Structural and functional characterisation of chikungunya virus replication complex dynamics

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**Structural and Functional Characterisation of
Chikungunya Virus Replication Complex
Dynamics**

Tan Yaw Bia

SCHOOL OF BIOLOGICAL SCIENCES

2019

Structural and Functional Characterisation of Chikungunya Virus Replication Complex Dynamics

Tan Yaw Bia

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Masters of Science (SBS)

2019

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I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Asst. Prof. Wu Bin

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Asst. Prof. Luo Dahai

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Tan Yaw Bia

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ABBREVIATIONS

IMAC	Immobilised Metal Affinity Chromatography
Ni-NTA	Nickel-Nitrilotriacetic Acid
IEX	Ion- Exchange Chromatography
SEC	Size Exclusion Chromatography
GF	Gel Filtration
S200	Hiload Superdex 200 16/600 pg
S75	Hiload Superdex 75 16/600 pg
DTT	Dithiothreitol
TCEP	Tris (2-Carboxyethyl) Phosphine
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
GOL	Glycerol
NaCl	Sodium Chloride
Rluc	Renilla Luciferase
Nluc	Nano Luciferase
Cam	Chloramphenicol
Kan	Kanamycin
Amp	Ampicillin
Bla	Blasticidin
CHIKV	Chikungunya Virus
RRV	Ross River Virus
SINV	Sindbis Virus
VEEV	Venezuela Equine Encephalitis Virus
EM	Electron Microscopy

SDSPAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
GA	Glutaraldehyde
FPLC	Fast Protein Liquid Chromatography
nsP	Non-structural Protein
rpm	Rotation per Minute
DMV	Double Membrane Vesicles
mt	Mitochondrial
+ssRNA	Positive Sense Single Strand RNA
dsRNA	Double-Stranded RNA
Cryo-EM	Cryogenic Electron Microscopy
Cryo-ET	Cryogenic Electron Tomography
min	Minutes
s	Seconds
IPTG	Isopropyl β -D-1-thiogalactopyranoside
RC	Replication Complex
RBS	Ribosomal Binding Site
MCS	Multiple Cloning Site
GFP	Green Fluorescent Protein
RLU	Relative Luminescence Unit
CV	Column Volume
P123 and nsP4	P123-P4

ABSTRACT

Chikungunya virus (CHIKV) is a 12 kilobase (kb) positive sense single strand RNA (+ssRNA) mosquito-borne virus. Through mosquito bites, the infected victims develop Chikungunya Fever (CHIKF) associated with symptoms like acute fever and painful polyarthrititis. Unfortunately, there are no current effective antiviral drugs and vaccine treatments against CHIKV and many other +ssRNA viruses due to the inadequacy of biological information of its replication complex (RC) formations and the process of viral replications. Hence, this project aims to provide the structural and functional characterisation of the replication dynamics of CHIKV RC, made of 4 non-transmembrane non-structural proteins (nsPs; nsP1-4) contributing their enzymatic activities essential to viral replication. Recombinant CHIKV RC bound to its binding partners, such as 3' untranslated region (UTR) region of viral RNA, was reconstituted *in vitro* for its macromolecular 3D modelling and analysis through Electron Microscopy (EM). A quick and convenient bacterial cell-based luciferase system to study the CHIKV replication dynamics was established. The detection of the signal amplification of the Renilla Luciferase (Rluc) luminescence indicated the presence of viral replication activity from the replicase plasmid expressing CHIKV RC which acted on the 3' UTR region coupled to Rluc reporter gene in the reporter plasmid. This replicase-reporter system captured the importance of self-proteolytically regulated non-structural polyprotein processing and polymerase activity of RC required for the first event of viral replication to synthesise negative-strand RNA. Further biochemical and structural determination of the enzymatic domains of CHIKV RC will reveal the functional roles and spatial organisation of nsPs in the active assembly of RC. The expansion of the experimental database will eventually build a structural-aided drug discovery platform to identify the effective antiviral drug candidates to treat the infections of CHIKV and +ssRNA group viruses.

1. INTRODUCTION

1.1. Background of Chikungunya Virus

Originated from the disease outbreak in Makonde Plateau, the southern region of Tanzania in 1952, this virus was first identified by Robinson as chikungunya, a name that derived from local Makonde language meaning “that which bends up” representing the stooped posture of the infected people suffered from chikungunya fever (Robinson, 1955). The symptoms shown by CHIKV from arthritogenic “Old World” alphavirus often range from acute fever and rashes to severe polyarthritis that could persist for months to years, often in the group of infected people in chronic condition (O. Schwartz & Albert, 2010) while the “New World” alphaviruses like Venezuela Equine Encephalitis Virus (VEEV) are associated with encephalitis. The transmission of CHIKV via the bite of female *Aedes aegypti* mosquito from an infected human to human normally occurs in the tropical and subtropical region. However, the evolutionary genetic adaption of CHIKV (single mutation of the envelope protein, E1-A226V Mutation) allows transmissions through a new vector, *Aedes albopictus* spreading CHIKV further to temperate regions (Tsetsarkin, Vanlandingham, McGee, & Higgs, 2007). The E1-A226V CHIKV mutant first caused large-scale epidemics in 2005-2006 outbreaks at a furious rate in Reunion Island, French Polynesia reporting 266,000 cases affecting close to one-third of the population (O. Schwartz & Albert, 2010; Tsetsarkin et al., 2007). It spread to Sri Lanka with approximate total of 80,000 cases accumulated in between 2006 and 2008 and further to South East Asia countries like Singapore to experience the first major outbreak in 2008 affecting 1000 residents and subsequent re-emergence in 2013 (Hapuarachchi et al., 2010; Leo et al., 2009; Oon & Ng, 2014). The distribution of the CHIKV through new transmission vector, *Aedes albopictus* eventually expanded to the Caribbean and America causing over 1.37 million suspected cases (since 2013; data provided by WHO) (Tsetsarkin et al., 2007; Weaver & Forrester, 2015). Apart from mosquito vector, the increased frequency of international travel

and global warming keep the momentum of CHIKV to spread over the globe at sporadic pattern (Suhriebier, Jaffar-Bandjee, & Gasque, 2012). Up to the present, there are no official antiviral drugs or vaccine effective against CHIKV infection and replication but only symptomatic relieving drugs (Burt et al., 2017). With the wide global distribution of possible re-emerging arthropod-borne viruses (arboviruses) like CHIKV, zika virus (ZIKV), dengue virus (DENV) and yellow fever virus (YFV), this escalates the need to develop effective diagnosis, treatment and preventive measure to combat the rapid rising rate of infectious diseases (Morrison, 2014). Therefore, developing a comprehensive understanding of the viral replication process should be the main strategy for accelerating the drug discovery process to combat arthritogenic CHIKV effectively.

1.2. Viral Entry and Exit at Molecular Level

Zooming into the molecular level of viral entry during the infection in human cells, CHIKV was recently found to invade the cells first through direct binding on the cell-adhesion receptor, Mxra8 to strengthen virus particle attachment to the cell surface (Zhang et al., 2018), followed by rapid clathrin-mediated endocytosis in forming early endosome. Eventually, the maturation of endosome causes a change of its internal content into an acidic pH environment allowing CHIKV to fuse with endosomal membrane to release its viral genomic RNA into cytoplasm of infected host. The viral replication starts with translational expression of CHIKV viral non-structural proteins from the first segment of its genomic RNA for their assembly into a protein complex as a replication machinery. Subsequently, at the late stage of infection, the production of structural protein from the second viral genomic segment allows the packaging and maturation of the virion before exocytosis through inner leaflet of cytoplasmic membrane to

the outside of the cells to infect more cells (Hoornweg et al., 2016; O. Schwartz & Albert, 2010).

