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A flow-through chromatography process for influenza A and B virus purification

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A B S T R A C T

Vaccination is still the most efficient measure to protect against influenza virus infections. Besides the seasonal wave of influenza, pandemic outbreaks of bird or swine flu represent a high threat to human population. With the establishment of cell culture-based processes, there is a growing demand for robust, economic and efficient downstream processes for influenza virus purification. This study focused on the development of an economic flow-through chromatographic process avoiding virus strain sensitive capture steps. Therefore, a three-step process consisting of anion exchange chromatography (AEC), Benzonase® treatment, and size exclusion chromatography with a ligand-activated core (LCC) was established, and tested for purification of two influenza A virus strains and one influenza B virus strain. The process resulted in high virus yields (≥68%) with protein contamination levels fulfilling requirements of the European Pharmacopeia for production of influenza vaccines for human use. DNA was depleted by ≥98.7% for all strains. The measured DNA concentrations per dose were close to the required limits of 10 ng DNA per dose set by the European Pharmacopeia. In addition, the added Benzonase® could be successfully removed from the product fraction.

Overall, the presented downstream process could potentially represent a simple, robust and economic platform technology for production of cell culture-derived influenza vaccines.

1. Introduction

Each year about 10% of the world population is infected with influenza virus (Gerdil, 2003) (*Orthomyxoviridae* family) with about 250,000–500,000 deaths (World Health Organization, 2009). Especially for children below the age of four and people above the age of 65, the virus represents a serious health risk (Matthews, 2006). The best medical strategy against influenza is prophylactic vaccination and medical treatment. As the influenza virus undergoes constant changes by mutations and reassortments, annular revaccination is necessary.

Traditionally, egg-based processes have been used for production of influenza vaccines, in which the virus purification was achieved by processes containing zonal gradient centrifugation (Bardiya and Bae, 2005; Reimer et al., 1966). With establishment of cell culture-based processes (Genzel et al., 2006; Kistner et al., 1999; Tree et al., 2001), new downstream processing schemes were established over the past years (Brands et al., 1999; Kalbfuss et al., 2007a; Kalbfuss et al., 2007b; Palache et al., 1997).

One option is the use of chromatographic methods with the advantages of moderate buffer conditions, little shear stress and high selectivity (Präve et al., 1994). Most chromatographic separations involve at least one capture step. On the one hand this can reduce the costs for stationary phases, i.e. for processes with high amounts of impurities such as monoclonal antibody purification. On the other hand a high variability of products to be purified, for example influenza virus strains which are updated annually for vaccine production can significantly reduce yields and productivity of

Abbreviations: AEC, anion exchange chromatography; LCC, ligand-activated core chromatography; DBC, dynamic binding capacity; CV, column volumes; UF, ultra filtration; A/PR, influenza A/PR/8/34; A/Wis, influenza A/Wis/67/2005; B/Mal, influenza B/Mal/2506/2004; GMEM, glasgow minimum essential medium; FCS, fetal calf serum.

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processes as shown by Opitz et al. (2009). Here, the losses in the capture step were most likely due to changes in binding epitopes. As a result, process robustness need to be carefully evaluated by testing different influenza strains during process development. In addition, process modifications during production campaigns might be required.

The consequent use of chromatographic flow-through process units would avoid some of the problems addressed above. As elution steps with e.g. high amounts of salts can be omitted, the risk for reduction of immunogenicity and aggregation of the virus is also reduced. In particular for virus harvest with moderate DNA and protein contamination levels non-capture processes seem to be highly suitable for efficient virus purification.

So far, only one process for cell cultured-derived influenza virus purification was published which was able to reach the dose limits regarding DNA (10 ng) and protein (100 μg per strain) as required by the European Pharmacopoeia (2012) and the US Food and Drug Administration (FDA) (2012). This process comprised four chromatography unit operations involving a virus capture step, and was operated at 4 °C (He et al., 2011). Unfortunately, however, no information regarding the robustness in terms of batch-to-batch variation and the impact of changes in influenza A and B virus strains on yield was provided.

In addition, Iyer et al. (2012) proposed a process train based on two flow-through steps. However, due to missing information regarding the level of contamination per dose of vaccine, and the fact that one of the chromatographic materials used (primary amine membrane adsorber: ChromaSorb®) is no longer available from the manufacturer stated, evaluation and use of this process option is difficult. Nevertheless, comparable results might be achieved using similar materials from other suppliers. A purification scheme described by Kalbfuss et al. (2007b) using anion exchange chromatography (AEC) and size-exclusion chromatography (SEC) also avoided capture steps. However, as the authors discussed, further optimization of this approach is required as the DNA contamination level (10 ng) for manufacturing of human influenza vaccines according to the European Pharmacopeia was still exceeded by far. Similarly, a continuous SEC-based simulated-moving-bed process proposed by Kroeber et al. (2013), also failed to reach the required DNA limits for human vaccines while showing improved productivity.

