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Purification of cell culture-derived influenza A virus via continuous anion exchange chromatography on monoliths

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ABSTRACT

The continuously increasing demand for potent and safe vaccines and the intensifying economic pressure on health care systems underlines the need for further optimization of vaccine manufacturing. Here, we focus on downstream processing of human influenza vaccines, investigating the purification of serum-free cell culture-derived influenza virus (A/PR/8/34 H1N1) using continuous chromatography. Therefore, quaternary amine anion exchange monoliths (CIM® QA) were characterized for their capacity to capture virus particles from animal cells cultivated in different media and their ability to separate virions from contaminating host cell proteins and DNA. The continuous chromatography was implemented as simulated moving bed chromatography (SMB) in a three zone open loop configuration with a detached high salt zone for regeneration.

SMBs exploiting 10% and 50% of the monoliths' dynamic binding capacity, respectively, allowed the depletion of >98% of the DNA and >52% of the total protein. Based on the hemagglutination assay (HA assay), the virus yield was higher at 10% capacity use (89% vs. 45%). Both SMB separations resulted in a ratio of total protein to hemagglutinin antigen (based on single radial diffusion assay, SRID assay) below the required levels for manufacturing of human vaccines (less than 100 μg of protein per virus strain per dose). The level of contaminating DNA was five-times lower for the 10% loading, but still exceeded the required limit for human vaccines. A subsequent Benzonase® treatment step, however, reduced the DNA contamination below 10 ng per dose. Coupled to continuous cultivations for virus propagation, the establishment of integrated processes for fully continuous production of vaccines seems to be feasible.

1. Introduction

Influenza disease, caused by influenza viruses, affects up to 10% of the world's population every year. The most effective measure against an influenza infection is vaccination [1]. Due to the constant change of the influenza virus surface proteins, however, it is necessary to adapt the virus strains each year according to a WHO recommendation [2].

The steadily rising demand of vaccines and the strict time frame for the production of seasonal and pandemic vaccines lead to the introduction of cell culture production systems for virus propagation [3]. These systems were further developed over the years regarding the use of serum-free media and the establishment of suspension cultures [4]. Future technologies will include the use of high cell density cultures [5,6] and continuous cultivations [7]. In order to complement these advances in upstream processing, purification schemes have to be adapted accordingly.

One approach to handle large batch volumes in downstream processing (DSP), and to establish robust processes with high yields is the application of continuous unit operations [8]. In addition, continuous processes also improve the energy and material usage during production, and reduce the production footprint improving overall process efficiency. A large number of unit operations have already been reported for use in continuous

Abbreviations: CV, column volume; CSS, cyclic steady state; DSP, downstream processing; $DBC_{10\%}$, dynamic binding capacity (10% breakthrough); GMEM, glasgow minimal essential medium; HA assay, hemagglutination assay; HA, hemagglutinin antigen; IAV, influenza virus A/PR/8/34 (H1N1); MDCK, Madin Darby canine kidney; moi, multiplicity of infection; SMB, simulated moving bed; SRID assay, single radial immunodiffusion assay; toi, time of infection.

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DSP of biologicals [9,10]. For some complex methods like chromatography, however, further development and optimization is required. Currently, the most promising techniques for continuous chromatography are countercurrent simulated moving bed (SMB) [11,12] or periodic countercurrent chromatography (PCC) [13].

For larger target biomolecules like viruses chromatography matrices without diffusion limited mass transfer into the matrix pores like membranes [14] or monoliths [15] provide elevated capacities at high flow rates and pressure stability. Therefore, we focused in this study on SMB using CIM® QA disk monoliths to separate serum-free cell culture-derived human influenza virus A/PR/8/34 (H1N1) from protein and DNA contamination. The continuous capture of the virus from clarified harvest was implemented as a three zone open loop SMB. In combination with a subsequent Benzonase® treatment, protein and DNA contamination levels required for manufacturing of influenza vaccines for human use were achieved.

2. Material and methods

2.1. Preparation of virus broth

Human influenza virus A/PR/8/34 (H1N1; Robert Koch Institute, Berlin; hereafter referred as IAV) propagated in animal cell cultures was used for all experiments. Material for scouting experiments was produced in adherent MDCK cells (ECACC, No. 841211903) maintained in serum-containing GMEM (Gibco, 22100093) as described by Genzel et al. [16]. After three days the broth was clarified, inactivated, and concentrated as reported by Kalbfuss et al. [17]. Aliquots of 15 mL and 50 mL were stored at −80 °C before processing.

