Technical note “Comparison of influenza virus particle purification using novel magnetic sulfated cellulose particles with an established centrifugation method for analytics”

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Supporting information technical note:
The supporting information shows the host cell proteins in the purified influenza A virus samples (table S-1) and the list of identified viral proteins (table S-2).

Comparison of influenza virus particle purification using novel magnetic sulfated cellulose particles with an established centrifugation method for analytics

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ABSTRACT: A method for the purification of influenza virus particles using novel magnetic sulfated cellulose particles is presented and compared to an established centrifugation method for analytics. Therefore, purified influenza A virus particles from adherent and suspension MDCK host cell lines were characterized on the protein level with mass spectrometry to compare the viral and residual host cell proteins. Both methods allowed to identify all ten influenza A virus proteins, including low abundant proteins like the matrix protein 2 and non-structural protein 1, with a similar impurity level of host cell proteins. Compared to the centrifugation method, use of the novel magnetic sulfated cellulose particles reduced the influenza A virus particle purification time from 3.5 h to 30 min before mass spectrometry analysis.

Small-scale purification of influenza virus particles with centrifugation for analytics (CFA) is a time consuming and labor intensive process and can be hard to implement if high sample throughput is needed.

For purifying influenza virions in large scale, the application of sulfated cellulose pseudo-affinity based matrices, like Cellufine sulfate[1,2] (JNC Corp.) or the more recently introduced Capto DeVirS[3] (GE Healthcare Bio-Sciences AB), can be used. Furthermore, new matrices based on sulfated cellulose membranes were evaluated and showed good performance for the production of influenza or modified Vaccinia Ankara (MVA) virus vaccines.[4,5,6] The mode of action of this pseudo-affinity adsorption is not yet understood in detail, but the sulfated cellulose seems to mimic heparan sulfate proteoglycans (HSPG), which are naturally involved in pathogen-host interactions.[7] During this interaction the HSPG act as low affinity co-receptors for direct internalization or for increasing the likelihood of binding to more specific secondary receptors.[8] This mode of action is present in a wide range of viral, bacterial, and parasitic pathogens binding to cellular membranes.[8]

In this report, pseudo-affinity adsorption of influenza virus particles is combined with magnetic separation using novel magnetic sulfated cellulose particles (MSCP) for small scale purification of influenza A virus particles for analytical studies and for exploring options in downstream processing of virus harvests in vaccine manufacturing. The method is compared on protein level to an established small scale CFA method used to obtain highly purified influenza virions.[9,10,11]

EXPERIMENTAL SECTION

Three biological replicates and two technical replicates were performed for each virus sample. Solutions and media were prepared with purified water (MQ water) from a water purification system (Milli-Q Advantage A10, Millipore). Used chemicals, except chemicals for mass spectrometry (MS), had synthesis grade. All solvents and buffers labeled aqueous (aq) were prepared with MQ water. For mass spectrometric sample preparation and analysis an additional filter unit was added to the water purification system to remove trace organics (LCPAK0001, Millipore).

Cell lines, cell cultivation and virus infection. Adherent MDCK cells ($MDCK_{ADH}$) (ECACC #84121903) were cultivated under serum-free conditions (Episerf #10732022) at 37°C as described by Lohr et al.[12] The MDCK suspension host cell line $MDCK_{SUS2}$ was generated by cell line adaptation of $MDCK_{ADH}$ cells (K. Scharfenberg, FH Emden/Leer) and cultivated in chemical defined medium SMIF8 PGd 2x (protein- and peptide-free; Gibco, through contact with K. Scharfenberg) with addition of 23.8 mmol/l $NaHCO_3$, 85.6 mmol/l NaCl, 0.1% (v/v) Pluronic-F68, 0.001‰ (v/v) ethanolamine (98%), 1.6 mmol/l l-glutamic acid, 20.3 mmol/l d-(+)-glucose, 4 mmol/l glutamine and 4 mmol/l pyruvate.[13] Cells were infected with human influenza A/PR/8/34 (H1N1) (#3138, Robert Koch Institute (RKI), in-house generated adherent MDCK-derived viral stock, $TCID_{50}$ titer of 5.17×10^8 infectious virions/ml, HA titer of 2.63 $\log_{10}$ HA units/100 µl) with a multiplicity of infection (moi) of 0.025 and 2×10^{-6} units trypsin (#27250-018, Gibco) per cell. 250 ml vented shaker flasks or 850 cm^2 roller bottles (both VWR) were used for all infection experiments.[12,13,14]

At 72 hours post infection, supernatants were harvested and clarified for 20 min at 150 g (Avanti J-20XP, Beckmann Coulter) at room temperature (RT) to obtain clarified virus harvests (CVH).

