mL) | V (mL) | $\dot{q}_{\text{HA}}$ (kHAU/100 µL) | $\dot{q}_{\text{prot}}$ (µg/mL) | $\dot{q}_{\text{DNA}}$ (µg/mL) | $N_m$ |
| 1 | 37 | 3.31 | 185 | 37.1 | 69 | 1.73 (96%) | 33.1 (33%) | 6.62 (33%) | 4 |
| 2 | 28 | 4.27 | 167 | 20.2 | 51 | 1.89 (81%) | 32.5 (36%) | 4.95 (45%) | 3 |
| 3 | 47 | 2.72 | 200 | 53.9 | 86 | 1.17 (79%) | 33.0 (30%) | 7.52 (26%) | 5 |
| 4 | 47 | 3.24 | 146 | 30.5 | 86 | 1.50 (85%) | 32.3 (40%) | 5.39 (32%) | 5 |
| Mean ± SD | 3.38 ± 0.64 | 174 ± 23 | 35.4 ± 14.1 | 1.57 ± 0.31 | 32.7 ± 0.4 | 6.12 ± 1.17 | (85%) | (35%) | (34%) |

Virus concentrates from ultrafiltration were purified by size-exclusion chromatography. Three to five injections were necessary to process one lot. Average activities and concentrations are reported for each lot. Yields of analyzed species are given in brackets below. Mean values and standard deviations for all lots are provided at the bottom.
aVolume.
bSpecific HA activity.
cTotal protein concentration.
dDNA concentration.
eNumber of injections required to process lot.
beyond physiological conditions did not seem to affect HA activity. Adding NaCl up to 1.5 M to SEC-purified virus concentrate resulted in identical HA activities after incubation for 2 h at room temperature (data not shown). Ionic strength was therefore chosen to control the adsorption-desorption process in AEC.

Two chromatography media (Sepharose Q FF, Sepharose Q XL) were considered for the separation of hcDNA from virus. NaCl already used in stability experiments was chosen as displacer, the pH was stabilized at 7.3 using phosphate buffer. Based on experiments with HiTrap screening columns (data not shown), the feed (SEC-purified virus concentrate) was conditioned to an initial salt concentration of 0.4 M NaCl. Columns 7 and 8 were loaded with 1.6 cv of concentrate from the same batch. After washing with buffer containing 0.4 M NaCl, a linear gradient over 10 cv was run from 0.4 to 1.5 M NaCl. The flowthrough and eluate of each run were fractionated and analyzed offline for HA activity and hcDNA (Fig. 6). In contrast to experiments with screening columns most of the virus still adsorbed to both anion-exchangers (little virus in flowthrough). Displacement by NaCl lead to elution of the virus over a wide range of salt concentration. Elution of the virus from Sepharose Q XL resulted in a split peak possibly indicating distinct viral populations or association with hcDNA. For both media, hcDNA eluted in a sharp peak overlapping with part of the virus peak. Higher NaCl concentrations were necessary to displace hcDNA from Sepharose Q XL leading to better separation from the virus. Respectively, Sepharose Q XL was selected for subsequent experiments.

Since results from experiments with screening columns were not transferable (most likely due to lower concentrations of virus and DNA or different concentration ratios in the feed material and the shorter column length), the optimum salt concentration to prevent adsorption of virus was determined in batch experiments. Sepharose Q XL was mixed with SEC-purified virus concentrate in a microtiter plate at various salt concentrations. The specific HA activity or hcDNA concentration was about half the activity or concentration found in column chromatography. The ratio, however, was identical. The amount of virus added corresponded to a load of 35 kHAU/mL of resin. After incubation for 2 h at room temperature samples were taken from the supernatants and analyzed for HA activity and hcDNA. The desorbed fraction (i.e., the fraction in the supernatant) was calculated for each salt concentration as the ratio of HA activity or hcDNA concentration to values at 1.5 M NaCl (Fig. 7). Only at the lowest concentration of 0.15 M NaCl virus adsorbed completely to the stationary phase whereas complete adsorption of hcDNA was observed up to a concentration of 0.7 M NaCl. At this concentration only 15% of the virus was adsorbed.

