Electronic Supplementary Material (ESI) for Nanoscale Advances.

Trafficking of JC virus-like particles across the blood-brain barrier

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Supplementary Methods

Comment [YD]: Answer to Question 2 from Referee 3.

1. Bioanalytical applications for quality control of JC VLPs.

The upscaled and optimized expression and purification process of the JC VLPs required utilization of different buffers at the individual steps. The used buffer systems containing 150 mM NaCl, 10 mM HCl, pH 7.4 with or without $CaCl_2$ (Goldmann *et al.,* 1999)[1] and HEPES buffer (Hoffmann *et al.,* 2018)[2] were reported to be acceptable buffer systems for JC VLP stability and characterization. These buffers helped to stabilize VLPs at the different purification steps and, as a result, helped to increase VLP yield at the different chromatographical steps of the manufacturing.

(1) Protein electrophoresis with Bioanalyzer

VLPs were analysed on 2100 Bioanalyzer (Agilent) using Protein 230 kit (Agilent) according to manufacturer instructions. System control, peak integration and calculations were carried out with Agilent 2100 Expert Software.

(2) Thermal Shift Assay (TSA)

5 µg of VLPs in duplicates were mixed with SYPRO® Orange Protein Gel Stain (Sigma Aldrich) and transferred to Hard-Shell® 96-Well PCR Plate (Bio-Rad). A temperature ramp from 22 to 95 °C with 1°C/min was run on CFX96 Touch™ cycler (Bio-Rad) to detect fluorescence signals at 450-490 nm as excitation and 560-580 nm as emission wavelengths. Data were evaluated using CFX Manager 3.1 (Bio-Rad), where melting temperature was calculated based on the steepest slope of the curve plotted against fluorescence signals over the temperature gradient.

(3) Nano Differential Scanning Fluorimetry (nDSF)

10 µL of VLPs in the concentration range between 0.05-5 mg/mL was run on Tycho™ NT.6 (NanoTemper Technologies GmbH) according to manufacturer instructions. The unfolding profile of protein over a temperature gradient was analyzed by measuring the intrinsic tryptophan fluorescence (using emission wavelength of 330 nm for folded protein and 350 nm for unfolded protein). The infliction temperature (Ti) was calculated based on the steepest slope of the curve plotted against the fluorescence ratio over the temperature gradient.

(4) Size Exclusion HPLC (SE-HPLC)

Size exclusion chromatography was performed with a Hitachi Chromaster HPLC system equipped with a diode array detector (5430, Hitachi) for UV monitoring at 280 nm. VLPs were run through SRT®-C SEC-2000 column (Sepax) in a mobile phase composed of 10 mM Tris–HCl, 150 mM NaCl, pH 7.5 at a flow speed of 0.75 mL/min. System control and peak integration were operated with Chormaster software (Hitachi). VLP fraction was verified by Dynamic Light Scattering (DLS), Western Blotting (WB) and Transmission Electron Microscope (TEM).

(5) Field-Flow Fractionation with Multi-Angle Light Scattering (FFF-MALS)

Field-Flow Fractionation with Multi-Angle Light Scattering (FFF-MALS) was performed with modules in the following order: Agilent1260 HPLC solvent delivery system and autosampler (Agilent Technologies), Eclipse Dualtec (Wyatt Technology), Agilent 1260 infinity variable wavelength detector (with UV monitoring at 280 nm), DAWN Heleos 8+ fitted with a Quasi Elastic Light Scattering system (QELS) (Wyatt Technology). A short channel with a 10 kDa regenerated cellulose membrane (Superon) was used. Field-Flow Fractionation (FFF) was set up using the following parameters: Spacer, 350 µm; permanent channel flow speed, 1.0 mL/min; Elution 2 min; Focus + inject, cross flow 2.0 mL/min, 9 min; Elution, 10 min at a cross flow speed of 2.0 mL/min, followed by a cross flow ramp from 2.0 to 0.05 mL/min for 25 min; Elution + inject 6 min; Total running time, 52 min; Mobile phase, 10 mM Tris-HCl, 150 mM NaCl, pH 7.5.

(6) Nanoparticle Tracking Analysis (NTA)

Nanoparticle Tracking Analysis (NTA) was performed using a NanoSight NS300 (Malvern Panalytical) equipped with a sCMOS detector and a 405 nm laser. Samples were diluted in 150 mM NaCl, 10 mM Tris, 2 mM $CaCl_2$, pH 7.4 as needed to give approximately 100-200 particles per frame and equilibrated to 25 °C. Data were recorded at the highest possible camera level (e.g. level 16: slider shutter 1300, slider gain 512) at 25 frames per second in order to increase sensitivity for small nanoparticles with inherently low amounts of scattered light. For each sample 5 x 30 s were recorded. In between each 30 s run the solution was manually advanced within the flow cell in order to screen another fraction of the sample.