Influenza virus purification and concentration with centrifugation for analytics. CFA for virus purification was performed as described by Hennig et al.[11] Briefly, CVH was centrifuged at 4,000 x g for 35 min to remove cell debris, followed by a 10,000 x g centrifugation for 45 min to remove cell compartments (Avanti J-20XP, Beckmann Coulter). At each step, the supernatant was transferred into a new centrifuge tube and the pellet was discarded. Finally, influenza A virions were concentrated at 98,649 x g for 90 min by high speed centrifugation (Optima TM LE-80K, Beckmann Coulter).[9,10,11] The virus purification by the CFA method was car-

ried out simultaneously for all six CVH samples and took approximately 190 minutes.

Production of magnetic sulfated cellulose particles. **"**Magne™ Protein A Beads 20% Slurry" (#G8781, Promega) was used as a backbone for sulfation as described in patent EP14175925.[15] Briefly, 400 mg of the magnetic cellulose particles were added to a chlorsulfonic acid-pyridine reaction mixture (1:18.2) and incubated overnight at 35°C (Caution: Addition of chlorsulfonic acid to temperature-controlled pyridine below 0°C). Then the supernatant was decanted and the MSCP were washed with MQ water to remove any residual pyridine. Finally, the MSCP were resuspended in 20% ethanol$_{(aq)}$ to get a 50 vol% MSCP solution for storage.

Virus purification and concentration with magnetic sulfated cellulose particles. 1.75 ml of the 50 vol% MSCP in 20% ethanol$_{(aq)}$ solution were washed three times with 10 mM Tris-HCl$_{(aq)}$ pH 7.4. CVH was diluted with 10 mM Tris-HCl$_{(aq)}$ pH 7.4 1:3 to decrease the salt content of the sample for optimal virus binding to the MSCP. Fifteen ml of the diluted virus solution was incubated with the washed MSCP for 1 min under gentle mixing. Then the supernatant was discarded and bound influenza A virus particles were eluted from the MSCP with 1 ml of 0.6 M NaCl$_{(aq)}$ 10 mM Tris-HCl$_{(aq)}$ pH 7.4. Finally, the MSCP were regenerated with 15 ml 2 M NaCl$_{(aq)}$, 10 mM Tris-HCl$_{(aq)}$ pH 7.4 for 10 min and conditioned for the next virus purification by washing three times with 15 ml 10 mM Tris-HCl$_{(aq)}$ pH 7.4. The virus purification by the MSCP method was carried out simultaneously for all six CVH samples and took approximately 10 minutes.

LC-MS/MS based proteome analysis. The pelleted influenza A virions obtained after high speed centrifugation were lysed in 200 µl of aqueous lysis buffer (30 mM Tris-HCl, 150 mM NaCl, 1 mM $MgCl_2$, 5 mM EDTA, 2% SDS), inactivated at 80°C for 5 min and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Briefly, 55 µg of protein was precipitated overnight at -20°C in 96% ice cold ethanol. The solution was centrifuged at 14,000 x g, 0°C for 10 min and the supernatant was discarded. The protein pellet was dried and resuspended in 200 µl 8 M urea in 0.1 M Tris-HCl$_{(aq)}$ pH 8.5 (UA). Subsequently, the solution was incubated for 5 min on centrifugal filter units primed three times with MQ water (Pall Nanosep 10K Omega, molecular weight cut-off 10 kDa). After centrifugation at 14,000 x g and incubation at RT for 10 min, 200 µl of UA was added to the filter unit. After that, a centrifugation at 14,000 x g for 10 min was carried out. Then 100 µl 20 mM dithiothreitol in UA was added to the filter unit, mixed for 1 min at 600 rpm with a Thermomixer comfort (Eppendorf) and incubated without mixing at 56°C for 20 min before centrifugation at 14,000 x g at RT for 10 min. In the next step, 100 µl 50 mM iodoacetamide in UA were added and mixed 1 min at 600 rpm. The sample was incubated in the dark without mixing at RT for 20 min, followed by centrifugation at 14,000 x g at RT for 10 min. The proteins in the filter units were washed three times with 100 µl UA and three times with 100 µl 50 mM aqueous ammoniumbicarbonate (ABC$_{(aq)}$). After that, the filter unit was transferred to a fresh tube and 0.02 µg/µl trypsin (enzyme to protein ratio 1:50) in ABC$_{(aq)}$, containing additionally 5% acetonitrile (ACN) and 1 mM $CaCl_2$, were added. Then, the solution was incubated at 37°C overnight, followed by a centrifugation at 14,000 x g at RT for 10 min. Next, the filter unit was washed with 50 µl ABC$_{(aq)}$ followed by 50 µl MQ water. After that, all three flow throughs were harvested, combined, dried with a vacuum centrifuge (Alpha 2-4 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH), and stored at -20°C.

