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Development of a bio-analytical strategy for characterization of vaccine particles combining SEC and nanoES GEMMA

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Abstract

Commonly used methods for size and shape analysis of bionanoparticles found in vaccines like X-ray crystallography and cryo-electron microscopy are very time-consuming and cost-intensive. The nano-electrospray (nanoES) gas-phase electrophoretic mobility macromolecular analyzer (GEMMA), belonging to the group of ion mobility spectrometers, was used for size determination of vaccine virus particles because it requires less analysis time and investment (no vacuum system). Size exclusion chromatography (SEC) of viral vaccines and production intermediates turned out to be a good purification/isolation method prior to GEMMA, TEM (transmission electron microscopy) and AFM (atomic force microscopy) investigations, as well as providing a GEMMA analysis-compatible buffer. Column materials and different elution buffers were tested for optimal vaccine particle yield. We used a Superdex 200 column with a 50 mM ammonium acetate buffer. In addition, SEC allowed the removal of process-related impurities from the virions of interest. A sample concentrating step or a detergent addition step was also investigated. As a final step of our strategy SEC-purified or untreated vaccine-nanoparticles were further analyzed: (a) by immunological detection with a specific polyclonal antibody (dot blot) to verify the biological functionality, (b) by GEMMA to provide the size of the particles at atmospheric pressure and (c) by AFM and (d) TEM to obtain both size and shape information. The mean diameter of inactivated tick-borne encephalitis virions (*i.e.* vaccine particles) determined by GEMMA measurement was 46.6 ± 0.5 nm, in contrast to AFM and TEM images providing diameters of about 58 ± 4 and 52 ± 5 nm, respectively.

Introduction

The visualization of viruses, inactivated viruses (vaccines) and other pathogens as bacteria has drastically increased the knowledge about their structure and their infectious behavior. Morphological analysis of virus particles and nanobioparticles in general is usually

performed with methods like electron microscopy (EM), especially cryo-electron microscopy and X-ray crystallography.^{1,2} These two methods allow the visualization and characterization of nanobioobjects' structures at the Ångström level.³ However, a cost-intensive and time-consuming elaborated sample preparation is necessary and the data interpretation requires profound knowledge and sophisticated software, as well as high instrument investments. Transmission electron microscopy (TEM) of negatively-stained virus and vaccine particles derived from liquid samples is a powerful tool to obtain images of biological materials in the nm range, but besides good knowledge on staining methods for certain biological species high-investment costs are necessary.⁴ Data about general virion size and even some capsid substructures, the capsomers,⁵ can be provided by atomic force microscopy (AFM) by generating images of the scanned nano-object adsorbed on an even surface. In case a liquid cell is used for AFM measurements in solution, the nanoparticles have to be fixed on the substrate.^{6,7} For AFM analysis in ambient air of analytes in the nanometer range, sample preparation is not so sophisticated in the tapping mode.⁸⁻¹⁰ Apart from the different sample preparation methods for virus/vaccine particle analysis in ambient air or in solution, it has to be kept in mind that the biological activity of a particle highly depends on its structure, especially the structure in solution. Therefore, the different sample preparation methods may have an influence on the virus nanoparticle geometry (*i.e.* a hard-to-control bias is introduced), because for AFM the virus particles are adsorbed on the substrate in solution and then dried before recording. For TEM analysis the virions are adsorbed to a specially prepared grid and stained afterwards in solution, dried and then measured in an ultra-high vacuum.¹¹

Besides these visualization/microscopic methods for virus nanoparticle characterization, field-flow fractionation (FFF) combined with a multi-angle light scattering (MALS) detector was used recently for the determination of the size distribution in virus samples.¹² Even though the measurement was carried out under native conditions, data interpretation *via* MALS is not straightforward, because a certain bias is easily introduced. Other techniques such as dynamic light scattering (DLS), size exclusion chromatography (SEC) with different detection systems, ESI mass spectrometry and analytical ultracentrifugation can be used to size virus nanoparticles but with severe disadvantages.¹³⁻¹⁷

