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Alphavirus Purification

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ALPHAVIRUS PURIFICATION

FIELD OF THE INVENTION

The disclosure relates to methods for the purification of alphaviruses for use in vaccines.

BACKGROUND OF THE INVENTION

Regulatory agencies such as the World Health Organization establish standards and guidelines for the production of pharmaceutical compositions administered to humans, such as vaccines, that limit quantity and components of the compositions. Meeting these standards is particularly challenging with regard to production of vaccines containing biological agents, such as viruses, which must be propagated on cell substrates. Such vaccine preparations must be sterile (*i.e.*, free from independently replicating organisms) and may contain no more than 10 ng of host cell DNA per human dose, among other requirements. These standards are in place in order to ensure safety of the composition for human administration, but may introduce challenges in the development of processes used to produce such compositions.

Chikungunya virus (ChikV) is a positive-sense, single-stranded RNA virus from the genus *Alphavirus*, family *Togaviridae*. Chikungunya virus disease is mainly an outbreak disease and is associated with high attack rates. The virus is transmitted to humans via a mosquito vector and causes fever, rash, fatigue and severe polyarthralgia. Infections with ChikV generally resolve spontaneously and are not usually fatal, except in rare cases involving CNS infection, where the death rate is from 10-30%. Particularly at risk for ChikV CNS disease are infants under one year and adults over 65 years, with an infection rate of 25-fold and 6-fold higher than the general population, respectively, and with a rate of persistent disabilities estimated at between 30% and 45% (Gerardin, 2016). Furthermore, about 30 percent of all ChikV patients experience arthralgia for months to years after recovery. In some cases, neurological, renal, cardiac, respiratory or hepatic complications can also result.

There are currently no vaccines or medications available for the treatment or prevention of Chikungunya virus disease. Outbreaks in the past have occurred mainly in Africa, but the Central/East/South African (ECSA) genotype has recently expanded its geographical range, resulting in outbreaks in India, Asia, and even temperate Europe (Weaver, S., Arrival of Chikungunya Virus in the New World: Prospects for Spread and Impact on Public Health PLOS

Neglected Tropical Diseases (2014) 8(6): e2921). Although ChikV has been repeatedly imported into the Americas since 1995, no autochthonous transmission was reported until 2013 in the Caribbean. By 2015, the epidemic had spread to the mainland and caused more than 1,000,000 suspected cases in 43 countries in the Americas (Pan-American Health Organization (2015) Number of Cumulative Cases of Chikungunya Fever in the Americas). Further epidemics may be aided in part by the spread of the ChikV mosquito vector into non-endemic regions, as well as the ability of ChikV to adapt to local mosquito species (Vega-Rua et al., Chikungunya Virus Transmission Potential by Local Aedes Mosquitoes in the Americas and Europe, May 20, 2015, PLOS Neglected Tropical Diseases DOI:10.1371/journal.pntd.0003780). The high rate of contagion of Chikungunya virus disease, its potential for long-lasting complications as well as its geographical spread underscore the need for developing preventative measures, such as vaccines.

Vaccines against Chikungunya virus disease may comprise attenuated ChikV virus particles; i.e., ChikV virus particles which have been altered to reduce virulence, but maintain immunogenicity. To facilitate production, attenuated ChikV may be propagated on a cell substrate. It is known in the art that the adaptation of viruses to a particular host cell can change the surface charge of virus particles (Gardner et al., Deliberate Attenuation of Chikungunya Virus by Adaptation to Heparan Sulfate-Dependent Infectivity: A Model for Rational Arboviral Vaccine Design (2014) PLOS Neglected Tropical Diseases 8(2):e2719). As such, traditional purification technologies, such as ion exchange chromatography (see e.g. WO 0192552A2), which separate virus particles according to their surface charge may not be suited for purification of non-adapted ChikV or partial / fully adapted ChikV. Alternatively, another purification process not based on charge could be more advantageous.

SUMMARY

During the course of routine virus purification, it was observed that addition of protamine sulfate to a virus harvest produced on a cell substrate removed not only contaminating DNA derived from host cells, as expected, but surprisingly also virtually eliminated immature and otherwise non-infectious virus particles from the preparation. This finding provides a streamlined, gentle, reproducible and broadly-applicable process for obtaining highly-purified infectious virus particles for applications such as vaccine preparation; furthermore, the process is not dependent on the charge of the virus particle.

Disclosed herein are downstream processes for purifying ChikV virus particles from a crude preparation. The downstream process can be applied to either a ChikV virus which has not adapted for propagation on a particular cell substrate or for a partial / fully cell substrate adapted ChikV.

Aspects of the invention provide processes for the purification of infectious Chikungunya virus particles comprising the steps of (a) providing a crude harvest (a) comprising virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate; (b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising a protamine salt, preferably a protamine sulphate, to obtain a virus preparation (b); (c) contacting the virus preparation (b) with (i) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cut-off of the pores can enter the ligand-activated core and collecting the virus particles to obtain a virus preparation (d), or (ii) a solid-phase matrix comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cut-off of the pores can enter the ligand-activated core and separating the solid-phase matrix from the virus particles by filtration to produce a virus preparation (c); and further purifying the virus preparation (d) by sucrose density gradient centrifugation to obtain a virus preparation (e) comprising the infectious virus particles.

In some embodiments, the concentration of protamine sulphate in step (b) is about 1 to 10 mg/ml, more preferably about 1 to 5 mg/ml, more preferably about 1 to 2 mg/ml. In one embodiment, the concentration of protamine sulphate in step (b) is about 2 mg/mL. In one embodiment, the concentration of protamine sulphate is 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml. In a preferred embodiment, the concentration of protamine sulphate in step (b) is about 1.6 mg/ml.

In some embodiments, the residual host cell DNA of the virus preparation (e) is less than 1 µg/mL, especially less than 900, 800, 700, 600, 500, 400, 300 or 200 ng/mL, preferably less than 100 ng/mL. In a preferred embodiment, the residual host cell DNA of the virus preparation (e) is less than 10 ng/mL. In some embodiments, the residual host cell protein of the final virus

preparation (e) is less than 10 µg/mL, especially less than 9, 8, 7, 6, 5, 4, 3 or 2 µg/mL, preferably less than 1 µg/mL. In a preferred embodiment, the residual host cell protein of the virus preparation (e) is less than 100 ng/mL.

In some embodiments, the crude harvest (a) comprising the virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b). In some embodiments, the one or more pre-purification step(s) comprises digesting host cell genomic DNA in the crude harvest (a) comprising the virus particles and impurities by enzymatic treatment. In some embodiments, the one or more pre-purification step(s) comprises filtration, ultrafiltration, concentration, buffer exchange and/or diafiltration. In some embodiments, the one or more pre-purification steps is filtration using a filter having a pore size equal to or less than 1 µm. In some embodiments, the filter has a pore size equal to or less than 0.2 µm. In a preferred embodiment, the filter has a pore size of 0.2 µm. In some embodiments, the concentration and/or ultra/diafiltration and/or buffer exchange is performed by tangential flow filtration (TFF). In some embodiments, ultra/diafiltration of the crude harvest (a) comprising the virus particles and impurities is performed using a hollow fiber membrane having a cut-off of equal to or less than 300 kDa. In a preferred embodiment, the hollow fiber membrane has a cut-off of 100 kDa.

In some embodiments, the Chikungunya virus particle is a live virus, a chimeric virus, an attenuated live virus, a modified live virus, or a recombinant live virus. In a further step, the ChikV of the invention may be optionally inactivated. In some embodiments, the Chikungunya virus particle is an attenuated form of ChikV. For example, the virus may have reduced infectivity, virulence, and/or replication in a host, as compared to a wild-type virus. In some embodiments, the virus is a mutated or modified virus, for example the nucleic acid of the virus may contain at least one mutation relative to the wild-type virus. In some embodiments, the virus is a recombinant live virus, meaning a virus that is generated recombinantly and may contain nucleic acid from different sources. In some embodiments, the attenuated form of ChikV is derived from the LR2006-OPY1 ChikV infectious clone (La Reunion isolate). In some embodiments, the attenuated form of ChikV is the Δ5nsP3 mutant as described by Hallengård et al. (Novel Attenuated Chikungunya Vaccine Candidates Elicit Protective Immunity in C57BL/6 mice (2014) *Journal of Virology* 88(5):2858–2866) or an immunogenic variant thereof. The immunogenic variant of the Δ5nsP3 ChikV mutant is herein defined as having at least 80% sequence identity to the nucleotide sequence of the Δ5nsP3 mutant sequence, especially at least

81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or at least 99% sequence identity.