Therefore, in addition to chromatographic methods, nuclease treatment seems indispensable to achieve reliably accepted DNA contamination levels in downstream processing of human influenza vaccines. In fact, the majority of industrial vaccine production processes include a nuclease treatment step (Wolff and Reichl, 2008). The aim is not only to meet the required levels of residual DNA, but also to reduce viscosity of virus-containing material and to provide an additional safety step regarding virus inactivation (US Food and Drug Administration, 2012). With the expiration of patents for Benzonase® and the availability of new nuclease products such an enzymatic digestion step is now even more attractive for downstream processing of virus broths. However, the disadvantage is that the removal of nucleases can add to the complexity of downstream processing.

Recently, ligand-activated core chromatography (LCC) resin types have been made available that offer several advantages over conventional chromatographic media (GE Healthcare, 2012b). With an unfunctionalized outer shell and a strong and multi-modal ligand in the bead core, such resins are ideal for depletion of host cell proteins and removal of nuclease in one single step. Due to the spatial exclusion of virus particles from the binding sites (resin pore size: ~700 kDa; influenza virus particle size: 75–120 nm (Lange et al., 1999)), high virus recoveries can be achieved. Moreover, this resin type does not suffer from typical SEC drawbacks such as low productivity due to limited loading capacity and could possibly replace a typical SEC step at the end of a purification train.

In this study, the use of a flow-through process involving three unit operations for the purification of two influenza A strains and one influenza B virus strain with the focus on process robustness is described. As a first step, focusing on the removal of DNA, an anion exchange stationary phase (HiTrap™ Capto™ Q) was used, followed by a DNA digestion step using Benzonase®. In a third step, a novel LCC stationary phase (Capto™ Core 700) was chosen for the depletion of contaminating proteins and the removal of the added nuclease.

2. Materials and methods

2.1. Virus production

Three human influenza strains have been selected: A/Puerto Rico/8/34, H1N1 (A/PR) (Robert Koch Institute, Berlin, Germany), A/Wisconsin/67/2005, H3N2 (A/Wis) (#06/112, National Institute for Biological Standards and Control, London; UK), and B/Malaysia/2506/2004 (B/Mal) (#06/104, National Institute for Biological Standards and Control, London; UK). As host cells, adherent MDCK cells (#841211903, European Collection of Cell Cultures, Salisbury, UK) cultivated in serum containing GMEM medium (#22100-093, Life Technologies, Carlsbad, USA; FCS, #F7524, Sigma–Aldrich, St. Louis, USA) have been used as described by Genzel et al. (2004) and Opitz et al. (2009). Before addition of virus seed, the medium was replaced by serum-free GMEM medium (#22100-093, Life Technologies, Carlsbad, USA).

2.2. Virus harvest and preprocessing

Culture broths were harvested with a 5 μm and a 0.65 μm depth filter (#CFAP0508YY, #CFAP9608YY, GE Water & Process Technologies, Trevose, USA) in sequence, followed by a chemical inactivation with β-propiolactone (#33672.01, Serva Electrophoresis, Heidelberg, Germany; final concentration: 3 mM, 37 °C, 24 h). Subsequently, the broths were processed by a 0.45 μm membrane filter (#CMMP9408YY, GE Water & Process Technologies, Trevose, USA). Finally, the clarified broths were concentrated by UF-crossflow filtration (cut-off: 750 kDa; UFP-750-E-4MA, GE Healthcare, Uppsala, Sweden) as described by Kalbfuss et al. (2007a) and stored at −80 °C. This clarified, inactivated and concentrated material was used for all shown experiments and will be called 'virus material' hereafter.

2.3. Process set-up

All chromatographic experiments were performed with an ÄKTAexplorer 100 (GE Healthcare, Uppsala, Sweden). Virus fractions were monitored in-line with a static light scattering detector (BI-MwA, Brookhaven Instruments Corporation, Holtsville, USA). Virus material was centrifuged for 10 min at 9000 × *g* prior to loading onto columns. For screening studies and determination of dynamic binding capacity (DBC) the A/PR virus strain was used. All steps involving dialysis (buffer exchanges, preparation of samples for analytics) were performed with 3500 kDa cut-off membranes (#132720, Spectrum Labs, Los Angeles, USA).

2.4. Screening of AEC and LCC

The following AEC matrices were tested at a flow rate of 0.2–2 mL/min: 1 mL HiTrap™ DEAE FF (DEAE) columns (#28-9165-37, GE Healthcare, Uppsala, Sweden) and 1 mL HiTrap™ Capto™ Q (Capto Q) columns (#11-0013-02, GE Healthcare, Uppsala, Sweden). Before loading onto column, the virus material was