1.3. CHIKV Genome Structure and Viral Replication

The genomic RNA of CHIKV stores the essential information to orchestrate the whole CHIKV life cycle involving viral replication and virion assembly. The icosahedral chikungunya virion carries an approximately linear monopartite 12kb +ssRNA genome which is classified under the genus alphavirus and family of *Togaviridae*. CHIKV viral genome consists of two open reading frames (ORFs) flanking with 5' and 3' UTR. The two ORFs encode for non-structural proteins (nsPs) and structural proteins (SPs). The features of 5' UTR capped with 7-methylguanylated (cap0) structure, and 3' UTR with polyadenylated (polyA) tail mimic the host mRNA structure and hijack the host translational machinery such as ribosome to make nsP from the first ORF (Hyde et al., 2015; Strauss & Strauss, 1994). Four sites of CSE essential for viral replication were identified in alphaviruses: 5' UTR, first 51 nucleotide (nt) replication enhancing element in nsP1 coding region of the first ORF, subgenomic (26S) promoter and 19 conserved nucleotide at 3' UTR followed by a minimum 11 adenosine at poly(A) tail (Frolov, Hardy, & Rice, 2001; Hardy & Rice, 2005; Hyde et al., 2015; Levis, Schlesinger, & Huang, 1990). The first ORF translated to produce these multimeric non-structural polyprotein precursors, P123 and P1234 for the formation of viral replication complex (RC) which is also known as replicase (O. Schwartz & Albert, 2010; Strauss & Strauss, 1994). The partially processed RC precursors, P123 and nsP4, recruit the positive sense viral genomic RNA to induce invagination of cytoplasmic membrane for the formation of RC within spherule (Hellström et al., 2017). Therefore, the presence of proteinase activity of nsP2 protease region plays crucial processing roles through *cis*- and *trans*-proteolytic-cleavage of these RC

precursors in a stepwise manner to form different RC species to regulate the viral replication dynamics. The short biological half-life of RC, which is made of P123 and P4 provides a brief window of time for negative-strand RNA synthesis which is used for later stage of viral replication. The precursor, P123 will then be cleaved into self-interacting nsPs (nsP1 and nsP23 or nsP2 and nsP3) and together with nsP4 to use the negative-strand RNA as template for the positive-sense genomic RNA synthesis and subgenomic RNA transcription for production of structural protein at second ORF during the late stage of viral replication (Levis et al., 1990; Pietilä, Hellström, & Ahola, 2017; Strauss & Strauss, 1994). This eventually leads to virion assembly with progeny viral RNA genome packaged within, virion maturation and budding off the host cell cytoplasmic membrane to infect other host cells (O. Schwartz & Albert, 2010).

1.4. Virus Replication Complex

To understand the viral replication process, the architecture of viral RC must first be structurally determined. Hybrid approaches such as combining EM of the RC ultrastructure and X-ray crystallography of protein subunits of the RC are required to build a high resolution model. The review below states the similarities among the RC ultrastructures utilised by four different classes of viruses such as +ssRNA viruses, Retroviruses, double-stranded RNA (dsRNA) viruses and DNA viruses and the differences in negative-sense single-stranded RNA (-ssRNA) viruses for viral replication and packaging of progeny RNA for virion production. The RCs from many different classes of viruses are often membrane-associated and has membrane-manipulating ability to compartmentalize as virus replication “mini-factories” to create a favourable environment for viral replication to produce replicate genetic materials for the virus propagation (Ahlquist, 2006; den Boon, Diaz, & Ahlquist, 2010); except for the cases of dsRNA viruses (Patton, Silvestri, Tortorici, Vasquez-Del Carpio, & Taraporewala, 2006)

and -ssRNA viruses (Ortín & Martín-Benito, 2015) where polymerase and RNA are encapsulated within viral protein creating a similar isolated environment favourable for viral replication. (Figure 1)

Despite the diverging evolution on the assembly mechanisms for various classes of viruses (described from 1.4.1 to 1.4.4) to form their viral RC ultrastructures with their unique compositions, they maintain the same goal of isolating their RNA in the viral RC to complete their life cycle to propagate without disruption from host cell responses (Ahlquist, 2006; den Boon et al., 2010). The mutation prone property in viruses also provides the biological flexibility to adapt to efficiently complete their life cycle in a variety of viral RC ultrastructures constructed from modified-membrane or viral protein for virus proliferations (Ahlquist, 2006; den Boon et al., 2010).

Figure 1. Comparison of viral RC among different classes of viruses: dsRNA, +ssRNA (BMV and FHV) and reverse-transcribing Retrovirus. They feature similar encapsulated compartment by viral protein (blue) or membrane-invagination (double black line) for viral replication in dsRNA and +ssRNA and virion assembly for Retrovirus with their positive-sense genomic RNA (yellow) and negative-sense antigenomic RNA (green) packed within. Polymerase (pol; red) are found to be associated with the self-interacting viral protein in dsRNA, BMV and Retrovirus. Virion assembly in Retrovirus shares similar protein composition of viral RC of BMV. (den Boon et al., 2010) The shell protein (blue) wraps the

viral RNA and polymerase in dsRNA viruses. Protein A, a crown-like structure (blue) uses a structurally unique replication complex (third from the left) to replicate viral RNA at the neck of spherule, was discovered recently in FHV RC by Cryo-electron tomography (Cryo-ET) by Ertel group in 2017 (Ertel et al., 2017).

1.4.1. +ssRNA virus

The RCs of +ssRNA viruses are tomographically identified at its ultrastructure level in the form of cytoplasmic or mitochondrial (mt) membrane spherules and interconnected vesicular structure derived from the endoplasmic reticulum (ER). This virus-induced membrane reorganization phenomenon is hypothesized to create an isolated environment optimal for viral replication to protect viral RNA and RC degradation by RNase and proteases from host and evade immune responses from host cell (den Boon et al., 2010; M. Schwartz et al., 2002; van Hemert et al., 2008). The spherule structures are often observed in the host cells infected by alphaviruses like CHIKV and flaviviruses like Zika virus (ZIKV) and Flock house nodavirus (FHV) (Cortese et al., 2017; Ertel et al., 2017; Pietilä et al., 2017). On the other hand, Coronaviruses like severe acute respiratory related-syndrome coronavirus (SARS-CoV) and Hepatitis C virus (HCV) caused the formation of a more complicated ER-derived DMV networks (Knoops et al., 2008; Romero-Brey et al., 2012). The best native spherule ultrastructure was solved by Cryogenic Electron Tomography (Cryo-ET) on FHV where its self-interacting protein A oligomerises to form this crown structure RC at the neck of spherule bound to the outer mt membrane to perform viral replication (Figure 2) (Ertel et al., 2017). The expanding volume of the spherule indicates the viral replication of the progeny genomic RNA from the negative-strand RNA initially synthesised within by protein A with template viral genomic RNA (Ertel et al., 2017) (Figure 1-2). There is currently no successful attempt of *in-vitro* reconstitution or co-purification of nsPs to assemble a functional RC with the RNA

ligands or host factors or lipid-membrane materials. It is likely due to the toxicity of the protein expressions and low solubility of certain nsPs or RNA-dependent RNA polymerases, which some of them have a transmembrane region or oligomeric structures.

Figure 2. Ultrastructure of spherule from flock house nodavirus (FHV) is the best model of viral RC currently available. (A-B) Overall ultrastructure of the spherule docked at the outer mt membrane (dark blue) features the protein A crown structure (light blue) at the neck of spherule with viral RNA (red) encapsulated within the invigorated membrane (white). (C) The side view and (D) top view of the protein A crown structure density map 3D reconstructed from Cryo-ET micrograph. (E) Cross-section of the crown with central turret (white) and interior cone structure is surrounded by 12 spikes (yellow) next to the outer mt membrane (red) and spherule membrane (blue). (F) The display of overall features of the crown density map at a tilted angle. The figure was adapted from Ertel et al. (2017).