The specific structure of one (*i.e.* single) inactivated virus particle, which is accessible with the above-mentioned microscopic methods, is more or less negligible in virus vaccine development. For considering the immunological effects, the structures of a few nanoparticles are of minor relevance in contrast to the statistical evaluation of thousands of vaccine particles, which are difficult to obtain with the mentioned microscopic methods in a statistically solid way even with sophisticated software. Besides detailed virus characterization during vaccine development (*i.e.* process development) and knowledge about the size distribution of candidate vaccines, it is of importance to gain information on *e.g.* fragment-to-virus or nanoparticle-to-aggregate ratios because all of these parameters may influence the immunological efficiency. It has been reported for an influenza vaccine that splits virions, whole virus particles as well as various states of aggregation elicit different immune responses in mice.^{18,19} As mentioned above, the estimation of the nanoparticle size distribution (within an image of one vaccine batch) based on a significant

number (>1000) of nanoparticles is a very challenging task. The diameters of all present nanoparticles have to be collected within one or more images and evaluated statistically to finally draw really valuable conclusions about *e.g.* aggregation behavior or virus stability.

In the late 1990s an instrument called nano-electrospray gas-phase electrophoretic mobility macromolecular analyzer (nanoES GEMMA, also later termed *macroIMS*, macro-ion mobility spectrometer and LiquiScan-ES, liquid scan-electro spray system), originally designed for nanoaerosol physics, was applied to analyze biomolecules like proteins, DNA fragments and other organic nanoparticles, thereby obtaining a correlation between electrophoretic mobility diameters (EMDs, *i.e.* particle diameter or size) and the molecular mass of analytes up to 2 MDa.^{20,21} Recently, macromolecules such as carbohydrates and non-covalent functional protein complexes, even virus and bacteriophage samples were analyzed by this method to elucidate EMDs and to try to determine the molecular mass of a human rhinovirus particle.²² Apart from the molecular mass of a single virion, the complex of monoclonal antibodies with the human rhinovirus and the ratio of antibodies per viral particle could be determined.²³ A brief introduction of the methodology will help to understand the presented results and details can be found elsewhere.^{20,22,24,25} For nanoES GEMMA, the compounds of interest are aerosolized out of a solution at atmospheric pressure *via* an aerosol generator unit. It is assumed that during this step the produced droplets are dehydrated completely (only non-volatile compounds might remain). Subsequently, these multiple charged nanoparticles are charge-reduced by using a Polonium-210 α -radiation source. The singly charged, dry aerosol particles are then separated according to their EMD in the nanodifferential mobility analyzer (nanoDMA) operating in the size range of *e.g.* 3–120 nm. The classified (*i.e.* separated) particles leaving the nanoDMA are transferred and counted in the ultrafine condensation particle counter (uCPC) and finally a GEMMA spectrum with the particle number concentration (number of particles per cm³) on the y-axis and the EMD (*i.e.* size in nm) on the other axis is generated.²²

The outstanding advantages of this method are (a) the analyses of the molecular mass of macromolecules which are not accessible by mass spectrometric methods, (b) the method is independent of the chemical nature of the nanoparticle, (c) the system runs without vacuum pumps at atmospheric pressure, (d) the measurement of un-biased particle diameters in the nm range which is hardly accessible by other methods (*i.e.* from 2.5 to 300 nm with the nanoDMA), (e) the resolving power is better than that of SEC and similar to that of FFF, (f) the required sample volumes are between 1 and 2 μ L (consumed volumes in the nL range), (g) the sensitivity for proteinous nanoparticles is 10³ times below UV detectors (214 nm) and (h) the method allows the straightforward possibility to analyze non-covalently protein complexes as intact viruses.^{26,27} There are certain drawbacks as certain sample solution requirements are necessary such as (a) low ionic strength, volatile buffers and protein concentrations below μ g mL⁻¹, (b) not enough resolving power in the size range above 15 nm and (c) a degree of uncertainty in the size determination (± 0.25 nm) of nanoparticles up to 60 nm. It has to be kept in mind that other techniques such as the already mentioned FFF, DLS, analytical ultracentrifugation and analytical size exclusion chromatography (SEC) can also be applied to large proteins and complexes but with severe disadvantages. Analytical

SEC for sizing is only applicable to nanoparticles with a molecular mass smaller than 5 MDa,²⁸ while intact viral particles are estimated to be in the range of several MDa up to 200–300 MDa.²⁹ In the present work SEC was only applied as a preprocessing step for rough purification and fractionation, as well as a buffer exchange system to have samples ready in a volatile buffer system, which is compatible with the nanoES process applied in the GEMMA instrument.