Preparative runs where conducted with column 8. The column was fed with SEC-purified virus conditioned to 0.65 M NaCl. A linear gradient was run from 0.4 to 1.5 M NaCl (−, calculated from conductivity). Flowthrough and eluate fractions were analyzed for HA activity ( ) and hcDNA (−). Co-elution of hcDNA was observed for both chromatography media. Split peak elution in the case of Q XL suggested two virus populations and possibly association of virus with hcDNA.

**Figure 5.** Stability of HA activity with respect to pH. Inactivated cell culture supernatant was titrated to different pH (3.0–10.6). Reactions were neutralized after incubation for 2.5 (■), 4.5 (□), and 23 h (□) at room temperature and HA activity was determined. Values normalized by the average activity at pH 7 are reported. HA activity was found to be stable only within a narrow range around physiological pH (7–8).

**Figure 6.** Selectivity of Sepharose Q FF and Q XL between virus and hcDNA. Columns were loaded with 1.6 column volumes (cv) of SEC-purified virus conditioned to 0.4 M NaCl. A linear gradient was run from 0.4 to 1.5 M NaCl (−, calculated from conductivity). Flowthrough and eluate fractions were analyzed for HA activity ( ) and hcDNA (−). Co-elution of hcDNA was observed for both chromatography media. Split peak elution in the case of Q XL suggested two virus populations and possibly association of virus with hcDNA.
about 6 µg/mL in the feed). Even at the highest load no breakthrough of hcDNA was observed. Consequently, no data on the dynamic binding capacity of the column can be provided. Adsorbed impurities were displaced by a step gradient to 1.5 M sodium chloride and two cleaning solutions (acidic and alkaline high salt solutions). The chromatogram of lot 4 containing offline HA activity and hcDNA data is given in Figure 8. The product yield (based on HA activity) seemed to depend on the amount of virus loaded. For loads greater than 160 kHAU/mL of resin product yields of 80% and more could be achieved similar to the result obtained in the batch adsorption experiment. The removal of hcDNA was efficient in all cases leading to 67-fold (1.8 orders of magnitude) reduction on average. The amount of protein was reduced to 67% of the initial amount.

**Analysis of Product Fractions**

Samples of product fractions from two independent downstreams (ultrafiltration and chromatography) were analyzed by SDS–PAGE and agarose gel electrophoresis. Since the results were similar, only gels of samples from one downstream (lot 4) are presented in Figure 9a.

The reduction of protein impurities (originating from the cell culture medium and from lysis of host cells) can be followed on the protein gel. In contrast, several viral proteins were enriched. Among these the hemagglutinin protein (HA) at approximately 66 kDa and the influenza virus matrix protein (M1) at approximately 26 kDa could be identified by tryptic digestion and tandem mass spectrometry (data not shown). Some of the high weight components between 100 and 250 kDa could be identified as oligomers of the HA and neuraminidase (NA) proteins indicating that the denaturation in Laemmli buffer was incomplete. Even though SEC was used to separate small colloids from the virus, small proteins appeared in the product fraction. Since SDS–PAGE is a denaturing technique, complexes (e.g., virions) and aggregates were broken down and their components released. Remarkably, a small protein (or group of proteins) <14 kDa that was not separated by SEC disappeared completely after the anion-exchange step.