1.4.2. dsRNA viruses and Retrovirus

For dsRNA virus-like reovirus, it concentrates their viral replication activities and virion assembly within an encapsulation of viral proteins consisting of viral protein $\lambda 1$, $\lambda 2$ RNA capping enzyme, and $\lambda 3$ polymerase (Patton et al., 2006; Reinisch, Nibert, & Harrison, 2000). The reovirus shares similar stoichiometric composition and interaction pattern of viral proteins to +ssRNA brome mosaic virus (BMV) where reovirus viral protein $\lambda 1$ and $\lambda 2$ which represent the BMV 1a protein and $\lambda 3$ polymerase represents the 2a polymerase region. (Ahlquist, 2006; Patton et al., 2006; Reinisch et al., 2000) The membrane targeting and manipulating ability of self-interacting BMV 1a protein induces membrane invagination to form spherule structure fully covered with 1a protein with 2a polymerase packed within resembles functionality of the Gag and pol protein of Retrovirus class for the virion complex assembly (den Boon et al., 2010; M. Schwartz et al., 2002) (Figure 2).

1.4.3. -ssRNA virus

Unlike the +ssRNA viruses, the -ssRNA viruses like Ebola virus and Influenza virus adopted a different replication mechanisms to replicate with its RNA in complex with oligomeric polymerase and nucleoprotein (NP) to form ribonucleoprotein-RNA complex (RNP-RNA) acting as template for viral RNA replication and transcriptions (Arranz et al., 2012; Ortín & Martín-Benito, 2015; Sugita, Matsunami, Kawaoka, Noda, & Wolf, 2018). The RNP-RNA will then serve as a scaffold for nucleocapsid formation with its progeny viral RNA condensed and packed within as virion (Ortín & Martín-Benito, 2015; Sugita et al., 2018). The first near-atomic resolution 3D structures of Ebola virus RNA-free RNP (RNP-apo) and RNP-RNA without chemical modification or fixation were reported by Sugita et al. (2018).

1.4.4. DNA viruses

Poxvirus is a unique virus strain among the DNA viruses which localised its RC in cytoplasmic space surrounded by rough ER membrane near to the ribosome instead of the nuclei of the infected host cell which is similar to the spherules or DMVs cellular feature found in +ssRNA viruses (Schramm & Locker, 2005). Hijacking microtubules and rough ER within the host cell, this enables the self-replication in a precisely coordinated manner for virus proliferation (Schramm & Locker, 2005).

1.5. CHIKV non-structural protein and their structural status

Without the enzymatic activities of four non-structural polyproteins (nsP1-4) in CHIKV RC, the complex molecular process of viral replication and transcription will not be catalysed to complete in regulated manner. Therefore, characterisations of the CHIKV nsPs are essential to understand the replication process, especially in the polymerase core domain of RdRp located in nsP4, which houses the machinery for RNA *de novo* synthesis and polyadenylation activity (Chen et al., 2017). There are numerous enzymatic roles found in the nsP2 as one of the largest nsPs (approximately 80 kDa) such as RNA triphosphatase, nucleoside triphosphatase, RNA helicase and cysteine protease. The protease domain of nsP2 is crucial for the self-proteolytic processing of polyprotein precursors to regulate the maturation of the RC. The cytoplasmic RNA capping protein, nsP1 possesses the guanylyltransferase activity for capping a cap0 structure to 5' UTR and membrane association palmitoylation site at the triple cysteine residue 417-419 and amphipathic membrane binding region predicted at tryptophan (W258) residue (Ahola, Lampio, Auvinen, & Kääriäinen, 1999; Laakkonen, Ahola, & Kääriäinen, 1996; Lampio et al., 2000). Acting as accessory protein for viral replication, nsP3 is essential to interaction with other nsPs for the formation of RC and structurally identified to interact with

negatively charged ligand such as ADP-ribose and RNA (Malet et al., 2009). The current structures available for nsPs are CHIKV nsP2 helicase region (PDB: 6JIM) (Law et al., 2019), CHIKV (PDB: 3TRK) and Venezuela encephalitis equine virus (VEEV) nsP2 protease (PDB: 2HWK/5EZQ/6CBM) (Compton, Mickey, Hu, Marugan, & Legler, 2017; Hu et al., 2016; Russo, White, & Watowich, 2006), nsP2 protease and nsP3 zinc binding domain (p23pro-zbd) from SINV (PDB: 4GUA) (Shin et al., 2012) and I-TASSER predicted structure of nsP4 RdRp from Chen et al. (2017). The structural study on nsP4 and nsP1 are challenging and limited as they are low in solubility due to the highly flexible region at the N-terminus of nsP4 and peripheral membrane association region at the nsP1 triple cysteine at residue position 417-419. The continuous effort of structural determination is still crucial to characterise the functions of nsP1 and nsP4 as part of the RC. Apart from structural study, biochemical characterisation of these nsPs enzymatic activities accompanied with the screening of ligands or inhibitors will aid to the rational design of drugs or vaccines effectively and specifically targeting nsPs. The druggable nsP targets are likely to be RNA helicase activity and protease activity of nsP2 and RdRp polymerase activity or disruption the interaction between nsPs for preventing the formation of stable RC.

1.6. The Interaction of CHIKV nsPs with Host Proteins

The limited set of the viral protein encoded by the CHIKV RNA genome, it is not surprising to observe host cellular protein to be recruited into viral RC and through interactions with individual nsPs localised at different organelles of the host cells to facilitate alphavirus replication. These host-cellular-protein-nsPs interactions seem to occur in nsP2 and between the intrinsically disordered region between nsP3 and nsP4. The inhibition of host cellular heat shock protein 90, which interacts with CHIKV nsP3 and nsP4, was found to disrupt the CHIKV

replication (Rathore et al., 2014). Another interaction occurs at nsP3 C-terminus flexible region with host cellular protein, G3BP to prevent stress granule formation and amphiphysin, which are essential for promoting viral replication (Fros et al., 2012; Neuvonen et al., 2011; Panas, Ahola, & McInerney, 2014). Translational activities shutoff of the host cell by nsP2 which can localise at nucleus with nuclear localisation signal helps to direct all translation activities to make viral proteins such as nsPs for replication and SP for virion assembly (Garmashova et al., 2007). nsP2 interferes on JAK/STAT signalling pathway and inhibits unfolded protein response to promote viral replication without triggering the antiviral response by host cell (Fros et al., 2010; Fros et al., 2015; Fros, van der Maten, Vlak, & Pijlman, 2013). When viral RNA genome and structural proteins are finally ready for virion assembly in the late viral replication stage, packaging signal sequence found in nsP2 region of the CHIKV genome instead of nsP1 like in other alphaviruses will aid to pack CHIKV RNA genome into virion (Kim, Firth, Atasheva, Frolova, & Frolov, 2011).

1.7. The Aim and Rationale of the Project

The expression of single non-structural polyprotein chain in CHIKV and other alphaviruses without non-transmembrane protein makes them an ideal and simple viral replication system to study. CHIKV nsPs are likely to have high solubility in expression systems like bacterial expression system (*Escherichia coli*). The first aim is to isolate nsPs in single polyprotein chain to capture the macromolecular architecture of the precursors, P123 and P1234. An alternative approach will be the reconstitutions of functional RC from its individual protein subunit, nsP. In this report, precursor P123 recombinant protein of 201.5 kDa was engineered for the optimal protein expression in *Escherichia coli*. The preparation of high purity of P123 was optimised to be analysed under EM to determine the structure information and its homogeneity. The

second aim is to develop a bacterial cell based reporter assay to study the dynamic of viral replication process. Luciferase-based reporter-replicase system was established in the bacterial system as the quick and convenient protocol to understand the viral replication mechanisms where the negative-strand synthesis first occurs. Biochemical and structural characterisation of the RC precursor P123 and P1234 through the reporter-replicase system and EM will provide novel insight into the initiation of the replication process. This might help to identify the exact protein target for drug discovery for the inhibition of the replication right at the beginning. Unlocking the structural and biochemical information of CHIKV RC will help us to understand better on the replication behaviour of +ssRNA viruses.