In the present study we introduce a new strategy based on a combination of SEC as a pre-treatment method and nanoES GEMMA that is capable of analyzing the size distribution of vaccine particle samples. The results are corroborated by AFM and TEM measurements as well as immuno-analytical investigations. For this development we used well-characterized and inactivated tick-borne encephalitis (TBE) virions which have a spherical shape with a diameter of approximately 50 nm and a molecular mass of about 22 MDa.^{30,31}

Experimental

Isolation and purification of whole virus particles

Inactivated TBE virus vaccine samples were provided by Baxter Innovations (Orth/Donau, Austria). Such TBE virion preparations were purified by sucrose-density centrifugation and showed $\sim 200 \mu\text{g mL}^{-1}$ total protein content.^{32,33}

Chemicals

All chemicals and solvents applied were of analytical grade and used without further purification. If not stated otherwise for all buffer formulations water of ultra-high quality with a resistivity of $18.3 \text{ M}\Omega$ (25°C) was used (Simplicity UV water purification system, Millipore).

SEC

Buffer exchange, desalting and purification of the virions were performed with a FPLC-System (Pump P-500, Valve V-7 and Fraction Collector Frac-100, Pharmacia Fine Chemicals) equipped with a Superdex 200 10/300 GL (GE Healthcare Bio-Sciences) SEC column. 200 μL or 500 μL samples were loaded onto the column and eluted with different buffer concentrations (20 mM, 50 mM or 100 mM) of ammonium acetate (Merck), pH = 7.4 adjusted with ammonia (Merck) with a flow rate of 25 mL h^{-1} . Prior to fractionation 5 μL volumes containing different amounts (0%, 0.001%, 0.01% in water) of Tween 20 (Sigma-Aldrich) were put into the tubes used for collecting the fractions. The volumes of the fractions (500 μL) were reduced in a vacuum centrifuge (Univapo) to approximately 50 μL prior to measurements, unless otherwise stated.

Furthermore a HiTrap desalting column (GE Healthcare Bio-Sciences) was used with 100 mM ammonium acetate, pH = 7.4 adjusted with ammonia. 200 μL of sample were injected at a flow rate of 60 mL h^{-1} and fractions of 100 μL were collected.

Immunological detection

SEC fractions were immunologically tested for the presence of virions. After volume reduction aliquots of 2 μ L of each fraction were dropped onto a nitrocellulose membrane (0.45 μ m, Bio-Rad Laboratories) which was first incubated with mouse-derived anti-virus (TBEV) antibodies kindly provided by Baxter Innovations. After a washing step with Tris-buffered saline (TBS)-buffer with 0.1% Tween 20, a commercially available secondary antibody, anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (Sigma-Aldrich), was added. For color development 5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine (BCIP) and nitro-blue tetrazolium chloride (NBT) color development solutions (Bio-Rad Laboratories) were prepared according to the manufacturer's manual and added to the membrane until color development was sufficient. The reaction was stopped by adding water.

Gas-phase electrophoretic mobility macromolecular analyzer (GEMMA)

Volume reduced SEC fractionations were measured on a nanoES GEMMA instrument consisting of an electrospray aerosol generator (Model 3480, TSI Inc), an electrostatic classifier (Model 3080, TSI Inc) equipped with a nanodifferential mass analyzer (nanoDMA, Model 3085, TSI Inc) as a separation device and an ultrafine condensation particle counter (CPC, Model 3025A, TSI Inc) for detection. Data were recorded by the *macroIMS* manager software (release version 2.0.1.0., TSI Inc). Measurements were performed with a filtered air flow of 0.5–1.0 Lpm, a concentric CO₂ sheath gas flow of 0.1 Lpm and a chamber pressure of 4.2 psid (pound per square inch differential). The voltage for the nanoES process was set individually for every sample to operate in the cone jet mode and ranged from 1.10–2.00 kV. A fused silica capillary with an inner diameter of 25 μ m (polyimide coated, O.D.: 150 μ m, TSI Inc) grounded to a conical tip was used for the spray process.

