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

Marlene Havlik\textsuperscript{a}, Martina Marchetti-Deschmann\textsuperscript{a}, Gernot Friedbacher\textsuperscript{a}, Paul Messner\textsuperscript{b}, Wolfgang Winkler\textsuperscript{a}, Laura Perez-Burgos\textsuperscript{c}, Christa Tauer\textsuperscript{c}, Günter Allmaier\textsuperscript{a}\textsuperscript{*}

\textsuperscript{a} Institute of Chemical Technologies and Analytics, Vienna University of Technology, Vienna, Austria

\textsuperscript{b} Department of Nanobiotechnology, University of Natural Resources and Life Sciences, Vienna, Austria

\textsuperscript{c} Baxter Innovations, Orth/Donau, Austria

\textsuperscript{*} Corresponding author:

Institute of Chemical Technologies and Analytics,

Vienna University of Technology, Getreidemarkt 9/164, A-1060 Vienna, Austria.

Tel.: +43 1 58801 15160; Fax: +43 1 58801 15199.

E-mail address: guenter.allmaier@tuwien.ac.at
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 and 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 provided the removal of process-related impurities from the virions of interest. A sample concentrating step or a detergent addition 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 deliver 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.
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.\textsuperscript{1-2} These two methods allow the visualization and characterization of nanobiobjects’ structures at the Ångstrom level.\textsuperscript{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 gain images of biological material in the nm-range, but besides good knowledge on staining methods for certain biological species high-investment costs are necessary.\textsuperscript{4} Data about general virion size and even some capsid substructures, the capsomers,\textsuperscript{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.\textsuperscript{6-7} For AFM analysis in ambient air of analytes in the nanometer range, sample preparation is not so sophisticated in the tapping mode.\textsuperscript{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, specially 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 special prepared grid and stained afterwards in solution, dried and then measured in ultra-high vacuum.\textsuperscript{11}

Besides these visualization/microscopic methods for virus nanoparticles 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.\textsuperscript{12} Even though measurement ensure 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.\textsuperscript{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 is 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 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 split virions, whole virus particles as well as various states of aggregation elicit different immune responses in mice.\textsuperscript{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 macro IMS, 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.\textsuperscript{20-21} Recently, macromolecules as carbohydrates and non-covalent functional protein complexes, even virus and bacteriophage samples were analyzed with this method to elucidate EMDs and to try to determine the molecular mass of a human rhinovirus particle.\textsuperscript{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.\textsuperscript{23} A brief introduction of the methodology will help to understand the presented results and details can be found elsewhere.\textsuperscript{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 produced droplets are dehydrated completely (only non-volatile compounds might remain). Subsequently, these multiply-charged nanoparticles are charge-reduced by a Polonium-210 α-radiation source. The singly-charged, dry aerosol particles are then separated according to their EMD in the nano differential mobility analyzer (nano DMA) operating in the size range of e.g. 3-120 nm. The classified (i.e. separated)
particles leaving the nano DMA 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 is run 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 nano DMA), (e) the resolving power is better than SEC and similar to FFF, (f) the required sample volumes are between 1 and 2 µL (consumed volumes in the nL range), (g) the sensitivity for proteinous nanoparticles is \(10^3\) 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 µg/mL, (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 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 presented work SEC was only applied as a preprocessing step for rough purification and fractionation, as well
as 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 pre-treatment method and nanoES GEMMA that is capable of analyzing the size distribution of vaccine particle samples. 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.\textsuperscript{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 \( \sim 200 \, \mu\text{g/mL} \) total protein content.\textsuperscript{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 MΩ (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. 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 and fractions of 100 µL were collected.

Immunological detection

SEC fractions were immunologically tested for virion presence. After volume reduction aliquots of 2 µL of each fraction were dropped onto a nitrocellulose membrane (0.45 µm, 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'-indolyphosphate 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 nano differential mass analyzer (nano DMA, Model 3085, TSI Inc) as 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 CO$_2$ sheath gas flow of 0.1 Lpm and a chamber pressure of 4.2 psid (pound per square inch differential). The voltage for the nano ES 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 µm (polyimide coated, O.D.: 150 µm, TSI) and grounded to a conical tip was used for the spray process.