3. MATERIALS AND METHODS

3.1. Preparation of plasmids

Table 1. List of Miniprep materials:

Axyprep™ Plasmid Miniprep Kit	Axygen® Inc.
RNase A	Axygen® Inc.
Nuclease Free Water	VWR™
Nanodrop™ 2000 Spectrophotometer	ThermoFisher Scientific
Ethanol, EMSURE® grade	Merck
LB Broth	Axil Scientific
LB Agar	Axil Scientific
NEB® 5-alpha Competent <i>E. coli</i>	New England BioLabs®
Sorvall™ Legend™ Micro 17 Microcentrifuge	ThermoFisher Scientific
Sorvall™ ST 16R Centrifuge	ThermoFisher Scientific

Axyprep™ Plasmid Miniprep Kit was prepared to include the RNase A provided and ethanol to the indicated buffer in the protocol before the experiment. Plasmids were transformed into thawed NEB® 5-alpha Competent *E. coli* cells according to the transformation protocol below. The transformed cells were spread evenly on LB Agar plate supplemented with appropriate antibiotics for colony selection. A single colony was selected from the LB Agar plate to grow in 5 mL LB broth media in 50 mL conical tube for 16 hours overnight. The overnight culture was harvested at $4000 \times g$ for 5 minutes (min) in Sorvall™ ST 16R Centrifuge. The pellets obtained was processed according to the protocol recommended by Axyprep™ Plasmid Miniprep Kit using Sorvall™ Legend™ Micro 17 microcentrifuge to isolate the plasmid which was resuspended in water. Plasmids are stored in -20 °C freezer.

3.2. Molecular cloning

3.2.1. Site-directed mutagenesis

Site-directed mutagenesis was carried out with KOD FX NEO (TOYOBO®) according to the protocol provided and SimpliAmp Thermal Cycler (ThermoFisher Scientific) with parameter shown in the table below:

Table 2. Thermal cycler parameters for site-directed mutagenesis

denaturation	denaturation	annealing	extension	Final extension
95 °C	98 °C	58 °C	68 °C	68 °C
1 cycle	20 cycles			1 cycle

3.1.2. Transformation

1. LB agar plates with appropriate antibiotics are pre-incubated in 37 °C incubator.
2. Plasmid (25-100 ng) was added into the competent cell for incubation on ice for 30 min.
3. The competent cells were then transferred to 42 °C water bath to heat shock.
4. The competent cells were allowed to cool down on the ice for 2 min.
5. LB broth of 500 µL was added to competent cells for 1-hour recovery in 37 °C shaking incubator.
6. The competent cells were spread onto the pre-warmed LB agar plates.
7. The agar plates were incubated overnight before single colony selection.

3.3. Preparation of bacterial culture media

LB broth Miller (Axil Scientific) premixed power consists of 10g/L Tryptone, 10g/L NaCl and 5g/L Yeast Extract were dissolved in 25g per L of double-distilled water.

Terrific Broth (TB) media was prepared with 24g/L Yeast Extract (Axil Scientific), 12g/L Tryptone (Axil Scientific) and 8 mL of Glycerol enriched with 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4 in 1 L of double-distilled water for recombinant protein expression.

3.4. Preparation of buffers

Table 3. Buffer list and composition

IMAC-A	2 × PBS, 500 mM NaCl, 5 mM β-Mercaptoethanol and 10 % glycerol
IMAC-B	2 × PBS, 500 mM NaCl, 300 mM Imidazole, 5 mM β-Mercaptoethanol and 10 % glycerol
IEX-A	25 mM HEPES pH 7.4, 10 % Glycerol, 5 mM β-Mercaptoethanol
IEX-B	25 mM HEPES pH 7.4, 1 M NaCl, 10 % Glycerol, 5 mM β-Mercaptoethanol
GF500	25 mM HEPES pH 7.4, 0.5 M NaCl, 5 % Glycerol, 2 mM Dithiothreitol (DTT)

All buffers were prepared with double-distilled water and filtered with 0.22 µm PES membrane filter (Sartorius) and degassed under vacuum for 10 min for protein purifications.

3.5. Design of CHIKV nsPs expression constructs

CHIKV non-structural protein (nsP) sequence was sourced from isolated CHIKV Strain S27 from African Prototype to build different constructs of the whole replication complex (RC) and combinations of its components. CHIKV-P123 construct is designed based on this nsP sequences and cloned into pNIC-CH2 by LIC cloning with C-terminus hexahistidine (His₆)-tag. CHIKVP123-V1 is derived from CHIKVP123 by inactivation the protease active site (C1013S), removal of palmitoylation site (CCC417-419GSG), insertion of His-STREP-TEV-SUMO at the N-terminus through multiple site-directed mutageneses. The constructs like CHIKV-P123-V2 were first cloned from pUC57-CHIKVsyn into the pNIC-CH2 vector by LIC cloning as described above then further modified to remove palmitoylation site and replace C-terminus His₆-tag to 2xStrepII-3xFLAG tag to create CHIKV-P123-V3. All plasmids were prepared with Axygen® Axyprep™ Plasmid Miniprep Kit.

3.6 Construction of the luciferase-based reporter-replicase system

The reporter-replicase system was built with a Rluc reporter plasmid and a replicase expression plasmid in a bacterial cell environment. The transcription of reporter plasmid was driven by inducible T7 promotor as RNA template consisting of CSE (5' UTR, N-terminal 77 amino acid residue of nsP1 with 51 and CSE inclusive, 26S promoter, 3'UTR) for viral replication by T7-inducible replicases for CHIKV. The aim is to investigate the synthesis of the negative strand antigenomic RNA and the progeny genomic RNA.

3.6.1. Replicase component

The CHIKV nsP sequence was optimised to human codon for synthesis and subcloned into pUC57 and pUNO1 vectors through BamHI and SmaI cloning sites (Bio Basic Inc., Singapore). Mutation C1013S (which inactivates the nsP2 protease) and an additional stretch of N-terminus fusion tag comprising GL-STREPII-His6-SUMO were introduced during gene synthesis step. CHIKVsyn was constructed as the replicase plasmid component (CHIKV-P1234-C1013S) in the reporter-replicase system. The replicase plasmid was further modified site-directed mutagenesis with oYB_GNN_Forward and oYB_GNN_Reverse primers to produce GNN mutant to abolish the polymerase activity at nsP4 to make CHIKV-P1234-GNN. For the reversed mutation into wild-type (WT), T7-P1234-WT was created using oYB_WT_forward and oYB_WT_Reverse primers in site-direct mutagenesis to reverse the C1013S mutation to reactivate the nsP2 protease activity. The primer used in site-directed mutagenesis is listed below.

3.6.2. The Rluc reporter component

Reporter 1 (Rep01) gene cassette was constructed with T7 promoter (-17/+1), 5' UTR of CHIKV (nt 3-76 from CHIKV genome) followed by nsP1 (first 77 residues; 1-77), subgenomic 26S promoter, ribosomal binding site (RBS), enhanced Green Fluorescent Protein (eGFP), multiple cloning site (MCS), N-His6-tagged Rluc in reverse ORF (rHis6-rRluc), reverse ORF direction RBS (rRBS; sequence: CTCCTT), 3'UTR (498 nucleotides from CHIKV genome), polyadenylation tail of 50A (A₅₀) and T7 terminator.

