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High-throughput characterization of virus-like particles by interlaced size-exclusion chromatography

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ABSTRACT

The development and manufacturing of safe and effective vaccines relies essentially on the availability of robust and precise analytical techniques. Virus-like particles (VLPs) have emerged as an important and valuable class of vaccines for the containment of infectious diseases. VLPs are produced by recombinant protein expression followed by purification procedures to minimize the levels of process- and product-related impurities. The control of these impurities is necessary during process development and manufacturing. Especially monitoring of the VLP size distribution is important for the characterization of the final vaccine product. Currently used methods require long analysis times and tailor-made assays. In this work, we present a size-exclusion ultra-high performance liquid chromatography (SE-UHPLC) method to characterize VLPs and quantify aggregates within 3.1 min per sample applying interlaced injections. Four analytical SEC columns were evaluated for the analysis of human B19 parvo-VLPs and murine polyoma-VLPs. The optimized method was successfully used for the characterization of five recombinant protein-based VLPs including human papillomavirus (HPV) VLPs, human enterovirus 71 (EV71) VLPs, and chimeric hepatitis B core antigen (HBcAg) VLPs pointing out the generic applicability of the assay. Measurements were supported by transmission electron microscopy and dynamic light scattering. It was demonstrated that the iSE-UHPLC method provides a rapid, precise and robust tool for the characterization of VLPs. Two case studies on purification tools for VLP aggregates and storage conditions of HPV VLPs highlight the relevance of the analytical method for high-throughput process development and process monitoring of virus-like particles.

1. Introduction

In recent years, promising prophylactic and therapeutic vaccination prospects for public health threats have arisen from the development of virus-like particles (VLPs). VLPs are protein assemblages which are produced by recombinant expression of viral structural proteins [1,2]. Thus, the structure of highly pathogenic viruses such as HIV [3], Influenza [4] and Ebola [5] can be mimicked or tailor-made nanocarriers for antigenic epitope presentation [6–9] can be designed. Due to production in genetically modified organisms, the analysis of product-related and process-related impurities is important during development and manufacturing of VLP vaccines [10,11]. Process-related impurities such as host cell proteins (HCPs) and DNA can be rapidly assessed by methods standardized in the biopharmaceutical industry for therapeutic proteins [12]. In contrast, quantitative analysis of product-related impurities such as aggregates is more challenging and mostly tailor-made for each vaccine due to the large size and complexity of VLPs. Traditionally, VLP characterization is often done by transmission electron microscopy (TEM) requiring high investment costs, extensive sample and instrument preparation work, and specialized staff. A rapid technology for VLP characterization is dynamic light scattering (DLS) [13–15]. The method allows the determination of hydrodynamic particle diameters by measuring the fluctuations of light scattering from particles in solution. However, DLS is less sensitive in resolving aggregates [16]. Currently used quantitative methods for VLP aggregates are asymmetrical flow field-flow fractionation (AF4) [17,18,16], disc centrifugation particle size analysis [19], electrospray differential mobility analysis [18], and size-exclusion chromatography (SEC) [20]. These techniques are very time-consuming with analysis times ranging from 30 to 60 min per sample [17,18,16,20]. SEC is the most widely used technique for aggregate quantification in the biopharmaceutical

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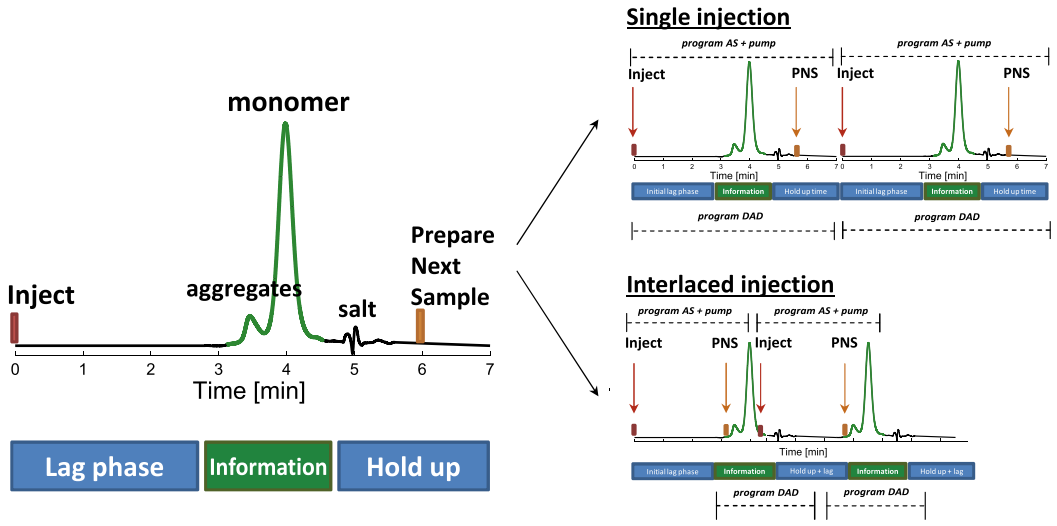