By selecting a sheath gas flow of 10.0 Lpm for the nanoDMA an electrophoretic mobility diameter (EMD) range up to 80.6 nm can be scanned. The actual measured size range was from 3.08 to 80.6 nm to measure impurities or smaller constituents as well as the whole vaccine virions. For data interpretation the median of 10 scans of each sample was used.

AFM

The measurements were carried out in air in tapping, constant amplitude mode using silicon cantilevers with integrated silicon tips (NanoWorld, Arrow type: NC) on a NanoScope III multimode SPM instrument (Veeco Instruments). For AFM measurements 10 to 20 μ L of the samples were dropped onto freshly cleaved mica platelets. After 5 min the mica surface was rinsed with double distilled water and blown dry with nitrogen. Depending on the scanned area, scan rates of 1 or 2 Hz were used. AFM recordings were corrected with the Research Nano-Scope 7.30 Software (Veeco Instruments) and height or length measurements were performed with the SPIP 5.1.6 software (Image Metrology).

TEM

Samples were adsorbed on copper grids, coated with formvar and carbon, for 1 min. Negative staining was performed using 1% uranyl acetate (pH = 4.5) for 1 min.³⁴ Samples

were examined on a Tecnai G² 20 instrument (FEI) transmission electron microscope with an acceleration voltage of 80 keV at various magnifications. Particle diameters were measured with the Photoshop CS 5 software (Adobe Systems).

Results and discussion

For virion characterization, excipients and additives used during virus nanoparticle preparation and in the final formulation have to be removed to prevent interference affecting the measurement itself or promoting artifact generation leading to difficult data interpretation or even misinterpretation. One fast and easy way for interference (particular small molecules and their aggregates) removal is SEC, which can also be used to change unfavorable buffer systems. In the case of electrospray-based instrumental approaches like the nanoES GEMMA, volatile buffers have to be used which are not commonly used in the production process or in commercial vaccines.

For finding the appropriate elution buffer for SEC and subsequent GEMMA analysis, different volatile buffer systems with 20 mM, 50 mM and 100 mM ammonium acetate with pH = 7.4 were tested and evaluated. This buffer system is quite unusual for virus vaccine preparations and the stability of the isolated particles was a concern. For this, Tween 20 was added as a stabilizing agent to the elution buffer or to the collected fractions. To check the presence of virus particles in single SEC fractions, dot blots were established as a quick immunological testing procedure. Fig. 1 shows the results for 500 µL of virus preparation separated on a Superdex 200 10/300 GL SEC material and 100 µL prepared on HiTrap desalting devices using various buffer concentrations and a defined concentration of Tween 20 provided either in the collection tubes (5 µL 0.01%) or in the elution buffer (0.0001%). Due to the highly specific anti-virus antibody, the dot blot clearly showed which eluting conditions were favorable to obtain high virus yields. It could be shown that 20 mM ammonium acetate is not sufficient for eluting high amounts of virus particles from the SEC column (Fig. 1A), whereas 100 mM ammonium acetate showed clear spots in five fractions (Fig. 1D).

To achieve higher TBE vaccine particle yields, Tween 20 was used as a stabilizing agent. The results obtained by direct addition of this detergent to the elution buffer were compared to those obtained when Tween 20 was added to the collection vials before fraction collection. The results indicated that an elution buffer containing Tween 20 throughout the SEC process is not favorable (Fig. 1C), but the addition of Tween 20 to each sampling tube before collection led to more intensive spots in the immunological screening (Fig. 1B and E). In analogy to Western blotting, it was assumed that the established immunological method gives relative quantitative information, such that more intensive spots correlate with higher virus particle concentrations. Finally, an ammonium acetate concentration of 50 mM with pH 7.4 was chosen as it had a much sharper elution profile with Tween 20 added to the fraction collector's tubes before fractionation. Using this setup, the vaccine particles could be separated from unfavorable process additives and/or necessary excipients, and the buffer could be changed to a volatile system with a controllable sample dilution effect. These results were compared to another SEC material or a better device, namely a HiTrap desalting

column. However, since the separation efficiency was worse and the virus particles eluted distributed over several fractions (Fig. 1F), this approach was rejected.

