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Asymmetrical flow field-flow fractionation coupled with multi-angle laser light scattering for stability comparison of virus-like particles in different solution environments

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ABSTRACT

The stabilities of two commercially available virus like particles, CHO-HBsAg expressed by Chinese hamster ovary (CHO) cells and Hans-HBsAg expressed by *Hansenula polymorpha* (Hans), were compared using AF4-MALLS under different treatment processes. The initial molecular weight and hydrodynamic diameter of CHO-HBsAg measured with AF4-MALLS were 4727 kDa and 29.4 nm, while those of Hans-HBsAg were 3039 kDa and 22.8 nm respectively. In salt solution of 2 M ammonium sulfate, the molecular weight and size of CHO-HBsAg had little change, and its antigenicity remained 95%, while those of Hans-HBsAg changed greatly, resulting in aggregation and 75% antigenicity loss. In freeze–thaw operations, Hans-HBsAg aggregated heavily. Most of the aggregates precipitated and the rest soluble aggregates reached 10^5–10^6 kDa in molecular weight. The antigenicity of Hans-HBsAg decreased to 26.9% after five freeze–thaw cycles. For CHO-HBsAg, there was no obvious aggregation in freeze–thaw, and the antigenicity retained above 98%. In heating process, Hans-HBsAg gradually aggregated to large particles with temperature and the antigenicity decreased to 10% when the temperature reached 80 °C. In contrast, CHO-HBsAg would not aggregate with temperature, remained 92% antigenicity at 80 °C. The study demonstrated that CHO-HBsAg appeared to be a superior vaccine antigen in term of particle stability and constant antigenicity, which are important in production, transportation and storage.

1. Introduction

Asymmetrical flow field-flow fractionation (AF4) is a mild separation technique where a field is applied to a fluid suspension or solution pumped through a long and narrow channel which contains only one permeable wall serving as accumulation barrier. The field is perpendicular to the direction of flow in order to cause separation of the particles present in the fluid. The separation is matrix-free, thus avoiding any solid–liquid interaction that may affect the particle size in other analytical methods [1,2]. Availability of commercial AF4 instrument coupled with sophisticated analytical equipment such as multi-angle laser light scattering (MALLS), mass spectrometry and luminescence detection has widened the applications in biotechnology.

One obvious application is the analysis of virus-like particles (VLPs) used as vaccines and prospective gene delivery agents. Chuan et al. [3] used AF4-MALLS to quantitatively determine the variability across different preparations of VLPs, as clearly demonstrated by the difference in size distributions of VLPs from insect cells and those following in vitro self-assembly. Mohr et al. [4] reported the development of a miniaturized high-throughput strategy to evaluate the performance of various excipients in enhancing the stability of murine polyomavirus (MuPyV) VLPs against aggregation. AF4-MALLS was also applied to the characterization and quantitation of type A influenza virus particles to assess its usefulness for vaccine preparation [5].

Hepatitis B surface antigen (HBsAg), a virus-like particle, can be produced in various biological systems such as yeast (*Saccharomyces cerevisiae* or *Hansenula polymorpha*) [6,7] and mammalian cells (Chinese hamster ovary, CHO) [8,9], and have been used to

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develop Hepatitis B vaccines. However, processing HBsAg in a real manufacturing environment may encounter the problem of aggregation due to their large size and complex structure. The aggregation may occur during processes such as separation, purification, lyophilization. It may also happen during transportation and storage [10–12]. There has been evidence that aggregation resulted in the loss of vaccine potency [13,14]. The aggregation was induced by process environment, and its degree may be dependent on different expression systems such as CHO cells (CHO-HBsAg) and *Hansenula polymorpha* (Hans-HBsAg). The CHO-HBsAg owned glycosylation and the whole composition is closer to that of plasma-derived vaccine than yeast-derived HBsAg [7,15]. Zhou compared the molecular weight, size and monomer number between CHO-HBsAg and Hans-HBsAg by high-performance size exclusion chromatography (HPSEC) and multi-angle laser light scattering (MALLS) [16]. The data obtained support the assumption that the higher immunogenicity of CHO-HBsAg is related to its more favorable macromolecular assembly structure. Besides, Diminsky et al. [8] studied the effect of storage temperature and HBsAg lyophilization on the structure, stability and immunogenicity of CHO-HBsAg, suggesting that the vaccine's physical, chemical and immunological characteristics are sufficiently stable at high temperature to reduce the need for "cold chain" transportation. Unfortunately, these results were not compared with yeast-derived HBsAg which occupies the major market. Among the yeast carries, *Hansenula polymorpha* is reported to be most efficient in fermentation productivity [17].