Fig. 1. Schematic illustration of SEC chromatograms for single- and interlaced-injection mode of an analyte containing aggregates and monomer. Information phases are marked by green colored bars, lag and hold-up phases by blue colored bars. Timelines for the program of the autosampler (AS) and pump and the diode array detector (DAD) are presented for two sequent injections in single- and interlaced-injection mode. (For interpretation of references to color in this figure legend, the reader is referred to the web version of the article.)

industry [21]. SEC methods have been successfully applied for process monitoring of capsomere vaccines [22], recombinant fusion protein vaccines [15], and human hepatitis B virus surface antigen (HBsAg) VLPs [23–26]. Recently, rapid size-exclusion ultra-high performance liquid chromatography (SE-UHPLC) methods have been realized for monoclonal antibodies by performing interlaced sample injections with analysis times of 2–6 min [27,28]. Fig. 1 shows a schematic drawing of the principle of interlaced SEC (iSEC) methods. The 'information phase' (*green*) in a SEC run is the time range including the elution of relevant species (aggregates, monomer). The longest phase in a classical single injection SEC method run is the 'lag phase' (*blue*), which is the time range from injection to elution of the first species. The 'hold- up' (*blue*) phase refers to the time from the end of the information phase to the column's void time defined by the elution of small molecules such as salts. In order to reduce the total analysis time of SEC methods without changing the performance of 'information phases', the 'lag phase' can be eliminated by injecting subsequent samples prior to the complete elution of previous sample components. This operation is referred to as interlaced injection mode.

In this work, we present the development and application of an iSE-UHPLC method for recombinant protein-based VLPs. The feasibility of the assay was evaluated for human papilloma (HPV) VLPs [29], human enterovirus 71 (EV71) VLPs [30], murine polyomavirus (MuPyV) VLPs [7], human B19 parvo (B19 VP1/VP2) VLPs [31]., and chimeric hepatitis B core antigen (HBcAg) VLPs [8]. Two case studies are presented for the application of the iSE-UHPLC during downstream process development and stability studies. The designed method allows a rapid assessment of VLP dispersity and is well-suited for high-throughput pharmaceutical process development of VLPs.

2. Materials and methods

2.1. Disposables

For precipitation screenings, sample storage, fractionation by FPLC and UHPLC, 350 μL-polypropylene plates (Greiner Bio-One, Kremsmünster, Austria) were used. Stability studies with HPV VLPs were performed in 1.5 mL-polypropylene Eppendorf® Safe-Lock Tubes (Eppendorf, Hamburg, Germany). Frozen VLPs were thawed and centrifuged in the same tubes at 18,000 × *g* and 4 °C for 10 min.

2.2. Chemicals and buffers

For the SE-UHPLC method, K_2HPO_4 was obtained from VWR BDH Prolabo (Radnor, Pennsylvania, USA). MOPS was purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). The SEC standard proteins thyroglobulin from bovine thyroid and uracil were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Alfa Aesor (Ward Hill, MA, USA), respectively. All other chemicals were obtained from Merck KGaA (Darmstadt, Germany). All buffer solutions were prepared with ultra pure water drawn from a water purification system provided by Sartorius (Goettingen, Germany). UHPLC analysis was conducted with buffers composed of 0.2 M K_2HPO_4 and 0.25 M KCl. The pH value was set at pH 7.0 for characterization studies with HPV VLPs and pH 7.4 for the SEC analysis of other VLPs. Semi-preparative purifications of B19 VP1/VP2 VLPs were performed by aqueous two-phase extraction and precipitation with PEG 4000 as described previously [32] and by anion-exchange membrane chromatography [33]. PBS buffer at pH 7.4 was used as mobile-phase buffer for chromatography experiments with a semi-preparative SEC column.

2.3. Virus-like particles

The general applicability of an interlaced SE-UHPLC method for VLP characterization was evaluated with several purified VLPs differing in size, morphology, expression hosts, and number of viral proteins. An overview of analyzed VLPs is given in Table 1.

Purified HPV VLPs (HPV type 33 [29]) derived from yeast cells were kindly provided by Merck & Co (Kenilworth, NJ, USA) at a concentration of 0.8 mg/mL in a buffer containing histidine and polysorbate 80 (pH 6.2). MuPyV VLPs were produced at shaker flask scale in *Escherichia coli* cells, purified as published by Middelberg et al. [7], and dialyzed into PBS (pH 7.4) yielding a concentration of 0.3 mg/mL. EV71 VLPs derived from *Spodoptera frugiperda Sf*9 insect cells were kindly supplied by Sentinext Therapeutics (Penang, Malaysia) in a Tris buffer (pH 7.5) at a concentration of 0.1 mg/mL. Chimeric HBcAg VLPs with fused tumor epitopes were expressed in *E. coli* and generously provided by BioNTech Protein