Peptides were analyzed by LC-MS/MS using an UltiMate 3000 RSLCnano splitless liquid chromatography system (Dionex Corp.) coupled online to a LTQ-Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific). After injection, peptides were loaded isocratically on a trap column (Dionex Acclaim, nano trap column, 100 µm i.d. x 2 cm, PepMap100 C18, 5 µm, 100 Å, nanoViper, Dionex Corp.) with a flow rate of 7 µl/min of the chromatographic liquid phase (98% MQ water, 2% ACN), 0.05% trifluoroacetic acid) for desalting and concentrating. Chromatographic separation was performed on an Acclaim PepMap C18 RSLC nano reverse phase column (2 µm particle size, 100 A pore size, 75 µm inner diameter and 250 mm length, Dionex Corp.) at 40°C column temperature. A flow rate of 300 nl/min was applied using a binary A/B solvent gradient (solvent A: 98% MQ water 2% ACN, 0.1% formic acid; solvent B: 80% acetonitrile, 10% MQ water, 10% trifluorethanol, 0.1 % formic acid) starting with 4% B for 4 min, continuing with a linear increase to 55% B within 29 min, followed by a column wash with 90% B for 5 min and a re-equilibration with 4% B for 25 min. For mass spectrometric acquisition, a data dependent MS/MS method was chosen. Precursor scanning was performed in the orbital trap of the hybrid MS at a resolution of 30,000 and an *m/z* range of 350 - 2,000 in positive ion mode. Subsequently, fragmentation and fragment ion scan were performed in the linear ion trap of the hybrid MS, with a mass range and a scan rate with "normal" parameter settings, for the top 20 most intense precursors selected for collision induced dissociation. Finally, LC-MS/MS data was analyzed using the Central Proteomic Facilities Pipeline (CPFP).[16] iProphet was used to combine searches made with Mascot.[17] Oxidation of methionine residues and carbamidomethylation of cysteine residues were set as variable modifications; one missed cleavage was attributed to trypsin; peptide mass tolerance was set to 10 ppm, fragment mass tolerance to 0.5 Da. Peptide identifications were validated within CPFP using PeptideProphet.[18] The UniProtKB/Swiss-Prot database were searched against the subsets *Canis familiaris* and influenza A virus (strain A/Puerto Rico/8/1934 H1N1).

Results and Discussion

A standard centrifugation method for influenza virion purification and concentration, used for example for glycan analysis, is a consecutive stepwise "g-force gradient centrifugation".[9,10,11] Compared to this established CFA method, which requires four centrifugation steps, the presented novel MSCP method is able to purify and concentrate influenza A virions with only one centrifugation step in less than 20% of the time.

After purification and concentration of the influenza A virus samples generated in $MDCK_{ADH}$ and $MDCK_{SUS2}$ host cells with the two different methods, we chose an optimized filter aided sample preparation (FASP) for the generation of tryptic peptides from the lysates for LC-MS/MS analysis based on Wiśniewski et al.[19] This method can be applied to samples containing a high concentration of detergents, which is neces-

sary to fully lyse the virions for the subsequent steps. Using this FASP approach, additional precipitation steps can be avoided and the total protein concentration, ranging from 0.2 to 200 µg, can be kept high. Therefore, this method is suitable to analyze the proteomes of influenza virions which may contain, in addition to host cell derived membrane proteins, other internalized host cell proteins. With both, the MSCP and the CFA method, all ten most abundant proteins of the influenza A virus could be detected as shown in table 1 and figure 1. Compared to previous studies[20,21], the viral proteome also included low abundant proteins like the influenza matrix protein 2 (M2) and the non-structural protein 1 (NS1). This corresponds to results obtained by Hutchinson et al.[21] who showed that NS1 is an internal component of the influenza virion due to its resistance to protease treatment. In addition, numerous host cell proteins were detected in all purified influenza A virion samples. For some of them, like annexin A2 and glyceraldehyd-3-phosphate dehydrogenase, it was demonstrated that they can be located inside the influenza virion.[20] Overall, we identified 56 to 69 host cell proteins, depending on the host cell line and purification method. Our findings include membrane-bound and cytoplasmic proteins that belong to different functional categories of animal cells. An detailed overview of the identified host cell proteins is given in supplementary table S-1.