In some embodiments, the molecule entering the core of the solid-phase matrix has a molecular weight less than 700 kDa. In some embodiments, the ligand of the ligand-activated core of the solid-phase matrix is capable of binding the molecule that enters the ligand-activated core via cationic-, anionic-, hydrophobic- or mixed interactions. In some embodiments, the ligand of the solid-phase matrix is octylamine, diethylaminoethyl, quarternary ammonium, or sulfonate. In a preferred embodiment, the ligand of the ligand-activated core of the solid-phase matrix is octylamine. Non-limiting examples of solid-phase matrices that may be compatible with the processes described herein include, without limitation, CAPTO® Core 700, CAPTO® DEAE, CAPTO® MMC, CAPTO® Q, CAPTO® S, FRACTOGEL® TMAE, Hyx T II, Q SEPHAROSE® Fast Flow. In some embodiments, the solid-phase matrix is CAPTO® Core 700. In some embodiments, the solid-phase matrix is used as a slurry and at a final concentration between 0.5% (v/v) and 10% (v/v), preferably 0.6%, 0.7%, 0.9%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, most preferably 1%. In some embodiments, the solid-phase matrix is incubated with the crude harvest comprising the virus particles at refrigerated temperatures (about 2°C to 8°C) or ice bath temperatures (about 0°C) with stirring for at least 5 minutes, preferably at least 10 minutes, preferably at least 15 minutes, preferably at least 30 minutes, preferably at least 1 hour, most preferably 15 minutes.

In some embodiments, the process of the invention results in an enrichment of infectious virus particles from the crude harvest comprising infectious virus particles and non-infectious virus particles and other virus products such that the enrichment of the infectious virus particles is at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, preferably at least 80%, especially 85% relative to the total virus particle content of the crude harvest (a) comprising the virus particles and impurities.

In some embodiments, the residual impurity of the final virus preparation with respect to all impurities in the crude harvest is less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, preferably less than 5% as determined by SEC-HPLC (Size Exclusion Chromatography – HPLC).

In some embodiments, the filtration step of the virus preparation (b) after contact with the solid-phase matrix is performed using a filter having a pore size equal to or greater than 1 µm. In

some embodiments, the filter has a pore size equal to or greater than 0.2 μm . In a preferred embodiment, the filter has a pore size of about 0.2 μm , such as 0.22 μm .

In some embodiments, the Chikungunya virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero- α His cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line. In some embodiments, said cell line is a duck cell line. In some embodiments, said cell line is a diploid avian cell line. In some embodiments, said cell line is EB66 cell line. In a preferred embodiment, said cell line is a Vero cell line.

Aspects of the invention provide a use of any of the processes described herein for manufacturing a composition for immunization against a Chikungunya virus infection. In a preferred embodiment, the composition is a vaccine. In preferred embodiments, the vaccine is administered to the subject once, twice or three or more times. In a preferred embodiment, the vaccine is administered once or twice. In a preferred embodiment, the vaccine is administered only once.

Other aspects provide compositions comprising the virus particles obtainable by any of the processes described herein for treating and/or preventing a Chikungunya virus infection.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings are not intended to be drawn to scale. The Figures are illustrative only and are not required for enablement of the disclosure. For purposes of clarity, not every component may be labeled in every drawing. In the drawings:

Figure 1 shows the process of flavivirus maturation in the host cell. Full maturation of the particles requires proteolytic cleavage of the precursor membrane glycoprotein (prM) by the host protease furin. Not all prM molecules are cleaved, resulting in the release of mature, mosaic or immature-like conformations from the cells. Mosaic and immature forms are generally not infectious—only mature virions are infective and have hemagglutinin (HA) / TCID₅₀ activity. (Figure adapted from Plevka, et al., Maturation of flaviviruses starts from one or more icosahedrally independent nucleation centres, EMBO reports (2011) 12, 602–606).

Figure 2 shows schematically the genome of the ChikV, including non-structural and structural proteins (labeled “CHIKV”) as well as a schematic representation of the Δ 5nsP3 attenuated Chikungunya virus used to exemplify the purification process of the current invention (labeled “ Δ 5nsP3”). The triangle indicates the approximate location of the deletion in the nsP3 coding region. (Figure adapted from Hallengård et al. 2014, *supra*.)

Figure 3 shows a flow-chart of an exemplary ChikV vaccine downstream purification process from the crude harvest to formulation of the DS, a preferred embodiment of the process of the invention.

Figure 4 shows the 214 nm, 260 nm and 280 nm absorbance of the individual SGC fractions of a representative purification run of the process of the invention (A); the SEC-HPLC analysis of the final pooled fractions containing purified infectious ChikV virus particles (B); and a silver-stained SDS-PAGE gel showing the protein content of the virus preparation following different steps of the process of the invention (defined in the table below the figure) (C). The SGC purified pool consisting of SGC fractions F7-F11 is shown in lane 12.

Figure 5 relates the SEC area (mAU*min; right axis) and TCID₅₀ results (log TCID₅₀/mL; left axis) of ChikV production harvests before and after PS treatment. The grey portions of the bars indicate large losses in SEC area following PS treatment, but no corresponding change in the total number of infectious particles (indicated by black portions of the bars) (A); SEC profile of virus preparation before and after PS addition, showing a complete removal of large size virus aggregates by PS treatment as well as a reduction in HCP and LMW impurities (B).

Figure 6 shows electron micrographs of ChikV harvest before and after PS treatment.

Figure 7 shows relative amounts of ChikV particles and other components by SEC-HPLC analysis at the different steps of the process of the invention including, from top to bottom: crude harvest (a); 10X concentrated harvest; diafiltrated harvest; PS treated material; CC700-treated material and SGC purified pool.

DETAILED DESCRIPTION OF THE INVENTION

Disclosed herein are processes for the purification of infectious Chikungunya virus particles, i.e., mature, functional virus particles. The processes disclosed are characterized by the removal of undesired by-products of virus production on host cells, such as non-infectious virus particles and aggregated and immature virus by-products. The processes provided herein allow the production of highly-purified live Chikungunya virus preparations comprising mostly infectious virus particles. During the course of the invention, it was observed that protamine sulphate, added to remove contaminating DNA during virus purification, resulted not only in removal of contaminating DNA, but also in the loss of a high percentage of total virus particles present in the preparation. Surprisingly, however, quantification of total infectious virus particles by TCID₅₀ before and after PS treatment revealed that the absolute number of infectious virus particles did not change following this loss of total virus particles. This observation suggested that treatment with PS can facilitate selective removal of non-infectious, aggregated and immature viral by-products, leaving behind the infectious Chikungunya virus particles. Because by-products produced during virus growth on host cells may have different (and undesirable) immunological properties or other unwanted side-effects or safety issues, a simple and robust way to remove these by-products is of high importance for the quality and safety of the final product.

Protamines are small arginine-rich nuclear proteins, present in high amounts in the sperm of fish, which have an important role in DNA packaging during spermatogenesis. Protamine sulfate (or “protamine” or “PS”) can form a stable ion pair with heparin and is thus commonly used during certain surgeries when the anti-coagulation effect of heparin is no longer needed. In large doses, protamine sulfate administered alone can also have a weak anticoagulant effect (“Protamine sulfate”. Wikipedia: The Free Encyclopedia. Wikimedia Foundation, Inc. 30 Sep 2015 Web. 26 Nov 2015 <https://en.wikipedia.org/wiki/Protamine_sulfate>). Protamine Sulphate is additionally routinely used in biotechnology applications such as DNA precipitation (e.g., removal of host cell DNA from cell culture processes), purification of DNA binding proteins and retroviral-mediated gene transfer.