Reporter 2 (Rep02) gene cassette was designed to have the same 5'UTR as Rep01, RBS, MCS, reverse ORF rHis6-rRluc, and the most conserved region of 3'UTR and 27 adenosine in the poly(A) tail. Rep02 gene is flanked with T7 promoter and T7 terminator for T7 polymerase transcription. All plasmids were prepared with Axygen® Axyprep™ Plasmid Miniprep Kit

Primer List

oYB_WT_Forward	GATACCTTCCAGAACAAGGCCAACGTGTGC TGGGCCAAGTCACTGGTCCCCATTCTGGAA
oYB_WT_Reverse	TTCCAGAATGGGGACCAAGTGACTTGGCCCA GCACACGTTGGCCTTGTTCTGGAAGGTATC
oYB_GNN_Forward	CTGACCAAGTCCGCCTGCGCCGCCTTTATC GGGAACAACAACATCATCCACGGCGTGGTC
oYB_GNN_Reverse	CTCGTCGGAGACCACGCCGTGGATGATGTT GTTGTTCCCGATAAAGGCGGCGCAGGC

3.7. Bacterial cell-based Reporter-Replicase Rluc Assay

The plasmids for bacterial cell-based luciferase assay as described in Table 3 were co-transformed into *Escherichia coli* BL21 (DE3) strain (Merck) for subsequent antibiotic based colony selection on LB agar plate containing appropriate antibiotics. The co-transformant colonies were cultured in 2 mL starter culture before inoculation in 10 mL LB media culture

supplied with appropriate antibiotic at starting $OD_{600} = 0.25$. The cultures were allowed to grow in 37 °C 250 rotation per minute (rpm) shaking incubator till the point of induction at $OD_{600} = 0.6$. The samples were then induced at 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG). Time-point based optimization to obtain the best Rluc signal output was first conducted. Eight sample fractions were collected at an interval of 1 hour from the beginning of IPTG induction to the fifth hours and 16th and 24th hour. After this optimisation, the experiment was subsequently repeated with the exact same experimental design as described before but induced for a fixed time of 2 hours post induction incubation. Fractions of 3mL for both uninduced and induced of the co-expression cultures were harvested by $4,000 \times g$ centrifugation in Refrigerated ST 16R benchtop centrifuge (ThermoFisher Scientific) to obtain bacterial pellets. Pierce Renilla Luciferase Glow Assay Kit (ThermoFisher Scientific) was applied to all co-expression samples. The pellet from each sample was resuspended in 200 μ L 1 $\times$ Cell Lysis Buffer from the kit and lysed by multiple freeze-thaw cycles and 20 s sonication with the pulse of 2 s on/off rate at 40 % amplitude. 20 uL lysates of different samples were mixed with 25 uL 1 $\times$ working solution from the assay kit containing luciferase substrate Coelenterazine. The mixture was incubated for 10 min as instructed in the assay kit before plate-reader reading. The measurement of luminescence intensity of luciferase activity was recorded in Synergy-H1 (Bio-Tek ®) with 1.0s integration time, a gain of 100, reading at the height of 1.00 mm within a non-treated 96-well half-area black flat bottom plate (Corning). The plate reader readings were exported to Excel sheets (Microsoft Office) and analysed them by plotting bar charts across different samples versus relative luminescence unit (RLU). If replicase makes the negative strand RNA from the transcripts of reporter plasmids, N-rHis6-rRluc and rRBS in reverse direction ORF will be synthesized in reverse complimentary fashion into a forward direction ORF which was translated by bacterial ribosomes to express Rluc to oxidise its substrate, coelenterazine to produce bioluminescence.

Table 4. Rluc Co-expression Reporter Gene List

pUC57 Reporter Plasmid	pUNO1 Expression Plasmid	Mutation Effect	Reference
T7-Rep01			this project
T7-Rep01	T7-CHIKV-WT		this project
T7-Rep01	T7-CHIKV-C1013S	C1013S mutation is to remove the activity of nsP2 protease active site to create RC precursor, P1234	Hellström et al. (2017) & this project
T7-Rep01	T7-CHIKV-GNN	This mutation abolishes the polymerase activity of GDD active site of the polymerase for RNA synthesis.	Utt et al. (2016) & this project

3.8. Recombinant Protein Expression

Plasmids expressing recombinant proteins of CHIKVP123-V1, -V2 and -V3 were transformed into *Escherichia coli* BL21 Rosetta™ II^{T1R} (DE3) strain (a gift from SBS Protein Production Platform in-house strain, with bacteriophage resistant). All CHIKVP123 protein constructs were expressed in phosphate enriched Terrific Broth media supplemented with 1 % (v/v) glycerol (Nacalai Tesque), 50 µg/ml Kanamycin (Gold Biotechnology) and 34 µg/ml Chloramphenicol (Gold Biotechnology). 1 % (v/v) starter culture was inoculated in every 800 mL TB for growing at 37 °C at 220 rpm in INFORS Multitron Standard shaking incubator. When OD₆₀₀ measurement was detected above 2.0, the TB culture was induced at a final concentration of 0.5 mM IPTG (Gold Biotechnology) to grow overnight for 24 hours at 18 °C in the shaking incubator. *Escherichia coli* cells were harvested after 24 hours of expression by 7000 rpm centrifugation in Sorvall Lynx 6000 Superspeed Centrifuge (Thermo Fisher Scientific). Cell pellets were stored at -80 °C.

3.9. Bacterial Recombinant Protein Purifications and Optimisations

The method written here summarised the most optimal purification protocols. The bacterial cell pellet was resuspended at 1:10 weigh to volume ratio in IMAC-A buffer supplemented with $1 \times$ EDTA-free Protease Inhibitor Cocktail (MedChemExpress) and 0.1 % Tween-20 (Bio Basic Inc.). The resuspension was briefly sonicated for 5 min with 70 % amplitude at a pulse rate of 5 seconds (s) on and 5 s off on ice bath at 4 °C before homogenisation and lysis at 4 °C pre-cooled PANDAPLUS2000 (GEA) homogeniser at 1000 bar for six re-circulations through the homogenisation valve. The lysate was clarified by ultracentrifugation at 40000 rpm at 4°C in Type 45 Ti Rotor in Beckman Coulter Optima XPN-100. Clarified lysate was incubated with IMAC-A pre-equilibrated His-Pur Nickel-Nitrilotriacetic Acid (Ni-NTA) (ThermoFisher Scientific) resins of 10mL bed volume for 1.5 hours at 4°C. Washing process of the protein bound Ni-NTA beads packed in gravity flow Econo-Pac® column (Bio-Rad) started with increasing concentration of Imidazole (Sigma Aldrich) from 10mM Imidazole to 30mM Imidazole with 10mM increment per 10 column volume (CV) wash. The target protein was eluted with elution buffer (IMAC-B) which was composed of IMAC-A with additional 300mM Imidazole. Subsequent 2-stage protein purification was conducted on AKTA Pure 25M Fast Protein Liquid Chromatography (FPLC) System (GE Healthcare) where all samples were 0.22 µm filtered before injection into the system. Continuing with Ion-exchange Chromatography (IEX, IMAC eluates were adjusted to 300mM NaCl with IEX-A buffer for loading onto HiTrap Heparin 5 HP (GE Healthcare) column attached to FPLC System at 1.5 mL/min followed by 2.5 mL/min washing step with 400 mM NaCl and 1.0 mL/min elution step at 850 mM NaCl in a user-defined method mode. IEX-eluted fractions were pooled and injected to HiLoad Superdex 200 pg 16/600 Size Exclusion Chromatography (SEC) column for isolation of target protein based on its size with high purity and homogeneity. SEC fractions were either first stored at 4°C for EM analysis or concentrated with IEX protocol as mentioned above using

Heparin 1 HP column. The protein fractions from SEC or IEX concentrated protein was later flashed frozen with liquid nitrogen for -80°C storage. For each purification step, protein quality was tracked and analysed by Coomassie Brilliant Blue stained Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel visualisation and data on FPLC chromatograms. The target protein concentration was spectrophotometrically identified on Nanodrop 2000 (Thermo Fisher Scientific) based on molecular weight calculated on ExPASy Server Tool, ProtParam (Gasteiger et al., 2005).