A breakdown of the protein hits is given in figure 1. As discussed before, it cannot be necessarily assumed that all of the host cell proteins were incorporated specifically into the influenza A virions. Besides the ten influenza A virus proteins (represented by "◆" in figure 1), a total number of 37 host cell proteins was identified for both host cell lines and methods. For each individual method, some host cell proteins could only be found with one host cell line used (see occurrence column in the supplementary table S-1). For example, testin was only identified using the $MDCK_{ADH}$ host cell line while claudin-3 was identified only for the $MDCK_{SUS2}$ host cell line. Overall, similar proteins were identified by both purification methods for both host cell lines. These results in 75 hits for the MSCP method and 78 hits for the CFA method for both host cell lines, showing that both methods have a similar purification performance (see figure 1). For the MSCP method a total number of 60 protein hits were identified for both host cell lines. Additionally, six hits for $MDCK_{ADH}$ only and nine hits for $MDCK_{SUS2}$ only were identified. The CFA method identified 38 protein hits for both host cell lines. As before, a low number of proteins could only be identified for $MDCK_{ADH}$ (18 hits) and for $MDCK_{SUS2}$ (22 hits).

Most likely, the purity of the samples used in the MS analysis could be improved with at least one additional washing step for both methods. However, an additional washing step in the CFA method will increase the sample preparation time by about 90 min due to the necessary high speed centrifugation step. In contrast, one additional washing step in the MSCP method will increase the sample preparation time by only a few minutes.

Table 1. List of identified viral proteins from the influenza A virus sample produced in $MDCK_{ADH}$ host cells after MSCP purification. The percentage coverage represents the total number of all amino acids in the protein sequence covered by the identified peptides. Data of the other purified influenza A virus samples is shown in the supplementary table S-2.

Protein name	Short name	Percentage coverage	No. of unique peptides
Haemagglutinin	HA	57.9	58
Matrix protein 1	M1	77.0	65
Matrix protein 2	M2	55.7	8
Neuraminidase	NA	33.5	20
Non-structural protein 1	NS1	82.2	34
Nuclear export protein	NEP	33.1	5
Nucleoprotein	NP	72.1	82
Polymerase acidic protein	PA	51.0	40
Polymerase basic protein 1	PB1	29.9	24
Polymerase basic protein 2	PB2	48.2	42

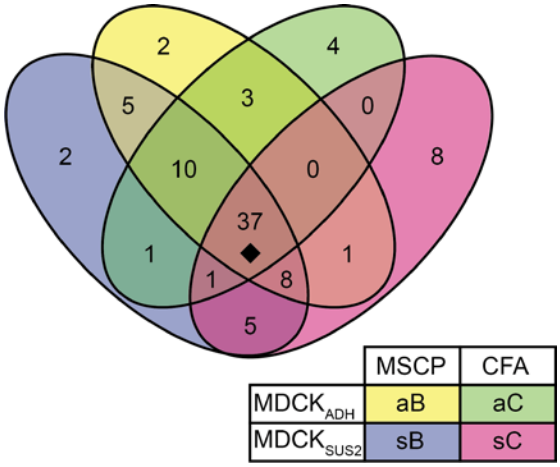

Figure 1. Comparison of the MSCP and CFA method for identified host cell protein hits, including the additionally identified ten viral proteins ("◆"). The Venn diagram shows the breakdown of the protein hits for the two host cell lines and both methods. Abbreviations: aB ($MDCK_{ADH}$, MSCP method), sB ($MDCK_{SUS2}$, MSCP method), aC ($MDCK_{ADH}$, CFA method), sC ($MDCK_{SUS2}$, CFA method).

Conclusion

The comparison of the two influenza virus particle purification methods clearly shows that the MSCP method and a previously established centrifugation method for analytics (CFA) performed equally well for purifying human influenza A/PR/8/34 (H1N1) virus particles produced in $MDCK_{ADH}$ and $MDCK_{SUS2}$ host cells. However, the MSCP method allowed a 7-fold reduction of the sample purification time.

With this time saving potential and the option to use the MSCP method for a wide range of other viral, bacterial, and parasitic pathogens, the MSCP method can be a very powerful tool for high throughput purification for analytical purposes. In addition, it can be assumed that the MSCP purification method could be applied for small and large scale production of viral vaccines.

ASSOCIATED CONTENT

Supporting Information

Detailed data on the host cell proteins in the purified influenza A virus samples (see table S-1) and the list of identified viral proteins (see table S-2) are shown in the separate supporting information. The Supporting Information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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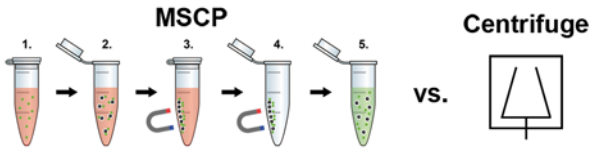
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