Protamine is obtained from salmon sperm or produced recombinantly and is used as a sulphate salt. The four major peptides, which constitute almost the entire nitrogen-containing

material in salmon protamine, have been fully characterized and found to be polypeptides of 30–32 amino acids in length, of which 21–22 residues are arginine. The average molecular mass is in the range of 4250 Da for the following sequence: PRRRRSSSRP VRRRRRPRVS RRRRRRGGR RR (SEQ ID NO: 1). Herein, protamine is also referred to as protamine salt, or preferably protamine sulphate.

The present invention relates to the use of protamine sulphate (PS) in a process of purification of a live virus, wherein the protamine sulphate facilitates the removal of impurities from a crude virus harvest, including non-infectious virus particles and aggregates. As seen in Fig. 1, virus production in the host cell can result in the release of virus products which are not mature, infectious particles, which can also be considered impurities according to the present invention. As such, the present invention also relates to the enrichment of infectious virus particles from a crude harvest containing a mix of virus particles and other viral products in various stages of maturation.

The use of protamine sulphate can follow crude cell lysis or any further step after cell lysis wherein the virus particles are further enriched or concentrated and/or other impurities are removed and/or buffer components are exchanged. The further steps may comprise filtration or concentration of the crude cell lysate.

The protamine sulphate may comprise the sequence PRRRRSSSRP VRRRRRPRVS RRRRRRGGR RR (SEQ ID NO: 1) or a variant thereof wherein the amino acid sequence comprises from 28–35 amino acids, preferably 29–34, more preferably 30–33 amino acids, most preferably 31 or 32 amino acids. The protamine sulphate preferably comprises at least 19 arginine residues, more preferably at least 20 arginine residues, more preferably at least 21 arginine residues, even more preferably at least 22 residues, most preferably 20 or 21 arginine residues. Further, other protamine sulphate-like compounds or variants thereof may be used. Therefore, the use of the term “protamine salt” herein shall serve to encompass natural variations on SEQ ID NO: 1, preferably, but not limited to, the protamine sulphate forms.

The process comprising the use of protamine sulphate of the invention can be applied to purification of any live virus for use in pharmaceutical compositions, for example, a vaccine. The Chikungunya virus to be purified may be any live Chikungunya virus, any live attenuated Chikungunya virus or any live Chikungunya chimeric virus, preferably a live attenuated

Chikungunya virus. In one embodiment, the ChikV is also be later inactivated. In a preferred embodiment, the virus is inactivated with formaldehyde.

In a preferred embodiment, the live attenuated Chikungunya virus is the protective ChikV-ICRES1- Δ 5nsP3 described by Hallengård *et al.* (Novel Attenuated Chikungunya Vaccine Candidates Elicit Protective Immunity in C57BL/6 mice (2014) J. Virology, 88(5):2858–2866). Briefly, the ChikV genome carries a positive-sense single-stranded RNA genome of 11 Kb containing two open reading frames encoding nonstructural proteins (nsP1 to nsP4) and structural proteins (C, E3, E2, 6K, and E1), respectively (see Fig. 2, top construct). The attenuated virus Δ 5nsP3, based on the La Reunion ChikV strain LR2006-OPY1, was obtained by the substitution of amino acid residues 1656 to 1717 of the P1234 polyprotein with a small linker (aa sequence AYRAAAG) in the hypervariable region of the nsP3 protein (see Fig. 2, bottom construct). The Δ 5nsP3 ChikV mutant was shown to be infectious, highly immunogenic and protective against challenge with wild type ChikV (Hallengård, *et al.*, *supra* and Hallengård, *et al.*, Prime-Boost Immunization Strategies against Chikungunya Virus (2014) J. Virology, 88(22):13333-13343). In one embodiment, the live attenuated Chikungunya virus may be a variant of the ChikV-ICRES1- Δ 5nsP3 attenuated mutant virus.

A preferred embodiment of the process of the current invention is shown in Fig. 3.

Table 1. Overview of process buffers and stock solutions

Buffer	Composition	Final pH	Final conductivity [mS/cm]
A	0.5 M NaOH		n.a.
B	0.1 M NaOH		n.a.
C	25 mM Tris, 150 mM NaCl	7.4 $\pm$ 0.2	16.5
D	1 M Tris	7.4 $\pm$ 0.2	n.a.
E	4.5 M NaCl	n.a.	n.a.
F	1 M NaCl	n.a.	n.a.
G	1 % SDS	n.a.	n.a.
H	50 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4 $\pm$ 0.2	n.a.
I	35 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4 $\pm$ 0.2	n.a.
J	15 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4 $\pm$ 0.2	n.a.
K	10 x PBS	7.4 $\pm$ 0.2	n.a.
L	50 mg/mL Protamine sulphate	7.4 $\pm$ 0.2	n.a.
M	Drug substance formulation buffer (10mM Tris(hydroxymethyl)-aminomethan, 5 % Sucrose, 1% (10 mg/mL) rHSA)	7.4 $\pm$ 0.2	1.3

Table 2. Abbreviations

CC700	Capto™ Core 700
ChikV	Chikungunya virus
EtOH	Ethanol
EU	Endotoxin units
DS	Drug Substance
DP	Drug Product
DSP	Downstream Process
HCP	Host cell protein
hcDNA	Host cell DNA
hpi	Hours post infection
HPLC	High Pressure Liquid Chromatography
ID	Inner diameter
LDS buffer	Lithium dodecyl sulfate sample loading buffer
MALLS	Multangle light scattering
mAU	Milli absorbance units
PD	Process development
PFU	Plaque forming units
SEC	Size exclusion chromatography
SGC	Sucrose gradient centrifugation
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
TFF	Tangential flow filtration
TCID50	Tissue culture infectious dose 50%
UF/DF	Ultrafiltration/diafiltration
rcf	Relative centrifugal force
rHSA	Recombinant human serum albumin

SEQUENCES

The amino acid sequence of a typical form of protamine is provided by SEQ ID NO: 1:

PRRRRSSSRP VRRRRRPRVS RRRRRRGGRR RR

EXAMPLES

A downstream process was developed for the purification of infectious Chikungunya virus particles whereby non-infectious virus particles and aggregates are removed by the addition of protamine sulphate. The unexpected and novel purification properties of protamine sulphate (PS) were evaluated in purification processes for Chikungunya Virus (ChikV) as follows:

Example 1: Development of a purification process for live attenuated Chikungunya virus vaccine produced in Vero cells

A downstream purification process for the attenuated Chikungunya virus mutant “Δ5nsP3” (as described by Hallengård et al., 2014, *supra*) produced under standard cell culture conditions in Vero cells was developed. Briefly, the downstream process consists of crude harvest filtration followed by concentration and diafiltration on a tangential flow filtration (TFF) system. DNA and host cell proteins were reduced by precipitation with protamine sulphate and by batch adsorption, respectively. Sucrose density gradient centrifugation was done as a final polishing step. Out of 16 roller bottles 1×10^{12} total PFU were purified with an overall DSP process yield of 10 - 15 % (~1 log₁₀ TCID₅₀ loss). Sucrose gradient pool samples were characterized with regard to product-related impurities, such as hcDNA, HCP and endotoxins and met safety criteria.

Harvest of Vero cell culture medium containing ChikV Δ5nsP3

ChikV Δ5nsP3 was grown on Vero cells in roller bottles. A first harvest was performed after 24 hours post infection (hpi; day 1 harvest) and stored at 2-8°C until further processing. After the first harvest, fresh medium was added and the roller bottles were returned to the incubator. A second harvest was done after 48 hours post infection (day 2 harvest) and stored at 2-8 °C.

Filtration of crude cell culture harvest

At both harvest timepoints the crude harvest was immediately filtered using a 0.2 µm filter capsule (GE ULTA™ CG, 2 inch). The filtered harvest after 48 hpi was pooled together with the 24 hpi harvest and the pooled filtered harvest material was immediately further processed by ultrafiltration.