Concerning the hcDNA, a shift in the size distribution was observed in agarose gels after purification by SEC. Fragments below 500 bp were completely removed. The maximum of the intensity shifted from about 650 bp to 1,000 bp. Since similar amounts of hcDNA were loaded also

### Table IV. Yields and purity after anion-exchange chromatography on Sepharose Q XL

| Lot | Load (kHAU/mL) | V (mL) | cHA (µg/mL) | cprot (µg/mL) | cDNA (µg/mL) | Protein (µg/dose) | DNA (ng/dose) |
|-----|----------------|-------|--------------|---------------|--------------|------------------|---------------|
| 1   | 28             | 12.0  | 0.16 (63%)   | n.a.          | 0.02 (0.6%)  | 0.53             | 485           |
| 2   | 161            | 35.3  | 0.52 (84%)   | n.a.          | 0.07 (2.0%)  | 1.67             | 620           |
| 3   | 94             | 28.0  | 0.60 (58%)   | 18.2 (64%)    | 0.07 (1.0%)  | 1.93             | 513           |
| 4   | 239            | 56.0  | 1.05 (80%)   | 20.0 (69%)    | 0.10 (2.1%)  | 3.39             | 455           |
| Mean ± SD |       |       | 19.1 ± 1.3   | 0.06 ± 0.04   | 115          | 518              |

hcDNA in SEC-purified virus was removed by anion-exchange chromatography (run in negative mode, i.e., virus in flowthrough). Yields of analyzed species are given in brackets. Mean values and standard deviations for all lots are provided at the bottom. Statistics of HA activity were omitted due to differences in the load. Hemagglutinin concentrations were estimated from HA activities according to the correlation described in Materials and Methods. A dose containing 15 µg of hemagglutinin protein was assumed for calculation of impurities.

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**Figure 7.** Optimum concentration of NaCl for the separation of virus from hcDNA. SEC-purified virus was adjusted to different salt concentrations and incubated in the presence of Sepharose Q XL. HA activity (■) and hcDNA (○) were measured in supernatants after incubation for 2 h at room temperature. Fractions of desorbed virus and hcDNA (i.e., in supernatants) were calculated for each salt concentration. Best separation was achieved around 0.7 M NaCl when 85% of virus was found in the supernatant while hcDNA was quantitatively adsorbed.

**Figure 8.** Chromatogram of lot 4 containing offline HA activity and hcDNA data.

**Figure 9a.** Analysis of Product Fractions: SDS–PAGE and agarose gel electrophoresis.
larger fragments became visible that had been below the limit of detection before. The residual hcDNA after AEC could not be visualized due to the very low concentrations.

Particle volume distributions obtained by dynamic light scattering analysis revealed bimodal distributions with a first maximum around 100 nm and a second maximum varying from 300 to 500 nm (Fig. 9b). Whereas the diameter of the first maximum was stable, the diameter of the second maximum seemed to increase over the process. This could indicate some kind of aggregation of virions. Indeed, analysis of virus concentrates stored at 4°C resulted in an increase of the mean diameter within a few days (data not shown). This increase was accompanied by a decrease in HA activity.

Discussion

Size-Exclusion Chromatography

SEC was used to separate small solutes and colloids from the virus. Elution from all four chromatography media led to a characteristic double peak pattern in the UV trace similar to the profiles presented for Sepharose 4 FF (Fig. 3). Virus was eluted in the first peak (void fraction) whereas the second peak resulted from the elution of small solutes (total inclusion; most likely amino acids, nucleotides, etc.) Similar elution profiles have been reported for human influenza virus from chicken egg (Heyward et al., 1977), equine influenza virus from mammlian cell culture (Nayak et al., 2005), and turkey corona virus (TCoV) propagated in turkey embryo (Loa et al., 2002) despite the use of different chromatography media and feed material. In the case of vesicular stomatitis virus (VSV-G) produced by packaging cell lines three peaks were observed upon elution from Sepharose CL-4B (Transfiguracion et al., 2003). In all studies the virus was concentrated prior to injection.