By selecting a sheath gas flow of 10.0 Lpm for the nano DMA 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 µL 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 NanoScope 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. 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 are measured with the Photoshop CS 5 software (Adobe Systems).

**Results and Discussion**

For virion characterization, excipients and additives used during virus preparation and in the final formulation have to be removed to prevent interferences 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 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 high-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. 1 A), whereas 100 mM ammonium acetate showed clear spots in five fractions (Fig. 1 C).

To achieve higher TBE vaccine particle yields, Tween 20 was used as stabilizing agent. Direct addition of this detergent to the elution buffer was compared to results where 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. 1 C), but the addition of Tween 20 to each sampling tube before collection led to more intensive spots in the immunological screening (Fig. 1 B 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 better device, namely a HiTrap desalting column. However, since separation efficiency was worse and the virus particles eluted distributed over several fractions (Fig. 1 F) therefore this approach was rejected.

After reducing the volume of each SEC fraction to 50 µL in a vacuum centrifuge to gain higher virus particle concentrations, the immunologically-positive samples were analyzed by 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 concentration during SEC fractionation revealed an EMD for the whole virus particle of 46.6 nm (Fig. 2 A). By comparing the diameter described in the literature 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 half maximum, FWHM) in this size range for a monodisperse particle is 4.5 % at maximum flow rate of the applied nano DMA. 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 particle as well.

It was observed that the buffer concentration has a significant influence on the virus particle signals in the GEMMA spectra. 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 - 2.0 × 10⁶ particles/cm³) 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. 2 B, the mean virions 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.\textsuperscript{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 nanoES GEMMA is known to show slight particle compression originating from the electrospray process.\textsuperscript{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 the 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 nonvolatile 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 to the buffer concentration, confirming Dole’s evaporating-droplet model.\textsuperscript{36}

The apparent EMD of the virus vaccine was also influenced by the addition of different amounts of Tween 20 (Fig. 3 A), 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 for samples containing Tween (0.001% Tween: 46.3, 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. 3 B). 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 µL. 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. 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 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 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 are charge-reduced to singly charged species before they are classified/separated by the nano DMA. All these steps are performed at atmospheric pressure without any heat burden.

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 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. 4 A). 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. 4 D).

The AFM micrographs confirmed these findings showing plenty of round shaped particles in the untreated vaccine sample (Fig. 4 B and C) while 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. The
average diameter 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 in the starting material (Fig. 4 E and
F).

Although buffer exchange and rough particle fractionation can be easily achieved by
SEC for vaccine particles a drawback for 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. 5 B)
between vaccine virus particles were RNA strands (Fig. 5 A and B).\textsuperscript{38} This “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 nano ES 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 unbiased (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 ideal suited to monitor vaccine development, the production process and to perform quality control tasks.
References

Figures with legends

**Fig. 1** Immunological verification of TBE vaccine presence in SEC fractions. Dot blot of (A) 20 mM ammonium acetate, pH = 7.4 (B) 50 mM ammonium acetate, pH = 7.4, 5 µL 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 0.01 % Tween 20 solution in collecting tubes and (F) fractionated by 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 ammonium acetate concentration in the SEC elution buffer on EMD determined by 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 peak between 58 and 65 nm to the virion dimers and between 20 to 40 nm are 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 detergent concentration and of a vacuum centrifugation step on the measured EMD (size) by 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, (C) AFM with higher magnification and of TBE vaccine particles after SEC purification (elution buffer: 50 mM ammonium acetate, pH = 7.4, 5 µL 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).
Highlight of novel aspects

In order to characterize vaccine-nanoparticles during production and in the final formulation we developed a strategy combining SEC and nano electrospray GEMMA (nanoparticle number concentration-based ion mobility device)