Purification of ChikV Δ5nsP3 by tangential flow filtration (TFF)

The pooled filtered harvest material was further processed by tangential flow filtration (TFF) in order to concentrate the harvest, reduce host cell proteins and replace the depleted cell culture medium with a defined buffer system (buffer exchange). A Millipore TFF system (Millipore Pellicon II mini membrane holder) equipped with a 100 kDa cutoff PES membrane module (Pellicon2 Biomax, 1000 cm²) was used for concentration and buffer exchange. A Pellicon2 Biomax membrane module was mounted on the Pellicon II mini filter holder and the

device was connected to a peristaltic pump. The system was first rinsed with ultra-pure water and then sanitized by recirculation of 0.1 M NaOH for 60 min. In case the system was not used immediately, it was stored in 0.1 M NaOH until use. Prior to use the system was rinsed with 1 L of RO-water followed by buffer A until the permeate pH value was constant at $\text{pH } 7.4 \pm 0.2$.

Adjustment of the ChikV $\Delta 5\text{nsP3}$ harvest (pH, salt)

The pooled filtered harvest material was adjusted to a final concentration of 25 mM Tris and 150 mM NaCl using stock solutions of both components (see Table 1). This adjustment was done to increase buffering capacity and to reduce unspecific adsorption to the membrane. The necessary volumes of stock solutions D (1 M Tris, pH 7.4) and E (4.5 M NaCl) were calculated as follows:

Volume of stock solution D (1 M Tris, pH 7.4) added to pooled harvest = Volume of pooled filtered harvest / 40

Volume of stock solution E (4.5 M NaCl) added to pooled harvest = Volume of pooled filtered harvest / 30

Example: 4 L harvest obtained from 20 RB (850cm²) would require addition of 100 mL stock solution D (1 M Tris, pH 7.4) and 133 mL stock solution E (4.5 M NaCl).

The calculated volumes of stock solution D and Buffer E were added to the pooled filtered harvest under gentle stirring. The adjusted harvest was then stirred using a magnetic stirrer for 5 minutes at room temperature.

Concentration and diafiltration of the ChikV $\Delta 5\text{nsP3}$ harvest by TFF

In a first step the adjusted harvest material was concentrated approximately 10 fold. The feed flowrate was approximately 220 mL/min. The transmembrane flux at a transmembrane pressure of approximately 0.6 bar was in the range of 90 ± 5 mL/min per 1000 cm² membrane. After concentration, the cell culture medium was exchanged against 25 mM Tris, 150 mM NaCl, pH 7.5 by continuous diafiltration with 6 volume exchanges. The diafiltration buffer was supplied to the feed vessel from a measuring cylinder by a second peristaltic pump set to a flowrate of approximately 90 mL/min. Minor flowrate adjustments of the second peristaltic pump in the range of ± 10 mL/min were done manually to ensure a constant volume of harvest in the feed vessel. After 6 volume exchanges, diafiltration was stopped. The liquid remaining in the membrane module was recovered by pumping the module empty with air.

Sucrose addition to diafiltrated ChikV Δ5nsP3 material

After diafiltration, sucrose stock solution H (50% (w/w) sucrose solution) was added to the diafiltrated material to achieve a final sucrose concentration of 10% (w/w). The volume of buffer H was calculated as follows:

Volume of stock solution H added (mL) = Volume (mL) of diafiltrated ChikV material x 0.25
(dilution factor = 1:4) (i.e., final sucrose concentration is 10%)

Example: 400mL diafiltrated ChikV solution would require addition of 100mL stock solution H (50% sucrose).

The calculated volume of solution H was added to the diafiltrated ChikV Δ5nsP3 material under gentle stirring and the solution was then stirred using a magnetic stirrer for a further 5 minutes at room temperature. (At this stage of the process the material can be either immediately further processed or stored frozen (< -65°C, hold step).)

DNA reduction by protamine sulphate precipitation

A DNA precipitation step using protamine sulphate (PS) was performed to reduce hcDNA. Protamine sulphate stock solution L (50 mg/mL PS in PBS) was added to the diafiltrated ChikV Δ5nsP3 material to a final nominal concentration of ~1.6 mg/mL. The necessary volume of stock solution L was calculated as follows:

Volume of stock solution L (50 mg/mL PS) added = Volume of diafiltrated ChikV Δ5nsP3 material in 10% sucrose / 31

Example: 500 mL diafiltrated ChikV Δ5nsP3 solution in 10% sucrose would require addition of 16 mL stock solution L (50 mg/mL PS in PBS).

The protamine sulphate stock solution was added while stirring the ChikV Δ5nsP3 material using a magnetic stirrer followed by incubation at 2-8°C for 30 minutes. After incubation, the DNA precipitate was not removed. The material was immediately further processed by batch adsorption with Capto™ Core 700 chromatography media.

Batch adsorption with Capto™ Core 700

To reduce HCPs, a batch adsorption step with Capto™ Core 700 (CC700) chromatography medium was performed after DNA precipitation. CC700 slurry (50% slurry in buffer A) was added directly to the protamine sulphate treated material. The required slurry volume was

determined based on the volume of ChikV harvest material (d1+d2) and was calculated as follows:

$$\text{Volume of CC700 slurry added to PS-treated concentrated harvest (mL)} = \text{Volume of ChikV } \Delta 5\text{nsP3 harvest material (mL)} \times 0.02 \text{ (dilution factor = 1:50) (i.e., final concentration of CC700 is 1\%)}$$

After slurry addition, the material was incubated at 4°C for 15 minutes under constant agitation using a magnetic stirrer. After incubation, the CC700 solid matter was allowed to settle by gravity for 10 minutes. The ChikV $\Delta 5\text{nsP3}$ material was then removed from the top of the solution in order to avoid blocking of the filter by the CaptoCore particles. The remaining CaptoCore particles and the DNA precipitate were then removed from the solution by filtration using a 0.2 μm Mini Kleenpak EKV filter capsule (Pall). The resulting filtrate was further processed by sucrose density gradient centrifugation.

Sucrose density gradient centrifugation

Sucrose density gradient centrifugation (SGC) was used for final concentration and polishing of the ChikV $\Delta 5\text{nsP3}$ material. The ChikV material was loaded on top of a solution consisting of three layers of sucrose with different densities. The three sucrose layers were selected based on a preliminary study which showed the formation of a linear sucrose gradient and good separation of the virus particles from residual contaminants. The optimal volumes of the sucrose solutions were determined empirically. The volumes of individual layers for a centrifugation at 500 mL scale are shown in Table 3.

Table 3. Sucrose concentrations and volumes (500 mL scale).

Solution	Volume (mL)
Harvest with 10% sucrose	360
15% sucrose	40
35% sucrose	40
50% sucrose	60
Total volume	500

Preparation of the sucrose gradient

The sucrose gradient bottles (500 mL) were prepared by underlaying the individual sucrose layers. A 3.5 mm ID plastic tube was attached to 60 cm of peristaltic pump tubing. The plastic tube was mounted on a laboratory stand using a clamp and placed into the centrifuge

bottle. The nozzle of the plastic tube was touching the bottom of the bottle. Using a peristaltic pump set to a flow rate of 25 mL per minute, the ChikV Δ5nsP3 material and the sucrose solutions were pumped into the cylinder. A measuring cylinder was used as a feed vessel. The first solution pumped was the ChikV Δ5nsP3 material as it had the lowest density (10% sucrose (w/w)). Following the addition of the ChikV Δ5nsP3 material, the sucrose solutions were pumped in ascending order starting with the lowest (15%), followed by the 35% sucrose solution and finishing with the highest density sucrose solution (50%). After all sucrose solutions were transferred, the plastic tubing was carefully removed in order not to disturb the layers.