Noteworthy, strong tailing of the virus (detected by offline-measured HA activity) was observed upon elution
from Sepharose CL-2B. This finding suggests that part of the virus population (or deteriorated virions) was able to enter a fraction of the pores. In addition, mass transfer effects (due to the large size of influenza virions) may have enhanced tailing of the elution peak. A phenomena which was described by Li et al. (1998) using the general rate model of chromatography. Strong tailing was predicted for low values of the non-dimensional parameter η. The parameter η is proportional to the product of pore diffusivity and accessible pore volume fraction. In the case of particles close to the exclusion limit (i.e., low accessible pore volume fraction) the pore diffusivity (Knudsen diffusion) is also low due to a diameter ratio of particles and pores close to unity; thus leading to low values of η and tailing elution peaks.

The UV trace was completely misleading with respect to the elution of protein and hcDNA. Both eluted mostly between the two peaks solely indicated by a slight absorbance above zero (for comparison of total protein and UV absorbance see Fig. 3). Absorbance levels between the two peaks in elugrams of egg-derived human influenza virus (Heyward et al., 1977) and TCoV (Loa et al., 2002) seem to suggest a similar situation there.

hcDNA appeared to be slightly larger and consequently eluted earlier than host cell protein on average if one assumes that total protein correlating with HA activity is virus protein (Fig. 2). Agarose gel electrophoresis of samples taken from the product fractions of preparative SEC runs revealed an average hcDNA fragment length of 1 kb (compared to linearized DNA of the ladder) with some fragments ranging up to 2 kb (Fig. 9a). Potschka (1991) reported the length-size correlation $R_{SEC} = 0.24 \text{ (bp)}^{0.25} \text{nm}$ for linearized DNA at an ionic strength of 200 mM which is based on measurements with a calibrated SEC column. Applying this correlation, the fragment size of 1 kb translates into a hydrodynamic radius of 42 nm which is about the average radius of influenza virions (Knipe and Howley, 2001). Thus, complete separation of virions from hcDNA cannot be achieved by size-fractionation techniques.

**Table V.** Overview of SEC protocols reported in the literature.

| Virus  | Medium   | $H^a$ (cm) | $V_{col}^b$ (mL) | $V_{mg}^c$ (cv) | $u^d$ (cm/h) | Dur.$^e$ (h) | Prod.$^f$ (cv/h) | Yield$^g$ (%) | Reference                  |
|--------|----------|------------|-----------------|----------------|-------------|-------------|----------------|---------------|---------------------------|
| dX174  | CPG      | 100        | 79              | 0.10           | 153         | 1.22        | 0.084          | n.a.$^h$      | Geschwender et al. (1969) |
| TCoV   | Sephacyl S-1000 | 95        | 466            | n.a.           | 6           | 5.94        | 0.002          | n.a.          | Loa et al. (2002)         |
| VSV-G  | Sepharose CL-4B | 40       | 80             | 0.012          | 12          | n.a.$^h$    | n.a.$^h$       | 70            | Transfiguracion et al. (2003) |
| MoMLV  | Sepharose CL-4B | 53       | 106            | 0.07           | 15          | 6.4         | 0.011          | 53            | Segura et al. (2005)      |
| Influenza | CPG      | 100        | 64             | 0.05           | 94          | 1.9         | 0.025          | 80            | Heyward et al. (1977)     |
| Influenza | Sepharose CL-2B | 95       | 191            | 0.10           | 30          | 5.9         | 0.018          | 38            | Nayak et al. (2005)       |
| Influenza | Sepharose 4 FF | 31       | 62             | 0.15           | 60          | 1.0         | 0.15           | 85            | Current study             |

Productivities were estimated from column dimensions, injection volumes and flow rates reported. One run was assumed to last for the elution of 1.76 cv in addition to the injected volume.

$^a$Bed height.

$^b$Volume of column packing.

$^c$Injection volume.

$^d$Superficial flow velocity.

$^e$Duration of single run.

$^f$Productivity.

$^g$Product yield.

$^h$Not available.