3.10. EM data collection

The purified homogeneous CHIKVP123-V1 protein right shoulder of peak fraction (numbered 1 from Figure 5A) from SEC was selected for EM. (Figure 4A) The protein construct this fraction was incubated with and without 3' FAM fluorescein labelled RNA (R23) (5'-GUUUUUAUAUUUCAAAAAAAAAAAAAAAAAA-3'FAM) in 25mM HEPES pH7.4, 150mM NaCl, 5% Glycerol, 2mM DTT buffer at 4°C. CHIKVP123-V1/R23 complex was pre-incubated for an hour prior to negative staining. CHIKVP123-APO and CHIKVP123-V1/R23 were negatively stained by 1% Uranyl Acetate (UA) on glow-discharged homemade carbon-coated support Copper/Rhodium (Cu/Rh) 400 mesh grid (Electron Microscopy Sciences). The micrograph images were acquired during a data collection session via Tecnai User Interface (TUI) software connected to Tecnai T12 Spirit electron microscope (FEI) operated at 200kV using Eagle™ 2K CCD camera (FEI) at a total dose of 25e/Å at 49,000x magnification with integrated time 1 second per image. Using Tecnai Imaging and Analysis (TIA) tools, micrographs were exported to TIFF format for visual analysis and processing. EMAN2 processing software was applied to the 70 micrographs collected for processing into 2D-class averaging and 3D reconstructed model. The 3D model of CHIKVP123-V1/R23 was then

exported by UCSF Chimera software, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311 (Pettersen et al., 2004).

5. RESULTS

5.1. Construction of the luciferase-based reporter-replicase system

All constructs in Figure 3A were designed with T7 promoter and RBS to be transcribed and/or translated in *Escherichia coli* BL21 (DE3) strain expressing T7 RNA polymerases and ribosomes. Reporter plasmid, T7-Rep01 was primarily selected for experimenting with replicase plasmids, T7-P1234-C1013S, T7-P1234-WT and T7-P1234-GNN. T7-P1234-C1013S. The inactivated nsP2 cysteine protease active site replicase, T7-P1234-C1013S was hypothesised to represent early stage viral RC as polyprotein precursors forms (P1234 or P123 and P4) with ability producing mainly the negative strand RNA species by recognition of 3' UTR-poly(A) tail region. The T7-P1234-WT replicase expression represented the natural self-proteolytic processing of CHIKV-RC precursors by nsP2 protease activity as part of regulatory mechanism for viral replication. This T7-P1234-WT replicase was most likely to contribute more towards the late stage of replication for viral RNA genome production and subgenomic RNA. The T7-P1234-GNN replicase with impaired nsP4 RdRp activity was derived from the replicase plasmid, T7-P1234-C1013S by mutating its active site residues, GDD to GNN to abolish its RNA synthesis ability (Chen et al., 2017). The recognition of replicase on 3' UTR of T7-Rep01 produced the negative-strand RNA of the positive sense reporter via viral replication. Through the negative-strand RNA synthesis, the reverse-ORF from bacterial transcribed positive-sense T7-Rep01 mRNA was replicated by the replicase to its complementary negative-sense mRNA to switch on the expression of the reporter gene cassette, rHis6rRluc and rRBS. The Rluc expression rate indirectly reflected the synthesis rate of negative-strand RNA species from the T7-Rep01 3' UTR region by the functional RC precursors, which could be P1234 or P123 + nsP4. T7-Rep02 reporter will be tested in the future experiment with the same set of CHIKV replicase plasmids mentioned above for

validation of previously suggested minimal conserved region of 3' UTR that is sufficient to trigger the negative-strand RNA synthesis (Figure 3A) (Hardy & Rice, 2005).

Figure 3. The schematic representation of construct design. (A) The CHIKV Reporter-Replicase constructs comprise of T7-P1234-C1013S replicase expression plasmid which can recognise the 3' UTR region of both reporters T7-Rep01 and T7-Rep02 for the negative-strand RNA synthesis which leads to translation of Rluc reporter protein to provide Rluc luminescence signal in the Rluc reporter assay. (B) These protein constructs are designed for expression of CHIKV P123: CHIKVP123-V1, CHIKVP123-V2 and CHIKVP123-V3.

5.2. Design of CHIKVnsP constructs through protein engineering

All constructs in Figure 3B were designed to carry T7 promotor and RBS for T7-polymerase bacterial expression system. CHIKVP123 construct was initially self-digested into fragments of individual protein - nsP1, nsP2 and nsP3 (Figure 4; left most gel labelled nsP123). In this initial construct, CHIKV nsP2 active site cysteine residue number 1013 (counting from nsP1) was first mutated to serine (annotated as CHIKVP123-C1013S) to first successfully produce P123 full-length multimeric polyprotein at low solubility (Figure 4; nsP123-C1013S second gel from the left). The solubility was further improved by mutating nsP1 triple-cysteine palmitoylation site at residue position from 417 to 419 with SDM into glycine-serine-glycine (CCC417-419GSG). To further boost the expression yield and solubility, this protein construct was engineered to fuse with this series of His-STREPII-TEV-SUMO tag at the N-terminus to incorporate the SUMO-tag with solubilising effect. After multiple protein modifications, the construct was assigned to a new experimental sample name, T7-CHIKVP123-V1 (Figure 3B top panel). Despite these efforts, the degradation problem was reduced to minimal level but not completely eliminated to produce homogenous soluble protein at higher purification yield (Figure 4). Therefore, a second construct design T7-CHIKVP123-V2 cloned from humanised CHIKV nsP synthetic sequence (Figure 3B middle panel) was surprisingly soluble with dramatically improved expression in the *Escherichia coli Rosetta II* expression strain (Figure 4). Another construct, T7-CHIKVP123-V3 (Figure 3B bottom panel) was then created with fusion of STREP-II and FLAG tags at its C-terminus for the convenience of pulldown experiments and better protein purification experiments at high purity in the future (Figure 4).

Figure 4. Construct optimisation of CHIKVP123-V1 through protein engineering. The SDS-PAGE gels were the results of small scale expression and solubility test of the protein constructs, P123, P123-C1013S, P123-V1 and P123-V2, sequentially arranged from left to the right. The construct, P123 was initially not expressed in the full-length polyprotein chain due to active nsP2 protease activity cleaving them into individual nsP1-3 proteins as shown in nsP123 SDS-PAGE gel (first from the left). The P123-C1013S construct was achieved by SDM to abolish nsP2 protease active site activity to yield full-length P123 at the top band of the nsP123-C1013S SDS-PAGE gel (second from the left). CHIKVP123-V1 with palmitoylation site (residue CCC417-419) mutated increased solubility and yielded the most amount of protein as shown on P123-V1 SDS-PAGE gel. A synthetic gene construct P123-V2 shows further improvement of the yield of full-length P123 as observed on P123-V2 SDS-PAGE gel.

5.3. Bacterial Recombinant Protein Purification and Optimisations

The protein solubility, structural integrity and yield of CHIKVP123-V1 were increased by the introduction of protein solubilising SUMO tag with affinity tags (His6-STREP II-TEV-SUMO) fused at the N-terminus and the removal of palmitoylation site (CCC417-419GSG) to reduce its binding to the membrane while protease active site inactivation (C1013S) produced a full-length polyprotein P123. The engineering on the CHIKVP123-V1 construct cannot prevent degradation as observed on SDS-PAGE gel with an extra bands right below P123 target protein (top band; in between 250 kDa and 150 kDa protein standard reference) at peak 2 along with bacterial contaminant proteins which are possibly heat-shock protein (HSP90) and GroEL observed in between protein standard of 75 kDa and 50 kDa (Figure 5A middle top panel). The elution profile of CHIKVP123-V1 in Figure 5A was delayed to 66.60 mL, which could be overlapping of the chromatogram peak of target protein CHIKVP123-V1, degraded products and bacterial contaminant proteins. A new construct design, CHIKVP123-V2 and CHIKVP123-V3 from humanised synthetic gene sequence improved greatly on the expression yield with minimised degradation and contaminants as observed in gel with molecular weight of 201.5kDa in group 2 fractions (Figure 5B middle top panel). CHIKVP123-V2 has elution volume of 59.0 mL (Figure 5B chromatogram) while CHIKVP123-V1 eluted at approximately 66.6 mL elution volume (Figure 5A chromatogram) that is similar to that of CHIKVP123-V3 which eluted at 65.6 mL (Figure 5C chromatogram). CHIKVP123-V2 showed an isolated symmetrical peak 2 in the chromatogram of Figure 5B at better chromatographic separation with minimal overlapping with other peaks consisting of undesirable protein. Therefore, a higher CHIKVP123-V2 protein amount was recovered from the high purity peak 2 fractions due to better separation of target protein from other contaminants (Figure 5B). The purification of CHIKVP123-V1 construct had a lower recovery of highest purity protein species from Figure 5A chromatogram peak 1 but it was sufficient for EM negative staining purpose (Figure

5A). Despite the overlapping chromatogram peaks in the CHIKVP123-V3 purification profile and longer retention volume in gel filtration, CHIKVP123-V3 SDS-PAGE showed a majority of the full-length CHIKVP123-V3 purified product under the left shoulder of chromatogram peak of Figure 5C peak 1. The CHIKVP123-V3 protein was further polished with stringent washing step and eluted in a buffer containing 800mM NaCl with the highest purity and concentration at the end of Heparin 1 HP concentrating step showing a sharp peak in Figure 5D.