Centrifugation

Prior to centrifugation a Beckman Avanti JXN-26 centrifuge equipped with rotor Beckman 10.500 was pre-cooled to 4°C. The prepared SG bottles were carefully transferred into the pre-cooled (4°C) rotor so as to not to disturb the sucrose layers. The bottles were centrifuged at 10,000 rpm (~18,500 rcf) at 4°C for 17-20 hours. (In case a different centrifuge system with a different rotor would be used, the necessary speed and centrifugation times would need to be calculated based on the k-factor in order to achieve comparable centrifugation efficiency.)

Sucrose gradient harvest

Harvesting of the sucrose gradient was done manually using a peristaltic pump. A 3.5 mm ID plastic tube attached to 60 cm of peristaltic pump tubing was used for harvesting the sucrose gradient. The 500 mL bottle containing the centrifuged gradient was mounted onto a laboratory stand in a tilted position (~12°) using a clamp. The plastic tubing was then placed into the bottle touching the bottom edge of the bottle and was fastened in position using a clamp. This resulted in a small gap of 1-2 mm between the tubing inlet and the bottom of the bottle. Using a peristaltic pump set to a flow rate of 60 mL per minute, the gradient was harvested and manually split into 5 mL fractions. A third of the bottle volume was harvested and the rest was discarded. The fractions were immediately tested by measuring UV absorbance in a plate reader as described below.

Analysis of fractions by UV absorbance and SEC-HPLC

UV absorbance measurement was used as primary method for analysis of the sucrose gradient fractions. Absorbance at 214, 280 and 260 nm was tested immediately after fractionation was completed. Briefly, a 100 μ L sample of each fraction was transferred into a 96 well plate and absorbance at 214, 260 and 280 nm was measured using a plate reader. The absorbance values were plotted against the fraction number. A representative profile is shown in Fig. 4A. The ChikV Δ 5nsP3 containing fractions were indicated by a peak in all three measured wavelengths (Fig. 4A, grey shaded area). The presence of impurities was indicated by an increase of the UV214 signal after the main peak. The fractions comprising the main peak were pooled from the peak start to the valley of the 214 nm curve. This method can be used as single method for pooling ChikV Δ 5nsP3 fractions.

After identification of the virus containing fractions, the respective fractions were pooled. Pooling criteria for SGC fractions were based on UV 260 nm data, e.g. start of pooling at ~10% of peak maximum, end of pooling at ~30% of peak maximum. (Final pooling criteria at a manufacturing scale may need to be determined empirically.) The sucrose gradient pool was either stored at $< -65^{\circ}\text{C}$ or immediately further formulated to drug substance (DS).

Size exclusion chromatography

The final pooled SGC fractions containing purified infectious ChikV Δ 5nsP3 virus particles were analyzed for purity by SEC-HPLC. In brief, SEC was performed as follows: a Superose 6 10/300 Increase column (GE Healthcare) equilibrated with PBS + 250 mM NaCl, pH 7.4 at 1 ml/min and 25°C , was used to detect ChikV particles at 214 nm detection wavelength in the pooled samples. SEC-HPLC is a semi-quantitative (relative yield) and qualitative (purity) method that separates intact virus particles from virus aggregates and host cell proteins (HCPs). The method cannot distinguish between infectious and non-infectious virus particles due to their identical retention time.

As shown in Fig. 4B, there were identified two defined peaks by SEC: the ChikV Δ 5nsP3 peak and a peak corresponding to buffer components. The SGC step yield based on SEC-HPLC data for pooled fractions F6-F11 was estimated at ~70 %. The final purity of the ChikV Δ 5nsP3 SGC pool, based on SEC-HPLC analysis, was estimated at $>95\%$.

SDS-PAGE and silver stain

SDS-PAGE silver stain was performed in order to qualitatively assess sample purity throughout the purification process from the first crude harvest through SGC. Briefly, ChikV process samples analyzed by SDS-PAGE/silver stain were diluted 1:1.33 with LDS buffer and were heated to 70°C for 5 minutes. The samples were loaded onto 4-12 % Bis-Tris Gels (NuPAGE). Silver staining was done using the Silver Express staining kit (Invitrogen).

A silver-stained gel of a representative ChikV $\Delta 5nsP3$ purification is shown in Fig. 4C. The viral proteins E1, E2 and C are marked on the right-hand side of the gel. The final SGC pool (fraction 7-fraction 11) is shown in lane 12. Note that a defined HCP band migrating between ChikV protein E2 and C still appears after CaptoCore700 treatment that has been identified as a single band in SDS-PAGE. This impurity is removed by sucrose gradient centrifugation, but can still be seen in fractions 13 and 14 (corresponding to lanes 14 and 15 of Fig. 4C).

Enrichment of infectious ChikV $\Delta 5nsP3$ particles by PS treatment

Although generally used as a method of removing contaminating hcDNA, it was observed in the course of the present invention that PS treatment also removes virus aggregates and HCPs. Size exclusion chromatography (SEC-HPLC, as described above) was used to throughout the purification process to determine the purity of the ChikV virus relative to impurities which also generate UV absorption.

As can be seen in Fig. 5A, treatment with PS reduces not only host cell proteins and low molecular weight contaminants of the ChikV $\Delta 5nsP3$ preparation, but also reduces the SEC area corresponding to virus products, including aggregates as indicated. A surprising finding, however, was that even a reduction of the total SEC area by 86% (in a representative experiment shown in Fig. 5B, grey portion of bars) did not result in a concomitant reduction in infectious virus particles as measured by TCID₅₀ (left axis). Instead, even though a large percentage of virus particles were removed by PS treatment, the infectious particles were not. This observation indicates that PS treatment selectively enriches infectious virus particles from a larger pool of total virus particles present in the crude harvest.

TCID₅₀ was performed to quantify infectious virus particles during the course of the purification process and to assign an active virus titer to final drug substance and drug product samples. Briefly, Vero cells were seeded at 2×10^4 cells per well in 100 μ L medium (EMEM

with 2 mM L-Glutamine + 5 % FBS (+ 1 % antibiotic/antimycotic) in 96-well TC-treated flat-bottom plates and incubated overnight at 35°C / 5% CO₂. On day two, Vero cell monolayers were infected by adding 100 µL of 1:10 serial dilutions of test samples to each of quintuplicate wells seeded with Vero cells and incubated at 35°C / 5% CO₂. On day seven, plaques were counted by visualization under a microscope. The TCID₅₀ was calculated according to the Reed & Munch endpoint calculation method (Reed, L.J.; Muench, H. (1938) A simple method of estimating fifty percent endpoints" The American Journal of Hygiene 27: 493–497).

Furthermore, electron microscopy of ChikV samples before and after PS treatment showed that not only large aggregates but also smaller non-infectious virus-like particles (basically not fully assembled particles without RNA genome) are effectively removed by PS (Fig. 6).

Furthermore, this enrichment of infectious virus particles could also be observed when analyzing day one and day two crude harvests separately. As presented in Table 3, the SEC area (total virus particles) of the day 1 harvest remains roughly the same after PS treatment; whereas a large decrease in virus peak area is seen for the day 2 harvest. This observation was confirmed by MALLS analysis of the virus preparation, wherein it was seen that a higher percentage of virus particles were of the correct size following PS treatment. Similarly to the results shown in Fig. 5, day 1 and day 2 harvests showed no reduction in infectious particles as measured by TCID₅₀ following PS treatment, indicating that mainly non-infectious, immature and/or aggregated virus particles are removed during the PS treatment and infectious particles are enriched in the preparation.

Table 3. Overview of the process of ChikV Δ5nsP3 purification as described in Example 1. SEC-MALLS analysis of harvests before and after PS treatment shows the removal of larger virus particles (aggregates), an effect that is particularly pronounced for day 2 harvests.

	SEC	MALLS		Infectious particles
	Area [mAU*min]	Total particles/mL	% correct size (20-40 nm)	TCID ₅₀ log 10
Harvest 1 (H1)	57	1.17E+11	49%	10.2
Harvest 1 + PS	53	1.33E+11	81%	10.0
Harvest 2 (H2)	36	4.60E+09	3%	7.9
H2 + protamine sulphate	2	8.80E+09	59%	7.9
Combined Harvests (C)	67	2.60E+10	14%	9.9
C + protamine sulphate	24	8.00E+10	72%	10.1

Finally, an overview of the relative amounts of ChikV particles and other components as measured by SEC-HPLC at various steps throughout the entire virus purification process from crude harvest (a) to the final SGC purified pool can be seen in Fig. 7. In sum, not only are the vast majority of contaminants and undesired products removed by the process, ChikV particles are highly purified. As shown by the previously presented data, the final preparation is a highly enriched preparation of infectious ChikV particles.