Due to the separation principle, volume overloading of SEC columns causes peak broadening and has to be limited. In addition, operation is restricted to relatively low flow rates (typically less than 60 cm/h) in order to maintain mass transfer limitations at an acceptable level. SEC operations hence often pose a bottleneck in downstream processes (for a comparison of productivities see Table V). In this study a high load of 0.15 cv was applied to a short column (30 cm) operated at a rather high flow rate (60 cm/h). The resulting productivity of 0.15 cv/h was sixfold higher than the productivity estimated for Heyward et al. (1977) and more than eightfold higher than the productivity estimated for Nayak et al. (2005). Still an overall 19-fold reduction in total protein was achieved after SEC (see below). Significant savings due to the lower amount of chromatography media required or due to faster processing can be expected. The potential of operating at even higher flow rate (and thus increasing productivity further) has not been explored yet but may be done so in future studies.

Viral preparations under conditions deduced from the loading study constantly resulted in yields ≥80% (Table III). On average 96% of eluted HA activity (corresponding to 85% of loaded HA activity) could be recovered in the product fraction which is in very good agreement with the 95% originally targeted. The loss of 10% of loaded HA activity can be explained by denaturation, aggregation, or non-specific adsorption of virions to the stationary phase. A similar product yield of 80% (based on HA activity and infectious activity) was reported by Heyward et al. (1977) for the purification of influenza virus on CPG (Table V). In contrast, the yield obtained by Nayak et al. (2005) was much lower (38%) using Sepharose CL-2B which was a result of strong tailing of the virus peak. But also total recovery was lower with 70% compared to 89% in this study. Yields for vesicular stomatitis virus and moloney murine leukemia virus purified on Sepharose CL-4B were both lower with 70 and 53%, respectively. However, these yields were based on infectivity which may be less stable than HA activity.
On average, total protein could be reduced to 35% of the amount loaded. Overall reduction was found to be 19-fold after SEC (Table VI). A bias in this result, however, cannot be ruled out. Total recovery of protein was only 69% on average, which was ascribed to matrix interference. Components of the cell culture medium had been found to interfere with the protein assay before. For samples from supernatants or concentrates this was compensated by preparation of standard solutions in cell culture medium. Such compensation was not applicable to SEC eluate fractions, however, since their composition is undefined. Underestimation of the protein concentration was therefore likely upon co-elution with interfering substances leading to a total recovery of less than 100%. If interfering substances were assumed to be small, correct estimation of the total protein concentration would be expected for virus eluate fractions and underestimation for waste fractions. But this remains speculative. The situation for hcDNA was different. Here, a total recovery of 136% was found on average. A contribution from non-specific background fluorescence was ruled out by measurement of appropriate blanks (sample without dye). In contrast, repeated measurement of concentrates at various dilutions resulted in slightly higher hcDNA concentrations at higher dilution. It was thus concluded that interference by the cell culture medium was not compensated completely by the calibration procedure (same as for protein described above). As a consequence, underestimation of the hcDNA concentration in virus concentrates occurred and led to a total recovery of more than 100%. Hence, the estimation of hcDNA concentrations in virus eluate fractions would have been correct but the degree of hcDNA depletion underestimated.

**Anion-Exchange Chromatography**

Stability experiments revealed sensitivity of HA activity to acidic and alkaline pH. Interestingly, degradation was fastest around a pH of 5 and slowed down towards lower pH. It was therefore concluded that degradation in this range was not unspecified (unfolding, hydrolysis, etc.) but occurred due to a molecular mechanism responsible for endosomal membrane fusion upon infection. This mechanism was reported to involve an irreversible change in the conformation of hemagglutinin protein triggered by the low pH in endosomes (Knipe and Howley, 2001). A similar finding for low pH was reported by Scholtissek (1985). Infection of chicken embryo cells by infectious allantoic fluid was impaired if allantoic fluids were treated at low pH for 1 h before. The threshold pH investigated for 34 subtypes was reported to be in the range of 5–6. In contrast, high concentrations of NaCl did not show any effect on HA activity despite the dramatic change in osmotic pressure. This can possibly be explained by stabilization of the envelope by viral matrix protein.