A

B

C **D**

Figure 5. Purification profiles of different constructs. In Figure 5A-C, the asterisk marked on chromatogram peak number indicated the location of target CHIKVP123 protein constructs. (A) The protein engineering improvement of CHIKVP123-V1 created a more stable and homogenous construct. (B) CHIKVP123-V2 expressed from synthetic gene produced symmetrical peak 2 in the chromatogram at high purity based on the 10% SDS-PAGE gel visualization (B top panel). (C) The purification of CHIKVP123-V3 was optimized to include a final last step (D) to concentrate the protein using Heparin 5 HP column for EM.

5.4. EM analysis of CHIKVP123V1 with its RNA ligand, R23

The RC precursor protein-RNA complex, CHIKVP123V1-R23 was the product of *in-vitro* reconstitution of purified CHIKVP123V1 with RNA oligo named R23 which consists of a most conserved 3'UTR sequence of CHIKV genome with polyA tail. The CHIKVP123V1-R23 complex formed a doughnut-like ring shape particles visualised in a low-resolution 3D reconstructed model (Figure 6A) and 2D class-average (Figure 6B) with length measured in between 13Å and 15Å on the micrograph on Figure 6C. At this low resolution, the ligand R23 RNA was not visible in any part of the structure but was suspected to reside in the hollow middle pocket of the RC (Figure 6A). The particles tend to form clusters during the staining, as observed in Figure 6C. The challenge to prevent positive staining was to reduce UA staining concentration from 2% to 1% and avoid exceeding 25s staining duration.

Figure 6. Cryo-EM micrograph of CHIKV P123-V1 with its ligand 3'UTR RNA, R23. (A) The top view (top panel) and side view (bottom panel) of the reconstructed EM model at low resolution showing a hollow centre of the doughnut-like protein particle. (B) The 2D class average of 25 classes of good particles. (C) Doughnut-like shape particles of size 13-15Å were observed in clusters on the part of the raw micrograph captured at 2K resolution with 100nm scale bar shown below.

5.5. Bacterial cell-based Reporter-Replicase Rluc Assay

To investigate the viral replication mechanisms of the alphaviruses, the CSE component (3' UTR and poly(A) tail) crucial for first negative-strand RNA replication on Rep01 was attached next to Renilla Luciferase gene in reverse direction ORF for reporting the negative-strand synthesis (Figure 7A). The replicase plasmid T7-P1234-C1013S, which expressed the replicase, recognised the 3' UTR from the mRNA product transcribed from the reporter plasmid, T7-Rep01. This resulted in complementary negative-strand RNA synthesis and thus enabled the expression of Renilla Luciferase to generate the oxidative-bioluminescence signal in the assay kit. The optimisation of bacterial-cell based Renilla Luciferase (Rluc) assay was started with time-point based experiment by using bacterial co-expression of T7-P1234-

C1013S and T7-Rep01. In Figure 7B, the RLU intensity which mirrored the expression level of Renilla Luciferase peaked at the second hour post IPTG induction. The RLU level experienced a sharp drop at the third hour to almost half of its maximum intensity and maintained till the fifth hour before another drastic decrease at the sixteenth hour and a completely diminished RLU signal after 24 hours. This indicated that 2 hours of IPTG induction was sufficient to provide the best luciferase signal in the RLuc assays. This detected the trend of negative-strand RNA synthesis of the reporter RNA and its stability of the T7-rep01 transcript over a 24 hour time course (Figure 7B). The replicase, T7-P1234-C1013S (CHIKV-C1013S in Figure 7B) scored the highest luminescence intensity (RLU value) before and after induction, but the intensity dropped to almost half of maximum after induction (Figure 7B). On the other hand, other combination such as T7-P1234-WT with reduced luciferase activity close to the negative control (Rep01 only sample) agreed with the theory in which the negative-strand RNA synthesis required the precursors of CHIKV RC such as P1234 or P123 and nsP4 (P123-P4) to kick-start the *de novo* RNA synthesis. The T7-P1234-GNN mutant showed a luminescence reading close to that of negative control (T7-Rep01 only) as an indication of the loss of RNA synthesis activity at the mutated active site, GDD of the nsP4 polymerase domain (Figure 7C). This concluded that the negative-strand RNA synthesis must begin with the presence of the precursors of RC with functional nsP4 RdRp region and viral RNA 3' UTR region as template.

Figure 7. The Development of Bacterial Cell RLuc Reporter-Replicase Assay. (A) Here is the schematic presentation of a set of constructs: T7-Rep01 RLuc reporter with T7-P1234-C1013S replicase and its mutants, T7-P1234-WT and T7-P1234-GNN (black arrow left: nsP2 protease active site S1013C mutant to reverse to WT; black arrow right: nsP4 polymerase active site GNN mutant). (B) The optimisation of RLuc reporter assay using time-point based

experiment where the RLU data was shown on top of the bar chart. (C) Rep01 with T7-P1234-C1013S, T7-P1234-GNN and T7-P1234-WT for functional characterisation of the viral replication dynamics for the first negative-strand RNA synthesis. The uninduced (white bar) and induced (black shaded bar) samples are indicated in the right-hand panel legend. Each bar chart is labelled with RLU data.

6. DISCUSSION

6.1. Recombinant Protein Purification Profiles and Optimisations

The CHIKVP123-V2 and CHIKVP123-V3 constructs were expressed at a better yield, and lower degradation and contaminations than that of CHIKVP123-V1. This could be due to the humanized sequence where the rare codon can be more efficiently transcribed and translated by *Escherichia coli* BL21 Rosetta™ II^{T1R} (DE3) strain. With this, the bacteria might experience less stress, and thus, the bacterial degradation pathway and chaperon expression are less likely to act on CHIKVP123 protein to result in less bacterial protein contaminant in the purified P123 protein. The purification was ultimately optimized to a four-stage purification. The fourth stage of purification was using an affinity-chromatographic technique (Heparin 5 HP column) to concentrate protein in a milder condition for all CHIKVP123 constructs due to its tendency to form soluble aggregates (data not shown) in a sudden increase of protein concentrations during the concentration process of protein in the Merck Amicon concentrators. In this case, the CHIKVP123 protein was suspected of having the need to partner with its RNA ligands or other proteins such as nsP4 and host proteins for higher stability. The alternative solution to reduce the random degradation by bacterial proteases could be the protein expression of CHIKVP123 in BL21 (DE3) pLysS cells with reduced bacterial proteases. Switching to a different expression system like mammalian cell lines and insect cell lines might reduce the degradation and background contaminant protein to enhance the quality of purification of CHIKVP123. The strategy of protein purification could be changed in the future to purify CHIKVP123-V3 C-terminally fused to FLAG tag by using anti-FLAG agarose beads to simultaneously achieve greater purity of full length protein and higher concentration for cryo-EM.