After reducing the volume of each SEC fraction to 50 μ L in a vacuum centrifuge to gain higher virus particle concentrations, the immunologically positive samples were analyzed using the nanoES GEMMA to obtain information about the presence of whole virus particles, the EMD of virions and the size distribution pattern of the liquid samples. GEMMA spectra of the sample with different ammonium acetate concentrations during SEC fractionation revealed an EMD for the whole virus particle of 46.6 nm (Fig. 2A). By comparing the diameter described in the literature³⁰ with the obtained GEMMA spectra, we concluded that the peak with an EMD around 46.6 nm comprises whole TBEV. The resolving power (expressed as full width at half maximum, FWHM) in this size range for a monodisperse particle is 4.5% at the maximum flow rate of the applied nanoDMA. As described later, the fractions used for nanoES GEMMA experiments were also analyzed by means of AFM and TEM. Both microscopic methods for these samples showed whole virus particles as well.

It was observed that the buffer concentration has a significant influence on the virus particle signals in the GEMMA spectra. The peak height and maximum variations were observed. All samples were collected in sample tubes containing 0.01% Tween 20 with varying salt amount in the eluent. The highest particle concentration ($1.0\text{--}2.0 \times 10^6$ particles per cm^3) for the virus particle peak was measured by utilizing 50 mM ammonium acetate. 20 mM and 100 mM ammonium acetate gave significantly lower signal intensities. In Fig. 2B, the mean virion EMDs are presented which were calculated from the peak maximum. The mean EMD was determined to be 46.8 nm for the nanoparticles eluted with 50 mM ammonium acetate buffer, which is slightly smaller than the published diameter of approximately 50 nm measured by X-ray crystallography.³⁰ These data fit to each other, as particle diameters measured by X-ray crystallography are usually higher due to the incorporated water and manipulation steps during measurements, while the nanoES GEMMA is known to show slight particle compression originating from the electrospray process.^{26,35}

Interestingly, we found that the peak maxima in the GEMMA spectra shifted and the observed mean EMD of the virus particles increased with higher ammonium acetate concentrations. In Fig. 2B it is shown that beside 46.8 nm for nanoparticles eluted with 50 mM ammonium acetate, we found 45.6 nm for those eluted with 20 mM and we calculated 47.3 nm for those eluted with 100 mM ammonium acetate. This EMD shift can mainly be attributed to buffer residues (we applied the highest quality of ammonium acetate in terms of non-volatile residues as described by the manufacturer) sticking to the particles of interest after droplet solvent evaporation during the nanoES process, which shifts the EMD to a higher value in correlation with the buffer concentration, confirming Dole's evaporating-droplet model.³⁶

The apparent EMD of the virus vaccine was also influenced by the addition of different amounts of Tween 20 (Fig. 3A), the lower the detergent concentration the lower the EMD. All experiments were carried out with 100 mM ammonium acetate buffer with varying amounts of Tween 20 in the collection tube. As described above, the virus concentration was

lower when no Tween 20 was added to the sampling tubes in the fraction collector, but the observed mean EMD (46.2 nm) was lower than those of samples containing Tween (0.001% Tween: 46.3 and 0.01% Tween: 47.3). Again a thin surface coating of the virion with the nonionic polymeric detergent can be assumed.

The influence on the EMDs of the vacuum centrifugation step was also examined (Fig. 3B). After SEC using 100 mM ammonium acetate and sampling the interesting fractions in sample tubes containing 0.01% Tween 20, the particles were either measured directly without any concentration step or the volume was reduced to 50 μ L. The EMD of the untreated virus particles was 48.8 nm and with the vacuum centrifugation step it was significantly lower, namely 47.4 nm. This can be explained by the fact that aqueous ammonium acetate solution emits (*i.e.* falls apart) ammonia and acetic acid during evaporation in the electrospray process. This leads to less concentrated buffer solutions providing less residue particles attachable to the vaccine virion of interest and therefore smaller EMDs are measured. The resulting EMD data (47.4 nm) after vacuum centrifugation can be compared to the above-presented study, proving that this experiment is reproducible ($N = 20$).