Data analysis was performed in NTA 3.2 software (Malvern Panalytical) with sample viscosity set to ”water” for automatic calculation based on sample temperature. A suitable detection threshold (e.g. 4-6) was selected manually to best identify the nanoparticles. Blur size and maximum jump distance were determined automatically. The 5 x 30 s data per sample were treated as replicates and used for size distribution calculation as mean +/- standard error of the mean.

2. LSTc inhibition assay

This study was performed according to a previous report [3]. 5 mM of lactoseries tetrasaccharide c (LSTc) (V Labs, Inc.) was incubated on ice with 31.5 µg/mL VLPs in hCMEC/D3 cell media for 1 h. hCMEC/D3 cells plated in 96-well plates were chilled at 4 °C for 30 min and then treated with the VLPs-LSTc complex at 4 °C for 1 h. The cells were washed with PBS and then re-cultured at 37 °C with the media for 24 h. After that, the cells were fixed, permeabilized and stained with mouse anti-human JCV VP1 monoclonal antibody (1:1500, Abcam) and secondary Cy3-conjuaged goat anti-mouse IgG (1:800, Jackson ImmunoResearch). Cellular uptake

was quantified based on VP1 immunofluorescence by using High Content Analysis (HCA) as described before.

Supplementary Results

Methods	Characteristics	Target Parameters
Transmission Electron Microscopy (TEM)[1]	VLPs per grid mesh Shape of particles Diameter of particles Aggregates	>50 particles Round and enclosing 40-50 nm Not visible
Western Blot (WB)[1]	VP1 band VP1 degradation	40 kDa band is observed Minor or no degradation
Dynamic Light Scattering (DLS)[1]	PDI Single peak (volume based distribution) Z-average diameter Other peaks (volume based distribution)	<0.2 Yes 40-50 nm Not detectable
Bioanalyzer[2]	VP1 purity (40 kDa)	>90%
Thermal Shift Assay (TSA)[2]	Major melting peak (in HEPES-Buffer*) Minor melting peaks	57-60 °C Not detectable
Nano Differential Scanning Fluorometry (nDSF)[2]	Major infliction peak Minor infliction peaks	70-73 °C Not detectable
Size Exclusion HPLC (SE-HPLC)[2]	Aggregates Other impurities	<5% of total AUC <5% of total AUC
Field Flow Fractionation (FFF-MALS)[2]	Concentration of particles Size of particles Aggregates Other impurities	>1.0 x 10^{11} VLPs/mL 40-50 nm <5% of total AUC <5% of total AUC
Nano Track Analysis (NTA)[2]	Concentration of particles Size of particles	>1.0 x 10^{11} VLPs/mL 40-50 nm

* HEPES-Buffer: 20 mM HEPES, 150 mM NaCl, pH 7.4

[1] TEM, WB and DLS have been introduced in the Experimental section for characterization of VLPs.

[2] Details about these applications are described here in the Supplementary section.

Table S1. Bioanalytical applications for assessing physicochemical characteristics of JC VLPs and controlling the batch quality.

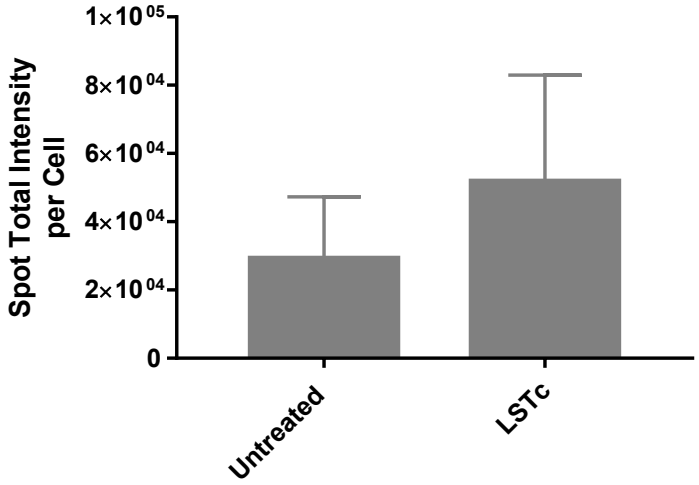

Figure S1. Inhibition study of lactoseries tetrasaccharide c (LSTc) on VLP uptake in hCEMC/D3 cells. LSTc is α-(2,6)-linked sialic acid, which was reportedly required for infectivity of native JC virus in human glial cells [3]. However, in comparison with the untreated control, LSTc showed no clear effect on inhibiting VLP uptake in the BBB cells.

Reference

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2. D. B. Hoffmann, J. Gruber, K. O. Böker, D. Deppe, S. Sehmisch, A. F. Schilling, N. Lemus-Diaz, M. Komrakova and S. Schneider, *Molecular Therapy-Nucleic Acids*, 2018, **12**, 443-452.
3. U. Neu, M. S. Maginnis, A. S. Palma, L. J. Stroh, C. D. Nelson, T. Feizi, W. J. Atwood and T. Stehle, *Cell host & microbe*, 2010, **8**, 309-319.