6.2. EM analysis of CHIKVP123-V1 with its RNA ligand, R23

The currently available reference 3D models for CHIKV RC such low-resolution tomographic ultrastructures like CHIKV spherules (Pietilä et al., 2017) and FHV protein A crown structure (Ertel et al., 2017) are insufficient to provide information to validate the structures of CHIKVP123V1-R23 complex in Figure 6. The low resolution CHIVP123V1-R23 complex 3D model built with considerably small dataset did not allow the fitting of any available crystallographic structure including the RNA ligand, R23. From my speculation of the crude doughnut-like structure of CHIKVP123V1-R23, RNA ligand might be wrapped around within the hollow region in the middle to interact with the nsPs surrounding it. Apart from the wet-lab methods, computer simulation such as online-server based tertiary structure prediction was equally difficult and frequently gave low confidence results with random loops due to the limited availability of homology models for CHIKV nsPs in the current database and the presence of some flexible regions within the complex. Therefore, the experimental structures from hybrid approaches combining X-ray crystallography and cryo-EM are still required to produce a highly accurate high resolution map model of the CHIKV RC. The determination of the crystallographic structure of individual nsPs or their domains, such nsP1 and nsP4, should first be conducted in the future. The protein constructs could be engineered through SDM to improve the sample quality for all structural approaches. The next critical step to take for high-resolution cryo-EM experiment would be the optimisation of sample preparation protocols through the modification of the protein expression and purification, the choice the right buffer, the selection of type of EM grid favoured by sample for vitrification, and the identification of ligands and other co-factors to provide the most stable and homogenous sample. With optimum sample preparation, the cryo-EM data collected throught automated software protocol will build a more accurate 3D-reconstructed high-resolution map model of CHIKV RC complexes like CHIKVP123V1-R23 and CHIKVP1234 from a higher sampling number of RC particle.

The X-ray crystallographic structure of individual nsPs (nsP1-4) will then be spatially fitted into the accurate electron density map model of CHIKV RC at high confidence to build a high-resolution EM structure. Given enough number of particles, the heterogenous protein populations could also be computationally sorted to identify all the possible conformations for their conformational changes or the presence of minor population of the conformation species. Simultaneously, the trials of different expression systems such as mammalian cell line and insect cell line could be conducted to express stable CHIKV RC and its homologue from other alphaviruses which are closely related to CHIKV as alternative to provide the RC ultrastructure and replication dynamics.

6.3. Bacterial cell-based Reporter-Replicase Rluc Assay

The decreasing RLU measurement after 5 hours, this suggests the instability of reporter transcript mRNA or polyprotein protein (P1234) aggregation or degradation due to a less favourable bacterial cell environment. The toxicity stress from IPTG induction acted on the bacterial cells could be another possible explanation for the fall in RLU signal due to cell death or negative feedback on the overall cellular activity on transcription and translation. However, this Rluc assay signal which peaked in two hours served well for the purpose to establish a fast method to understand the viral replication dynamics which involves nsP2 self-proteolytic processing of the CHIKV RC and the catalytic site of RdRp in nsP4. The P1234 might require some post-translational modifications like self-proteolysis of nsP2 to form a more stable mature RC. The palmitoylation of nsP1 for the association to the membrane, which was absent in the bacterial systems will not induce the invagination of the membrane to form spherules to have a stable environment for viral replication. Having not biologically adapted to the bacterial host system, the nsPs are unlikely to shut down the bacterial translational activity without

interacting with the *Escherichia coli* native proteins. Thus, this might not be able to suppress the expression of bacterial proteases and nucleases to prevent the destructions of the nsPs and T7-Rep01 mRNA transcripts. The polyprotein, P1234 could perhaps form P123 and P123-P4 upon bacterial protease degradation at the flexible region in between nsP3 and nsP4 cleavage junction. With the additional western blot experiment in the future, the result might explain which species of precursors, P1234 or P123-P4 or the mixture of them actually provided the ability of precursors to initiate the negative-strand synthesis. The first bacterial cell-based Rluc reporter-replicase assay was established here to report the first event of viral replication – negative-strand RNA synthesis and its dynamics. This experiment can be complementary or alternative to the other existing trans-replicase reporter assay system in mammalian or insect cells using different types of luciferases established by Bartholomeeusen et al. (2018); Spuul et al. (2011); Utt et al. (2016) which does not directly measure the negative strand RNA synthesis but only the positive strand RNA synthesis from 5' UTR and transcription of subgenomic RNA region from 26S promoter in the later stage of viral replication.

7. Conclusion and Future Direction

The spreading of epidemic cases of CHIKV at alarming rate urges the need to come out with some strategies to combat CHIKV with effective antiviral therapeutic treatments. The investigations of the CHIKV replicative mechanisms are crucial to understanding the enzymatic roles of each nsPs and how they are integrated together in the RC to perform viral replication. The rational design of protein constructs led to first successful bacterial expression and purification of multimeric polyprotein precursor of CHIKV RC, CHIKVP123 at high yield and homogeneity for structural characterisation under EM. The first 3D reconstructed EM model of CHIKVP123 in complex with its 3' UTR RNA (R23) at low resolution was reported here. The established bacterial cell-based reporter-replicase Rluc assay provided a fast and convenient platform to study the viral replication dynamics at different stages of CHIKV infection. The investigation of early stage viral replication of CHIKV reported in the reporter-replicase system highlighted the importance of the nsP2 protease activity for regulating the nsP processing. The Rluc assay of T7-P1234-GNN mutant result pointed out the essential role of the *de novo* polymerase activity of nsP4 for the synthesis of negative-strand RNA as first event of viral replication. The negative-strand RNA was used as template for the amplification of progeny positive-sense viral genomic RNA to be assembled into virion. These findings made nsP2 and nsP4 attractive druggable targets to inhibit the key enzymatic events of viral replications.

Moving forward to the future experimental planning, the identifications of the factors for the successful assembly of a stable RC will be the top priority. The structural integrity of the complex will likely be controlled by factors like the interactions between nsPs and the RNA ligands and probably with some other host factor proteins. ExpiCHO or Expi293 mammalian cell expression system could be used to express precursors of CHIKV RC or processed RC at a possibly higher yield, homogeneity and solubility but more time-consuming. Alternatively,

in-vitro reconstitution of RC from purified nsPs (nsP1-4) with RNA ligands, host factors and lipid membrane for Cryo-EM experiments could be another strategy to capture the stable CHIKV RC and possibly capture the conformational changes throughout the viral replications and transcriptions. The protein-protein interactions within nsPs and protein-RNA interactions in between RC and viral RNA and host cellular protein can be assessed through FPLC by identifying for the change in elution volume and the ratio of UV spectrophotometry value. Deploying more biochemical assay characterization experiment, like fluorescence polarization, should help to screen more RC-binding RNA ligands with high affinity and its binding footprint with the CHIKV RC. The development of enzymatic assay for RC or individual nsPs such as polymerase assay for nsP4 region and capping assay for nsP1 region will also bring greater understanding of their contributions to viral replications. With all these experimental approaches conducted in parallel, this will increase the success rate of isolation of CHIKV-RC with and without the RNA ligands for high-resolution structural elucidation by Cryo-EM.

As an enrichment experiment to the reporter-replicase Rluc assay, real-time PCR will be planned in parallel with the reporter-replicase assay for the quantification of the RNA species (negative strand and positive strand) produced by the CHIKV-WT replicase and its mutants. The reporter plasmids could be further experimented with its compatibility with other replicases from other alphaviruses such as Sindbis Virus (SINV), O'nyong-nyong Virus (ONNV), Ross River Virus (RRV), Semliki Forest Virus (SFV) and Venezuela Equine Encephalitis Virus (VEEV). The second reporter plasmid, T7-Rep02, could be applied to assess the minimal requirement for the 3' UTR region for negative-strand synthesis.

Upon the completion of structural, biochemical and virology characterisation of CHIKV RC, CHIKV will serve as a viral replication model and biological reference for a structure-assisted drug discovery platform for not just targeting CHIKV, but in general, the whole group of +ssRNA viruses.

8. References

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