Drug Substance (DS) Formulation

The pooled SGC fractions are diluted with DS formulation buffer M (10 mM Tris, 5% Sucrose, 1% (10 mg/mL) rHSA, pH 7.4 ± 0.2). The final target volume of DS should be in the range of approximately 2 L. Based on current data the estimated range of the dilution factor might be 1:20 to 1:50.

Final DS sterile filtration

The final DS was filtered under aseptic conditions in a laminar flow hood using a sterility grade 0.2 μm syringe filter (e.g. 0.2 μm Mini Kleenpak EKV filter capsule with 220 cm^2 filter surface, Pall).

Quantification of host cell DNA (hcDNA) host cell protein (HCP) and endotoxin

The residual host cell DNA content of the sucrose gradient pool samples was determined by using the qPCR based assay. The DNA content in SGC pool was determined to be ≤ 0.002 ng/mL. The presence of residual host cell protein (HCP) from Vero cells was determined by ELISA. Residual host cell proteins present in the sucrose gradient pool samples were quantified using the Vero Cell HCP ELISA kit (Cygnus, F500). The residual host cell protein content in SGC pool was determined to be ≤ 200 ng/mL.

Endotoxin content of the SGC pool and DS was measured by Endosafe[®]-PTS[™] system (Charles River). The system uses LAL reagents by a kinetic chromogenic methodology to measure color intensity directly related to the endotoxin content in a sample. Each cartridge contains precise amounts of a licensed LAL reagent, chromogenic substrate and an endotoxin control standard. Samples were diluted 1:100 in WFI. The SGC Pool F7-F11 was determined to be < 5.00 EU/mL; likewise, the Drug Substance was also determined to have < 5.00 EU/mL.

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The following specifications for impurities in final Drug product were proposed: hcDNA < 10 ng/dose; Endotoxins < 50 EU/dose; HCP < 200 ng/dose. These residual specifications would already be met in the highly concentrated SGC pool (~10 log TCID₅₀/mL), which provides a high margin of safety considering the high dilution factor of SGC pool to final DP of > 1:1000.

What is claimed is:

CLAIMS

1. A process of purification of infectious alphavirus particles, preferably Chikungunya virus particles, comprising the steps of:
 - (a) providing a crude harvest (a) comprising virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;
 - (b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising a protamine salt, preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);
 - (c) contacting the virus preparation (b) with (i) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cut-off of the pores can enter the ligand-activated core and collecting the virus particles to obtain a virus preparation (d), or (ii) a solid-phase matrix comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cut-off of the pores can enter the ligand-activated core and separating the solid-phase matrix from the virus particles by filtration to produce a virus preparation (c); and
 - (d) further purifying the virus preparation (c) by sucrose density gradient centrifugation to obtain a virus preparation (d) comprising the infectious virus particles, wherein the residual host cell DNA of the virus preparation (d) is less than 100 ng/mL and the residual host cell protein of the final virus preparation (d) is less than 1 µg/mL.
2. The process of claim 1, wherein the residual host cell DNA of the virus preparation (d) is less than 10 ng/mL and the residual host cell protein of the final virus preparation (d) is less than 100 ng/mL.
3. The process of claim 1 or 2, wherein the crude harvest (a) comprising virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

4. The process of any one of claims 1 to 3, wherein the one or more pre-purification step(s) comprises
 - (a) filtration using a filter having a pore size equal to or less than 0.2 μm ; and/or
 - (b) digestion of host cell genomic DNA by enzymatic treatment; and/or
 - (c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.
5. The process of any one of claims 1 to 4, wherein the concentration of protamine sulphate is 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml.
6. The process of any one of claims 1 to 5, wherein the molecule entering the core of the solid-phase matrix has a molecular weight less than 700 kDa.
7. The process of any one of claims 1 to 6, wherein the ligand of the ligand-activated core of the solid-phase matrix is capable of binding the molecule that enters the ligand-activated core via cationic-, anionic-, hydrophobic- or mixed interactions.
8. The process of any one of claims 1 to 7, wherein the ligand of the ligand-activated core of the solid-phase matrix is octylamine.
9. The process of any one of claims 1 to 8, wherein the solid-phase matrix is used as a slurry and at a final concentration between 0.5% (v/v) and 10% (v/v), preferably 0.6%, 0.7%, 0.9%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, most preferably 1%.
10. The process of any one of claims 1 to 9, wherein the solid-phase matrix is incubated with the protamine-treated virus preparation (b) at refrigerated temperatures (2°C to 8°C) with a stirring for at least 10 minutes, preferably 15 minutes, 30 minutes or 1 hour, most preferably 15 minutes.

11. The process of any one of claims 1 to 10, wherein the enrichment of infectious virus particles in the final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.
12. The process of any one of claims 1 to 11, wherein the filtration of step (c) of claim 1 is performed using a filter having a pore size equal to or less than 1 μm , preferably 0.2 μm .
13. The process of any one of claims 1 to 12, wherein the residual impurity of the final virus preparation is less than 10%.
14. The process of any one of claims 1 to 13, wherein the virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero- α His cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.
15. The process of claim 14, wherein said cell line is a Vero cell line.
16. The process of any one of claims 1 to 15, wherein the Chikungunya virus is a live virus, an attenuated live virus, a chimeric virus, a modified live virus, or a recombinant live virus.
17. The process of any one of claims 1 to 16, wherein the Chikungunya virus is the $\Delta 5\text{nsP3}$ attenuated mutant or an immunogenic variant thereof.
18. The process of any one of claims 1 to 17, wherein said process resulting in final virus preparation (d) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.
19. Use of the process according to any one of claims 1 to 18 for manufacturing a composition for immunization against a Chikungunya virus infection.

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20. The use according to claim 19, wherein the composition for immunization against a Chikungunya virus infection is a vaccine.
21. A composition comprising the virus particles obtainable by the process of any one of claims 1 to 18 for treating and/or preventing a Chikungunya virus infection.

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ABSTRACT

Described herein are processes for purifying infectious alphavirus particles.

