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Size distribution analysis of influenza virus particles using size exclusion chromatography

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ABSTRACT

Size exclusion chromatography is a standard method in quality control of biopharmaceutical proteins. In contrast, vaccine analysis is often based on activity assays. The hemagglutination assay is a widely accepted influenza quantification method, providing no insight in the size distribution of virus particles. Capabilities of size exclusion chromatography to complement the hemagglutination assay are investigated. The presented method is comparatively robust regarding different buffer systems, ionic strength and additive concentrations. Addition of 200 mM arginine or sodium chloride is necessary to obtain complete virus particle recovery. 0.5 and 1.0 M arginine increase the hydrodynamic radius of the whole virus particles by 5 nm. Sodium citrate induces virus particle aggregation. Results are confirmed by dynamic light scattering. Retention of a H1N1v strain correlates with DNA contents between 5 ng/mL and 670 ng/mL. Quantitative elution of the virus preparations is verified on basis of hemagglutination activity. Elution of hemagglutination inducing compounds starts at a flow channel diameter of 7000 nm. The universal applicability is demonstrated with three different influenza virus samples, including an industrially produced, pandemic vaccine strain. Size distribution of the pandemic H1N1v 5258, H1N1 PR/8/34, and H3N2 Aichi/2/68 preparations spreads across inter- and intra-particle volume and extends to the secondary interaction dominated range. Thus, virus particle debris seems to induce hemagglutination. Fragments generated by 0.5% Triton™ X-100 treatment increase overall hemagglutination activity.

1. Introduction

Vaccination is an effective cure against the pandemic outbreak of influenza. Various influenza vaccines from different manufacturers are based on inactivated whole virus particles. Influenza virus particles have a size of 80–120 nm. Hemagglutinin (HA) and neuraminidase are the two most abundant proteins on the surface of Influenza A virus particles. Currently, 17 different HA and 10 different neuraminidases have been characterized and give name to the different influenza A subtypes [1]. The amount of HA in an influenza vaccine is a crucial parameter for release of the biopharmaceutical. Guidelines by the European Pharmacopoeia require 15 μg of HA antigen for each of the present virus strains in a trivalent human vaccine [2].

Currently, HA quantification mostly relies on two methods: the single radial immunodiffusion (SRID) assay and the HA assay. SRID is based on antigen binding and is the gold standard for release of human vaccines [3]. The HA assay utilizes hemagglutination of erythrocytes [4]. Dilutions of the investigated virus sample are incubated with a chicken erythrocyte preparation. In case sufficient virus particles are present to induce hemagglutination, erythrocytes will float carpet-like in solution. Virus particle concentrations are estimated assuming hemagglutination requires one virus particle per erythrocyte. This assay dates back to the 1940s and is currently still accepted in research, development, quality control testing and the release of animal vaccines. Drawbacks of this method are the lack of standardization, its discrete format, deviations due to erythrocyte aging [5], secondary interactions of pH, buffer ions [6] and other compounds in complex virus samples. Virus inactivation agents have been shown to specifically affect HA activity of different strains [7,8]. Jonges et al. evaluated the use of different conditions and agents for viral inactivation. Heat and β-propiolactone lead to a decreased HA-titer. Comparatively smaller losses of the HA-titer can be observed after formalin treatment.

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Triton™ X-100 concentrations exceeding 0.2% lead to a complete loss of hemagglutination activity of H3N2. However, the determined HA-titer of H7N3 exceeds the initial value after treatment with 1% Triton™ X-100 [8]. The influence on HA activity extends to inorganic ions typically used during downstream processing. Calcium ions have been shown to mediate hemagglutination in presence of amyloid proteins [9].

Both methods, SRID and HA assay, are generally recognized and are being applied in research and development of purification processes. Kalbfuss et al. use the HA assay for mass-balancing of filtration. They conclude that roughly 40% of the HA activity is deposited on or inside the membrane, since it could neither be recovered in the permeate fraction nor in the retentate. They further suggest that this may represent cell debris [10]. Changes in HA activity due to fragmentation of initially spherical virus particles and aggregation might contribute as well. A recently published manuscript of our group describes discrepant mass balances, as well. Occasionally, the HA activity recovered from different anion exchange resins exceeds HA activity prior to chromatography by more than 100% [11]. In this case, non-conformity of activity was shown to be independent from the sample matrix. Thus, comparing the efficiency of different purification schemes based on HA activity may lead to an incomplete picture. This is in particular important when comparing data obtained with different virus feedstreams. Non-closing mass balances and reproducibility issues are addressed by another manuscript by Kalbfuss et al., where they suggest the use of a continuous HA assay format [12]. Undoubtedly, this assay format leads to a general improvement of the assay. However, matrix interference remains a problem. Consequently, buffer exchange steps might be necessary to quantify samples from process development.