In addition, adherent MDCK cells (ECACC, No. 841211903) were maintained in serum-free medium (EpiSerf; Invitrogen; supplemented with 2 mM L-glutamine, 2 mM pyruvate (Sigma Aldrich) and 20 mM glucose (Roth)). Cells were infected at a multiplicity of infection (moi) of 0.025. To facilitate IAV replication, porcine trypsin (5E−6 U/cell; Gibco, 27250018) was added at time of infection (toi). In addition, MDCK suspension cells (MDCK.SUS2, [4]) were cultivated in a 5 L bioreactor at 120 rpm in serum-free medium (Smif8; Gibco, by contact through K. Scharfenberg; supplemented with 4 mM of each L-glutamine and pyruvate; Sigma Aldrich) and infected at moi of 0.001. At toi, porcine trypsin (1E−5 U/cell; Gibco) was added. After three days, the serum-free cultures were harvested, directly clarified using two depth filters of 5 μm and 0.65 μm cut-off (GE water & Process Technologies), and chemically inactivated with 6 mM β-propiolactone (final concentration, 24 h at 37 °C; Serva Electrophoresis). Afterwards, the material was clarified again with a 0.45 μm filter (GE water & Process Technologies) and stored in aliquots of 15 mL and 50 mL at −80 °C.

2.2. Chromatography

All chromatography runs were performed on CIM® QA monoliths (BIA separations) which were cleaned after each run with a sequence of 1 M NaOH, deionized water, 0.5 M and 0.15 M NaCl in the respective running buffer. Individual experiments were performed using TRIS-buffer (50 mM TRIS, pH 7.4) or HEPES-buffer (50 mM HEPES, pH 7.5) with addition of 0.005 or 0.01% zwittergent (SB3-14; Sigma Aldrich) as described in the respective method paragraphs. All solutions were prepared with MilliQ® purified water. For sample loading, 0.15 M NaCl was used; for elution and regeneration of the matrix [18] an increasing salt concentration of up to 2 M was applied. Conductivity, UV absorbance (280 nm), and light scattering (Nicomp™ 380; Particle Sizing Systems) were monitored online to trace salt concentration/ionic strength, proteins, DNA, and IAV particles, respectively.

For each chromatography run, the cell culture harvest was thawed and applied as described above. For experiments with zwittergent-containing buffer, the respective zwittergent concentration (0.005% or 0.01%) was added to the feed material and incubated at room temperature for 2 h prior to loading.

2.3. Batch chromatography

Single column runs for scouting experiments were performed with an Äkta Pure system (GE Healthcare) using HEPES-buffer (without addition of zwittergent) at 3 mL/min on CIM® QA disks for all IAV harvests (adherent MDCK in GMEM and EpiSerf, suspension MDCK in Smif8). For elution, either a gradient increasing to 2 M NaCl (GMEM-based material) or steps of 0.6 M and 2 M NaCl were applied (serum-free broths). In addition, for the Smif8-based material, additional runs with 0.01% zwittergent in the feed and buffers were performed. All collected fractions were analyzed by offline HA assay to estimate yields.

The dynamic binding capacity ($DBC_{10\%}$) was determined using TRIS-buffer. The GMEM-based material was loaded with 3 mL/min onto a CIM® disk (0.34 mL column volume (CV)) and with 1 mL/min onto a CIMac™ (0.1 mL CV) until complete breakthrough. For the latter, the $DBC_{10\%}$ was also determined using a Smif8-derived IAV harvest with 0.005% zwittergent in the feed and buffer. The $DBC_{10\%}$ was defined as the amount loaded until breakthrough of 10% of the IAV concentration in the feed. The IAV were followed online via the light scattering signal. Flow through fractions were analyzed in addition with an HA assay to verify the online signals.

2.4. Continuous chromatography

SMB chromatography was operated as a three zone open loop process in which the CIM®s are regenerated (and the bound DNA removed) with a high salt concentration (2 M NaCl) in a detached zone (Fig. 1). Contaminating proteins are withdrawn through the raffinate and IAV eluted into the extract. The set-up involved two Äkta Purifier systems (GE Healthcare) as described by Kröber et al. [11]. One CIM® disk (0.34 mL CV) per zone and TRIS-buffer

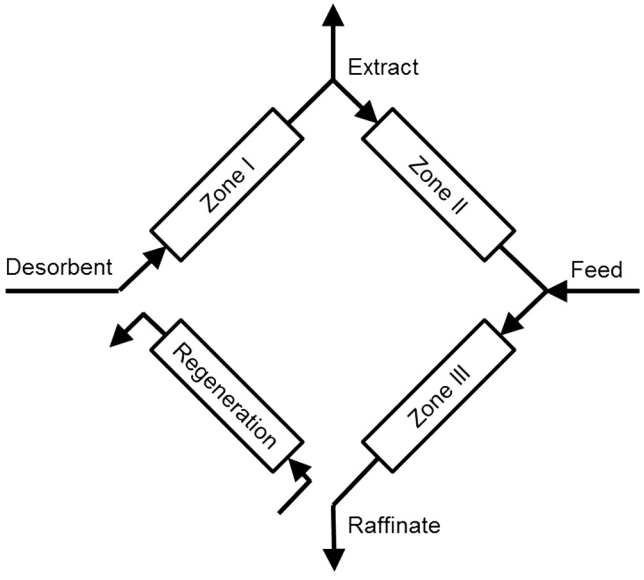

Fig. 1. Scheme of the three zone open loop simulated moving bed process used in this study. Influenza A virus adsorption by CIM® QA disk monoliths; detached regeneration zone; clockwise liquid flow and valve switching.

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