FIGURE 1

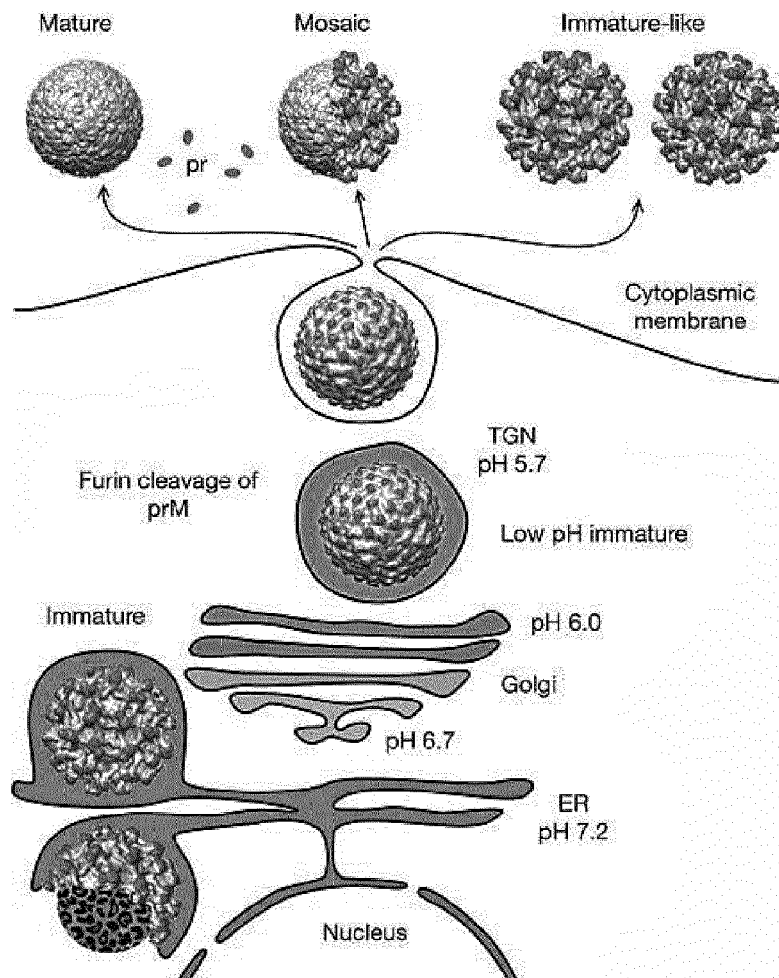


FIGURE 2

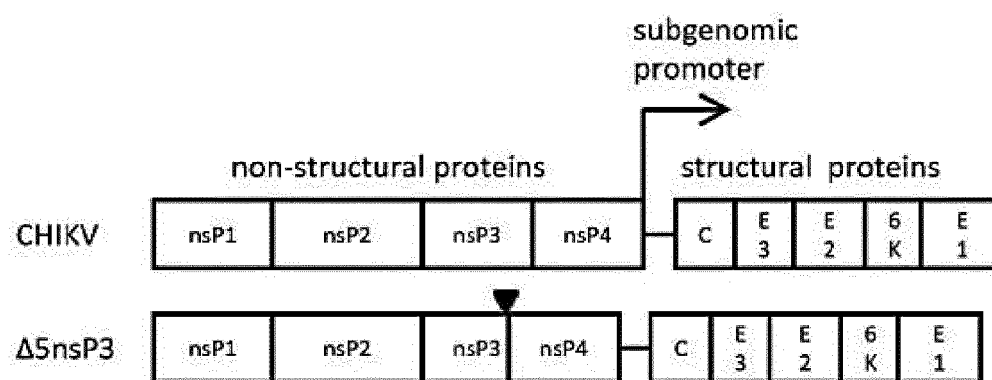


FIGURE 3

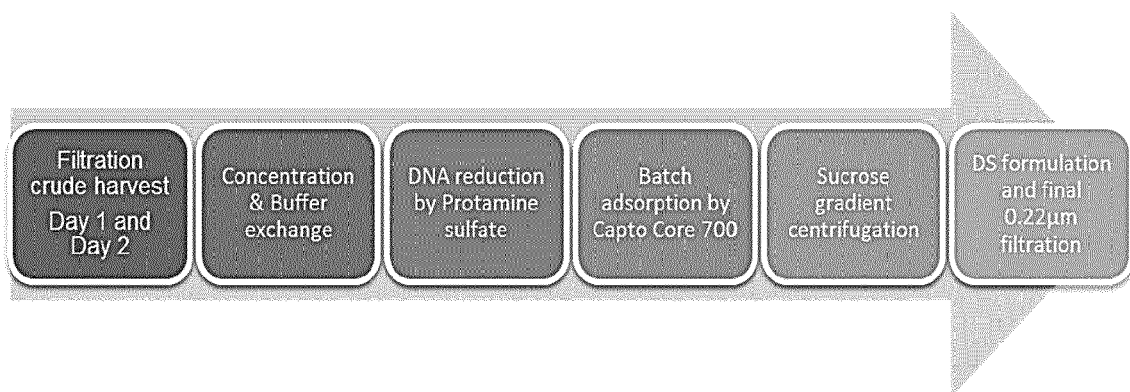


FIGURE 4A

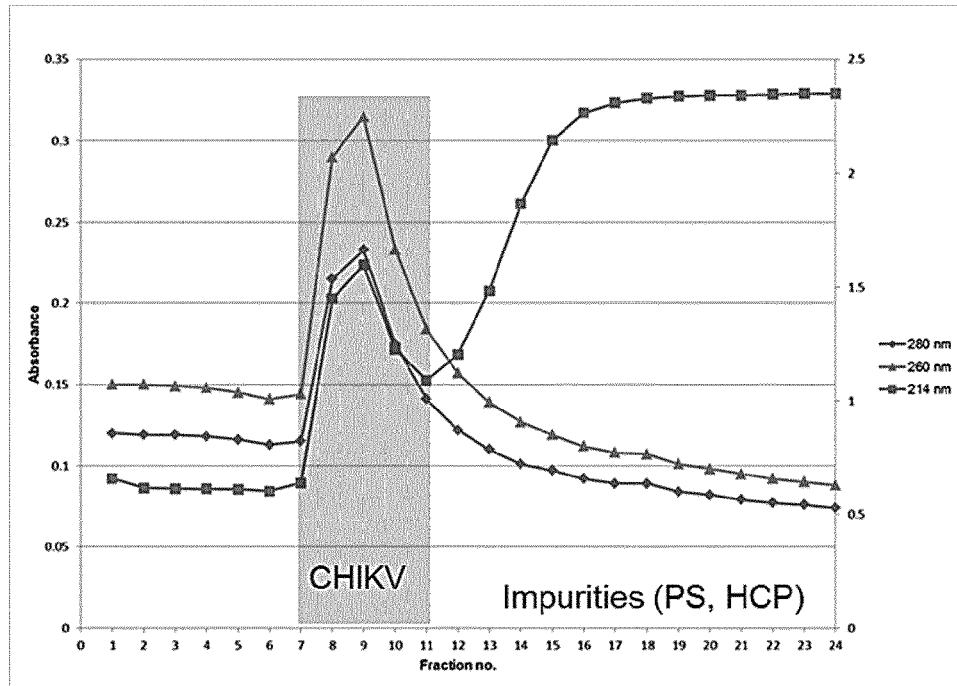


FIGURE 4B

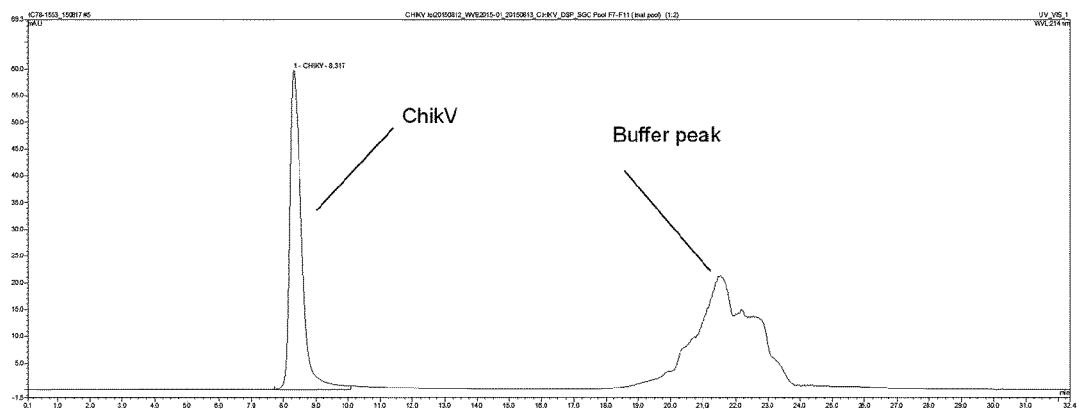
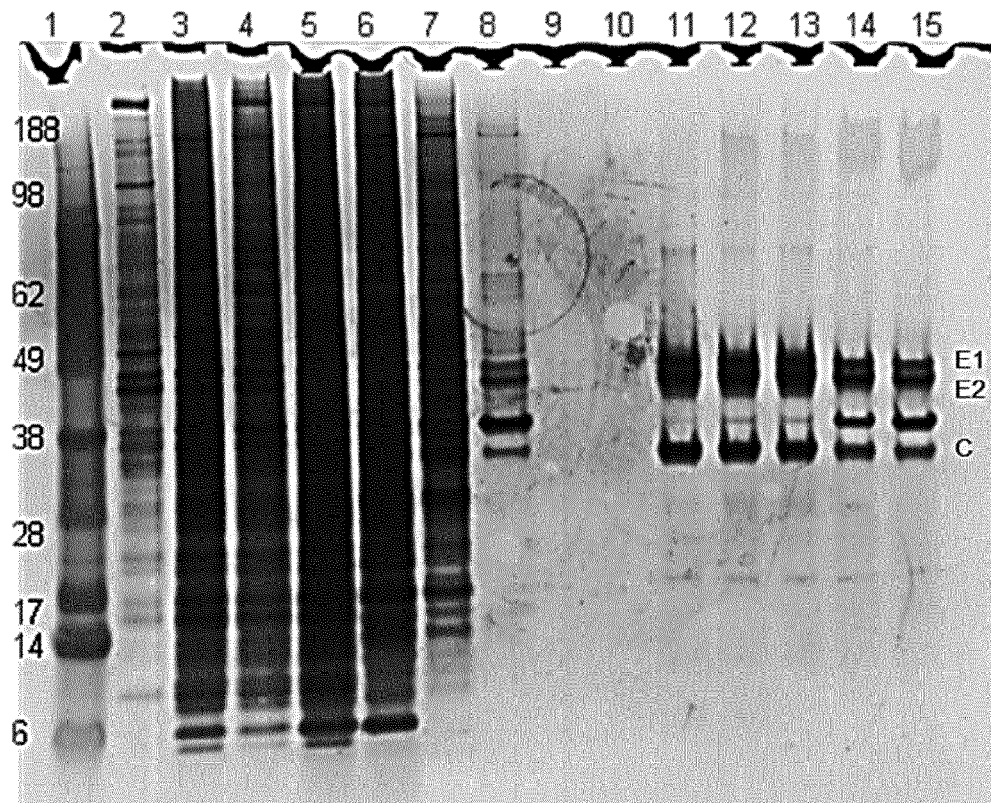