During the last years, enhanced process understanding and control has been pushed by the authorities [13]. This can be accomplished through process analytical technologies that are applicable to a wide design space of conditions potentially being used during the manufacturing process. Public institutions, such as the European Medicine Agency (EMA), encourage research in the field to complement the SRID assay [14]. Investigation of size, content, and immunogenicity of aggregates in the drug product or substance is recommended [14]. Numerous other techniques to either quantify influenza virus particles or the activity have been published. These include, but are not limited to: electron microscopy [15,16], surface plasmon resonance [17,18], enzyme linked immunosorbent assay (ELISA) [19], and reversed phase high pressure liquid chromatography [3]. Electron microcopy and surface plasmon resonance are comparatively expensive. Surface plasmon resonance further suffers from the same constraint as the SRID, since it requires an appropriate set of antibodies for new influenza strains. This holds true for most ELISAs. Reversed phase liquid chromatography is sample destructive and strong interference with β-propiolactone [5] limits its versatility. Except for electron microscopy, none of the previously listed methods allows to investigate the aggregate or fragment content of virus preparations. Alternative techniques to investigate the size distribution of virus samples are dynamic light scattering (DLS) or differential centrifugal sedimentation [20]. DLS is a mild, non-matrix assisted method, which is a great advantage with regards to potential secondary interactions. However, it does not allow sampling of different size fractions of the virus preparations for further activity analysis. In contrast, differential centrifugal sedimentation is afflicted with shear forces.

Analytical size exclusion chromatography (SEC) is a non-destructive technique that has been used for decades to analyze the aggregate and fragment content of therapeutic proteins [21]. Recently, several approaches to apply SEC to the aggregate analysis of virus and virus like particle samples have been published [22–25]. Earlier approaches in SEC of viruses did not pay much attention to this aspect [26]. Availability of SEC columns that allow for size distribution analysis of large virus particles, such as influenza, is limited. Due to the size of whole influenza virus particles, the estimated pore diameter distribution of such media should range between 300 nm to 1000 nm. Kalashnikova et al. found that behavior of protein coated nanoparticles in adsorption chromatography is mostly determined by the surface proteins [27]. This may hold true for non-adsorptive chromatography, as well. Hence, assessing pore volume and pore diameter by inverse SEC of such media is difficult, due to the limited availability of monodisperse proteins or protein-coated standards that have an appropriate hydrodynamic radius. An alternative method is provided by mercury porosimetry. Mercury intrudes at increasing pressure into the intra-particle and inter-particle space of dry porous particles. Pore diameters and pore volumes can be calculated from the required pressure and the corresponding mercury volume [28].

Herein, we present a complimentary analytical SEC method. The current study includes 3 different influenza A virus strains. The pandemic H1N1 vaccine strain 5258 is industrially produced in mammalian cells. Influenza A H1N1 PR/8/34 and H3N2 Aichi/2/68 originate from embryonated chicken cells.

2. Material and methods

2.1. Influenza samples

The pandemic H1N1 vaccine strain (5258) was produced in adherent Madin Darby Bovine Kidney (MDBK) cells at IDT Biologika GmbH (Dessau-Roßlau, Germany). A reference process has been published by Hundt et al. [29]. The H1N1v 5258 virus sample was inactivated by β-propiolactone treatment. The feedstream was volumetrically concentrated by a factor of 20 to a HA activity of 2000 HAU/100 μL. Samples for virus particle fragmentation experiments were pre-purified by preparative SEC. TOYOPEARL HW-65F (Tosoh Bioscience GmbH, Griesheim, Germany) was packed to a bed height of 22 cm. 100 mM sodium phosphate, pH 7.0 containing 200 mM sodium chloride was used as mobile phase at a flow rate of 150 cm/h. All chemicals were purchased from Sigma Aldrich/Merck KGaA (Darmstadt, Germany), unless otherwise stated. Besides this feedstream and SEC pre-purified feedstream, 15 H1N1v samples with different host cell DNA contents were available. These samples originate from method development experiments. HA-activity of these samples ranges from 6 HAU/100 μL to 280 HAU/100 μL.

Influenza A H1N1 PR/8/34 and H3N2 Aichi/2/68 were purchased from Charles River Avian Vaccine Services (North Franklin, USA). Both viruses were produced in embryonated chicken eggs and inactivated by formalin treatment. The virus samples were purified and concentrated to 2 mg/mL by density gradient centrifugation.

2.2. Mercury porosimetry

Mercury porosity of the TSKgel G6000PWxl stationary phase was accomplished with a PoreMaster 60-GT (Quantachrome, Odelzhausen, Germany). Porosimetry experiments were conducted according to the instructions of the instrument supplier. The resin was washed with deionized water and freeze-dried at −45 °C and 0.03 mbar for 24 h with a Zirbus VaCo 2 (Bad Grund, Germany) lyophilizer prior to the porosimetry experiments. 14.34 mL wet resin correspond to 1 column volume of TSKgel G6000PWxl (7.8 mm ID × 30 cm L). Mass and volume of the freeze-dried resin were determined and used for further calculation. 250 mg of the resin was filled into a penetrometer and connected to the mercury porosimetry instrument. The penetrometer was filled with mer-