Tested AEC media Sepharose Q FF and Q XL displayed different selectivity with respect to virus and hcDNA. Using Sepharose Q FF, virus and hcDNA did not seem to be separable at concentrations found in the feed. Possibly, separation could have been achieved using a different displacer (e.g., NO$_3^-$, HPO$_4^{2-}$, etc.) or taking advantage of mass transport effects (variation in flow rate). Since displacement is a competitive effect it is reasonable to assume that different displacers may yield different selectivity (depending on their affinity to the resin and their mobility). Under highly saturated conditions hcDNA may even displace the virus itself since the charge density (one negative charge per base pair) can be expected to be much higher than that of virions. At the laboratory scale, however, such conditions could not be achieved with the amount of feed available.

The effect of the flow rate (i.e., mass transfer effects) should in theory be negligible since both types of colloids (DNA and virions) are similar in size. DNA, however, is a random coil whereas virions are closer to a rigid sphere. It can be speculated that “tentacles” protruding from the DNA

### Table VI. Step and overall yields of investigated downstream process.

| Operation | HA/% | Turbid./% | Protein/% | DNA/% |
|-----------|------|---------|-----------|-------|
|           | Step | Over.   | Step      | Over. |
| Cultivation | —    | 100     | —         | 100   |
| DF        | 85   | 85      | 8.7       | 8.7   |
| MF        | 93   | 79      | 24        | 2.1   |
| UF        | 97   | 77      | —         | —     |
| SEC       | 85   | 65      | —         | —     |
| AEC       | 82   | 53      | 35        | 5.2   |

Step and overall yields are reported for the following unit operations: depth filtration (DF), microfiltration (MF), ultrafiltration (UF), size-exclusion chromatography (SEC), and anion-exchange chromatography (AEC). Performance of filtration operations has been reported previously (Kalbfuss et al., 2006). Losses during inactivation with β-propiolactone (between DF and MF) were not considered. A sufficiently high load in AEC and hence an average product yield similar to lots two and four was assumed. The first available measurement was defined as 100%. Turbidity was measured as extinction at 700 nm. Total protein and hcDNA were only measured after inactivation. Final process yields are given in bold type.

- Turbidity.
- Number of processed batches.
- Overall process yield after unit operation.
coil may facilitate adsorption (even if the DNA cannot enter the pores) and result in more favorable adsorption kinetics.

In contrast to Sepharose Q FF partial separation was achieved with Sepharose Q XL. Split peak elution of virus and co-elution of the second virus peak with hcDNA, however, indicated the presence of two distinct populations. Batch adsorption experiments demonstrated a similar behavior of a bimodal distribution of desorbed virus.

Conditioning of feed to 0.65 M NaCl resulted in the expected flowthrough of virus. Recoveries >80% were achieved with Sepharose Q XL for loads ≥160 kHAU/mL of resin which was in good agreement with the batch adsorption experiment (86% at 0.7 M NaCl). Reduced yields at lower loads most likely were the result of residual binding capacity of the stationary phase. After saturation most of the virus could be recovered in the flowthrough leading to high yields at high loads. Whether HA activity really is an appropriate measure for the load remains to be investigated.

On average, hcDNA was reduced to 1.5% of the amount loaded. Overall reduction was more than 500-fold (2.7 orders of magnitude) after AEC (Table VI). Interestingly, the residual amount of hcDNA per dose did not depend on the product yield. The product yield, however, was a strong function of the load. In addition to split peak elution and results of the batch adsorption experiment this is another indicator that part of the hcDNA was associated to virions and hence co-purified during chromatography. A similar situation had been reported by Konz et al. (2005) for the purification of adenovirus. It could be shown that hcDNA in virus-containing lysates was only completely digestable by DNase after treatment with detergent and high salt buffer. They further reported, that the addition of detergent to chromatography buffer was necessary to prevent aggregation of virions. These aggregates could be readily detected by dynamic light scattering measurements similar to aggregates observed in product fractions of different operations (Fig. 9b). It can be speculated that disruption of these aggregates would lead to higher purity (less hcDNA) and higher product yields.