Beside the newly introduced nanoES based method to study virion diameters, more common methods like AFM in the tapping mode on mica surfaces and TEM were used to visualize the biologically active virus fraction and corroborate the data obtained with the nanoES GEMMA. Variations in the measured diameters gained from the different methods (based on quite orthogonal concepts and also sample preparation protocols) could be attributed to the individual preparation methods and conditions during the measurements. For AFM, the TBE virus vaccine particles were adsorbed to the substrate (freshly prepared mica surface) in a solution and then dried (*i.e.* removal of the solvent system and of non-adsorbed particles) under ambient conditions before analysis under ambient pressure. For TEM analysis, the particles were adsorbed on a copper grid and stained afterwards in solution. After drying under ambient conditions the images were recorded in a high vacuum. In the nanoES GEMMA instrument the vaccine particles first undergo an electrospray process and then it is assumed that the aerosol droplets are completely dried as well as charge-reduced to singly charged species before they are classified/separated by the nanoDMA. All these steps are performed at atmospheric pressure without any heat burden.

The starting vaccine material (directly from the production process) and SEC fractions of this material showing positive immunological response by dot blot experiments and virion peaks of significant height with an EMD of ~ 46.4 nm by using the nanoES GEMMA were compared. The TEM micrograph of the starting material of the TBEV vaccine particles showed plenty of mainly round shaped particles of 52 ± 5 nm (calculated from 37 particles from several recorded images) in diameter (Fig. 4A). Getting accurate particle diameter from TEM images *via* manual measurements was rather challenging, because the contrast between the background and the nanoparticle itself is low and the edges are not very sharp. Nevertheless, it was possible to perform a statistical analysis. After SEC separation/fractionation, whole virus particles of comparable size distributions were found, although in lower numbers compared to the situation without SEC (Fig. 4D).

The AFM micrographs confirmed these findings showing plenty of round shaped particles in the untreated vaccine sample (Fig. 4B and C) while the quantity was again reduced after SEC fractionation. However the particle diameter of 58 ± 4 nm was calculated from 84 investigated particles from several recorded images and turned out to be even larger with this method compared to TEM analysis. This can be explained by the size and shape of the AFM tip leading to a well-known lateral enlargement of the soft nanoparticles and the type of scanning process.^{7,10,37} The average diameters of the virus particles in the untreated sample (starting material) and the SEC-fractionated sample were comparable leading again to the conclusion that the virions were not altered during SEC. Again the AFM micrographs of the TBEV particles after SEC showed fewer particles than those in the starting material (Fig. 4E and F).

Although buffer exchange and rough particle fractionation can be easily achieved by SEC for vaccine particles a drawback of this method is column aging. A continuous increase in the back pressure from 0.3 to 0.6 MPa could be observed and the particle elution volume decreased (especially if the column was in use for more than 12 months (despite correct storage between uses)), which resulted in higher TBEV particle concentrations in earlier fractions. As a consequence, the retention time and elution volume of the TBEV maximum decreased. Not only chromatography parameters and performance changed, but also alterations in the morphology of the vaccine particles were observed. The morphology of the nanoparticles in the AFM images looked like damaged structures (Fig. 5). The GEMMA spectra of a SEC fraction eluted by an aged SEC column showed an increasing amount of particles with an EMD below 20 nm and a decrease of the major virion peak size (46 nm). It can be assumed that the higher back pressure led to a high degree of virion disruption during SEC column passage. Regarding the size and the structure it can be supposed that the filamentous structures seen in the AFM image (Fig. 5B) between vaccine virus particles were RNA strands (Fig. 5A and B).³⁸ These “RNA structures” were seen in AFM images only. The freshly generated mica surface for AFM measurements is extremely planar, so smaller structures can be more easily seen, whereas the background of the TEM images seemed to be uneven due to the sample preparation and the sample excipients. Finally it has to be stated that aging of the SEC column can be easily monitored (*via* retention time shifts and peak broadening of reference compounds).