FIGURE 4C



Lane	Sample
1	Marker Seebblueplus2
2	CHIKV lot20150812_WVB2015-01_Filtered harvest 0.2μ_24hpi
3	CHIKV lot20150812_WVB2015-01_Filtered harvest 0.2μ_48hpi
4	20150813_CHIKV_DSP_UF/DF_Load
5	20150813_CHIKV_DSP_UF/DF_conc. 10x
6	20150813_CHIKV_DSP_UF/DF_conc.&dia. 11x
7	20150813_CHIKV_DSP_PStreatment
8	20150813_CHIKV_DSP_PS&CC700treatment
9	20150813_CHIKV_DSP_SGCFrac F5
10	20150813_CHIKV_DSP_SGCFrac F6
11	20150813_CHIKV_DSP_SGCPool F7-F10
12	20150813_CHIKV_DSP_SGCPool F7-F11 (final pool)
13	20150813_CHIKV_DSP_SGCPool F7-F12
14	20150813_CHIKV_DSP_SGCFrac F13
15	20150813_CHIKV_DSP_SGCFrac F14

FIGURE 5A

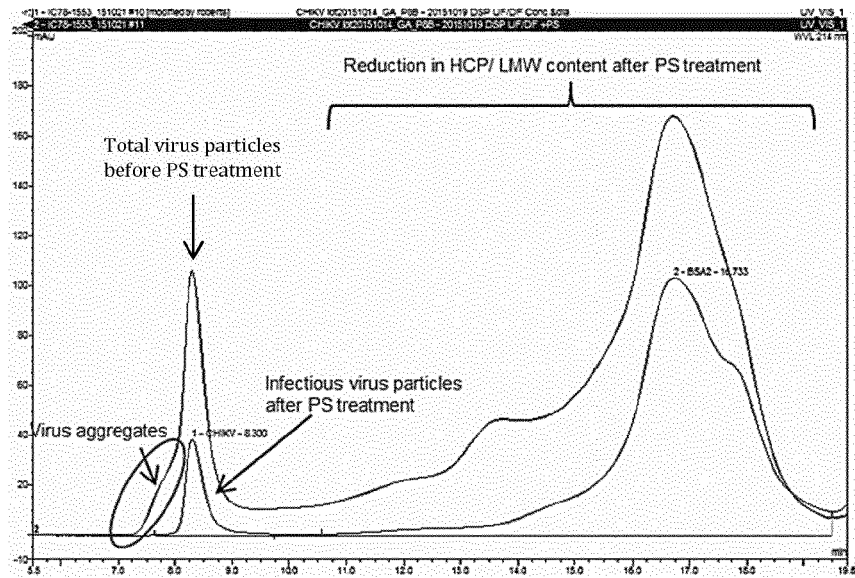


FIGURE 5B

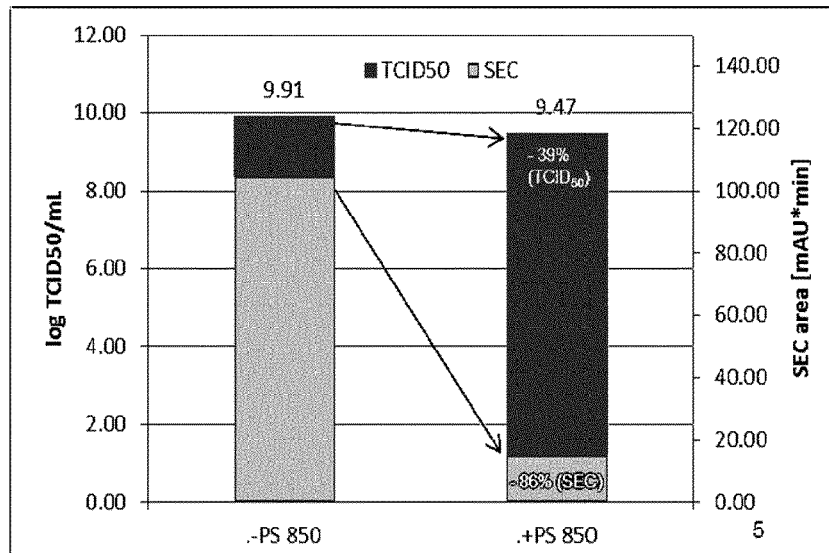


FIGURE 6

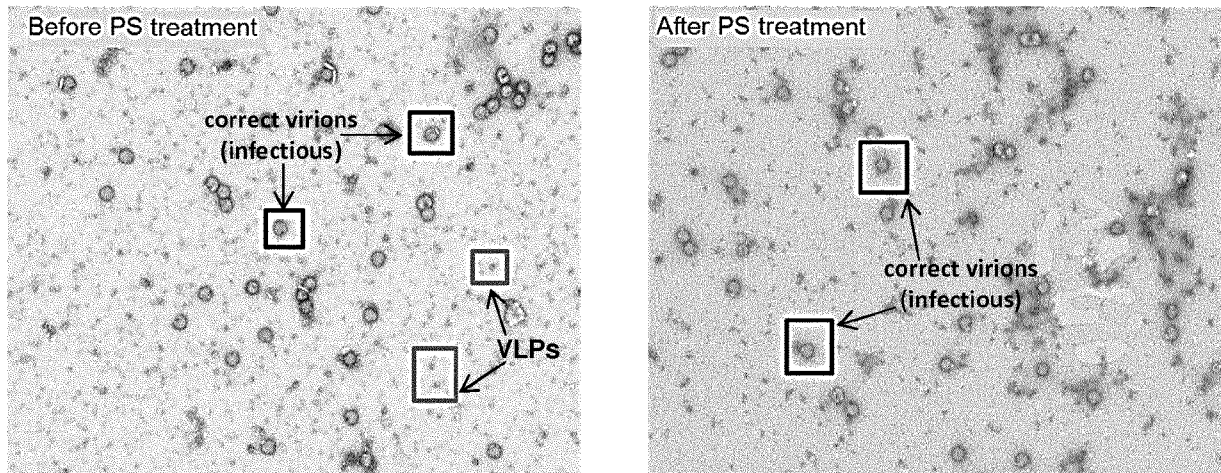


FIGURE 7