Surprisingly, the amount of total protein was reduced despite the high salt concentration, too. This loss can be explained by a loss in viral protein in the case of lot 3. In the case of lot 4, however, the reduction in total protein was higher than the loss in HA activity. Analysis by SDS–PAGE revealed that a small protein (or group of proteins) <14 kDa was removed during AEC (Fig. 9a). The separation behavior of this protein either suggests some kind of hcDNA association or a highly negative charge.

For precise calculation of the productivity, the dynamic capacity of the column for hcDNA would have been required. Still, a rough estimate of the productivity can be derived. For the purpose of plasmid purification, capacities around 1 mg/mL of resin had been reported testing various anion-exchangers (Ferreira et al., 2000). Assuming a hcDNA concentration of 6 μg/mL, the total capacity of the column calculates to 170 cv of loaded feed. Maintaining a constant flow rate of 200 cm/h and adding 1.7 h for equilibration, desorption, and cleaning (period of time required in experiments) a productivity of 11.2 cv of feed per hour can be achieved.

Process Performance

According to the European Pharmacopea 5 (2005, page 3406) 15 μg of hemagglutinin antigen per strain are required for one injectable dose of a human whole virion vaccine. The amount of total protein thereby should not exceed six times the total hemagglutinin content. Since hemagglutinin makes up for about one third of the viral protein (Oxford et al., 1981) this limit corresponds to a protein purity of at least 50% for whole virion vaccines. Regarding production in continuous cell lines the residual level of hcDNA should not exceed 10 ng per dose (European Pharmacopeia 5, page 455). With respect to the established cultivation system (Kalbfuss et al., 2006) these limits translate into at least 20-fold reduction of total protein and more than 10,000-fold (4 orders of magnitude) reduction in hcDNA assuming an overall product yield of 50%. In addition, more than 35-fold concentration of the virus will be required if the vaccine is assumed to contain three different virus strains (15 μg HA antigen each) and the volume of one dose should not exceed 2 mL.

The residual amount of hcDNA per dose was estimated to be in the range of 500 ng (Table IV) and thus clearly beyond the limit of 10 ng. Unless removal of hcDNA by AEC can be further improved, an additional operation seems to be required.

The amount of total protein per dose just exceeded the limit of 90 μg (assuming 15 μg of hemagglutinin per dose). Apparently the process failed to fulfill the requirements for protein purity. Since estimation of the HA concentration from HA activity is based on a number of assumptions this value should not be overstressed. Measurements with an ELISA under development indicated higher concentrations of hemagglutinin than would have been expected from HA activity alone. One possible explanation could be a higher content of hemagglutinin in cell culture-derived virions which is supported by the strong band appearing in protein gels (Fig. 9a). Another source of error is the incapability of the HA assay to distinguish between single virions and aggregates while particle volume distributions strongly suggested presence of the latter (Fig. 9b). In any case, analysis by SRID calibrated with an appropriate reference antigen is required for final judgement. But even if the burden of host cell protein turned out to be too high, purity could be enhanced at any time by decreasing the load in SEC accepting lower productivity.

Conclusion

Chromatographic purification (after concentration) of cell culture-derived influenza A virus resulted in more than
19-fold overall reduction in total protein and more than 500-fold reduction (2.7 orders of magnitude) in hcDNA. An overall product yield of 52% (based on HA activity) was achieved excluding the ultra-/diafiltration step required for final concentration. SEC was found efficient at the separation of host cell protein from virions. Purity, however, needs to be balanced with productivity for the purpose of process economy. The reduction in hcDNA was found to be insufficient with respect to requirements of the European Pharmacopeia 5. Further optimization of AEC or an additional purification steps seem to be required. So far the suggested process scheme remains fully scalable and is free of strain-specific unit operations (like immuno-affinity). Only standard unit operations (common to biotechnological downstream processes) were incorporated rendering the process suitable for industrial large-scale production.

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