Conclusions

A quick strategy with the new method nanoES GEMMA for evaluating TBEV vaccine particle integrity, mean size and size distribution in liquid vaccine samples was shown. SEC turned out to be a suitable method for buffer exchange and removal of additives/excipients from TBEV vaccine samples without significantly damaging the viral structure as long as the back pressure of the SEC column does not increase significantly. Different parameters such as buffer and detergent concentration, as well as a sample concentrating step by vacuum centrifugation have an impact on nanoES GEMMA analysis and the resulting determined EMD. But the choice of the buffer and detergent additives for nanoES GEMMA analysis also influences the performance of the SEC separation. Different buffer mixtures yielded different amounts of virus in the resulting fractions. Low ammonium acetate concentrations are preferred for the electrospray process in nanoES GEMMA. The vaccine

particle images recorded by AFM and TEM corroborated the GEMMA data but the image (size) analysis with the two microscopic methods had to be performed with a highly trained person despite software support. On the other hand, a GEMMA spectrum illustrates the mean size and size distribution of thousands of particles in minutes, *i.e.* good and un-biased (by operator) statistics. For the nanoES GEMMA analysis of purified TBE viral vaccine particles, just a few μL were necessary, whereas a volume of only a few nL were consumed for all measurements and one individual GEMMA spectrum was recorded within 2 min.

In summary, the developed strategy based on SEC and nanoES GEMMA is ideally suited to monitor vaccine development and the production process and to perform quality control tasks.

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Fig. 1.

Immunological verification of the presence of TBE vaccine in SEC fractions. The dot blot of (A) 20 mM ammonium acetate, pH = 7.4, (B) 50 mM ammonium acetate, pH = 7.4, 5 µL of 0.01% Tween 20 solution in collecting tubes, (C) 100 mM ammonium acetate, pH = 7.4, (D) 100 mM ammonium acetate, pH = 7.4; 0.0001% Tween 20 in elution buffer, (E) 100 mM ammonium acetate pH = 7.4, 5 µL of 0.01% Tween 20 solution in collecting tubes and (F) fractionated by a HiTrap desalting column with 100 mM ammonium acetate, pH = 7.4. C is a control sample directly deposited on the dot blot.

Fig. 2.

Influence of the ammonium acetate concentration in the SEC elution buffer on the EMD determined using the nanoES GEMMA. (A) GEMMA spectra at different ammonium acetate concentrations (the main peak corresponds to the intact virion particle (exhibiting a resolution of 5.5% (FWHM)), the peaks between 58 and 65 nm correspond to the virion dimers and those between 20 and 40 nm correspond to virion fragments). (B) Statistical evaluation ($N = 40$ per ammonium acetate concentration) of the determined sizes (EMDs). The point annotated with 42.4 nm indicates a statistical outlier and the bold line represents the median of the measured diameters.

Fig. 3.

Statistical evaluation ($N = 20$ for each parameter) of the influence of the detergent concentration and of a vacuum centrifugation step on the measured EMD (size) using the nanoES GEMMA. (A) No Tween 20 addition (0%) and addition of two different Tween 20 concentrations (0.001% and 0.01%) and (B) the application of a vacuum centrifugation step or without such a step. The point annotated with 46.3 nm indicates a statistical outlier and the bold line represents the median of the measured diameters.

Fig. 4. Microscopic images of TBE vaccine particles in the untreated sample (A–C), (A) TEM, (B) AFM, and (C) AFM with higher magnification and of TBE vaccine particles after SEC purification (elution buffer: 50 mM ammonium acetate, pH = 7.4, 5 μ L of 0.01% Tween 20 in collecting tubes). In fraction number 17 (D–F), (D) TEM, (E) AFM and (F) AFM with higher magnification.

Fig. 5.
Effect of aging of the SEC column material on TBE vaccine particle separation/purification. (A) AFM of fraction number 16 after SEC separation on the used column, (B) AFM with higher magnification of the same fraction showing a damaged virus particle (1) in the center and an intact one (2) at the top on the right. (C) GEMMA spectrum of fraction number 16 after SEC separation obtained from the older SEC column (12 months of use and correct storage).