In a real manufacturing process, the original structure of the HBsAg would change with conditions such as aggregation in different salt solutions or in chromatographic columns. Chen et al. [18] reported that Hans-HBsAg aggregated reversibly in high NaCl concentration and irreversibly in high ammonium sulfate solution. Greiner et al. [19] characterized the structural modifications accompanying the loss of HBsAg immunogenicity induced by three weeks incubation at 60 °C through physical techniques and further evidenced the close relationship between the structure and integrity of the Hans-HBsAg particle surface and its immunogenic properties.

To our best knowledge, there is no work comparing the differences of aggregation behavior and antigenicity between CHO-HBsAg and Hans-HBsAg under different process conditions. The purpose of this work is to utilize AF4-MALLS, the excellent technique measuring the molecular weight and size in solution environment, to compare the initial particle size and size distribution, and their change in different treatment processes. The study could provide database for the vaccine production, quality control and for evaluation of the safety and efficacy of HBsAg vaccine in preparation, transportation and storage.

2. Experimental

2.1. Reagents and materials

Ammonium sulfate ($(NH_4)_2SO_4$, 99%), disodium hydrogen phosphate ($Na_2HPO_4{\cdot}12H_2O$, 99%), sodium dihydrogen phosphate ($NaH_2PO_4{\cdot}2H_2O$, 99%) and sodium chloride (NaCl, 99.5%) were all analytical reagents. Milli-Q water of 18 MΩ (Merck Millipore, Bedford, MA, USA) was used for preparation of all solutions. Filter membranes were Durapore 0.1 μm from Sartorius (Sartorius AG, Goettingen, Germany). Purified HBsAg particles from Chinese hamster ovary and *Hansenula polymorpha* were kindly provided by North China Pharmaceutical Corporation (NCPC, Hebei, China) and Hualan Biological Engineering Company (Henan, China) respectively. The details for production and purification were depicted in Zhou et al. [16].

2.2. Instrument

The asymmetrical flow field-flow fractionation experiments were carried out using AF2000 MultiFlow FFF from Postnova Analytics (Landsberg, Germany). The AF4 channel was equipped inside the column oven and connected to three pumps during the whole separation. The cross-section of the channel is trapezoidal and the channel dimensions were 27.5 cm in length and from 2.0 to 1.0 cm in width. A spacer of 350 μm thickness was used for the separation and it was clamped between two plates. All separations were performed using a regenerated cellulose membrane with a cut-off of 10 kDa. The volume of the injection loop was 100 μL. Detection chains consisted of UV detector (Agilent Technologies 1100 series) tuned at 280 nm, a multi-angle light scattering detector (DAWN EOS, λ = 690 nm, Wyatt Technology Corp., USA) and a refractive index detector (OPTILAB DSP, Wyatt Technology Corp., USA).

2.3. Salt solution treatment

CHO-HBsAg and Hans-HBsAg solutions were mixed with ammonium sulfate solution at volume ratio of 1:1 and incubated for 1 h under room temperature after which the samples were analyzed by AF4-MALLS. The concentration of ammonium sulfate in data analysis was the final one in the VLP solutions.

2.4. Freeze–thaw treatment

CHO-HBsAg and Hans-HBsAg sample tubes were preserved to −70 °C in freezer for 2 h, after which the samples were thawed at room temperature. Freeze–thaw cycles repeated for five times, after which the samples were analyzed by AF4-MALLS.

2.5. Heat treatment

Solution samples of CHO-HBsAg and Hans-HBsAg were gradually heated from room temperature (20 °C) to 80 °C with interval of 10 °C by a metal bath with a temperature controller Model H_2O^3-PRO from Gingkobio Scientific (Beijing, China). Every temperature was maintained for 2 h and analyzed by AF4-MALLS.

2.6. AF4-MALLS methods

The separation was performed using an axial flow rate (flow rate through the detector) of 1.0 mL/min which was constant throughout the entire separation. Separations were started with sample injection and focusing for 4 min with the tip pump flow rate of 0.2 mL/min and focus pump flow rate of 1.8 mL/min. The cross-flow rate was constant at 1.0 mL/min during the injection and focusing step. The transition time from injection and focusing step to elution step was 1 min. Elution was started at cross-flow rate of 1.0 mL/min for 40 min and decreased to 0 in 5 min linearly. After the elution step, the cross-flow was kept at 0 for 5 min again to flush out any remaining sample components before the next separation was started. The mobile phase was 20 mM phosphate-buffer, pH 7.4. The RI and MALLS detectors were calibrated using sodium chloride and toluene, respectively. The MALLS detectors were normalized using BSA solution following the protocols recommended by Wyatt Technology. The 13th detector was occupied by a light fiber for dynamic light scattering measurement. The adopted *dn/dc* value of CHO-HBsAg and Hans-HBsAg were 0.165 mL/g and 0.164 mL/g as calculated by Zhou et al. [16]. Each curve shown is representative of triplicate samples.

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