

Structure and Dynamics of Picornavirus Capsids to Inform Vaccine Design

Abhay Kotecha

A thesis submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy



St. Catherine's College
Division of Structural Biology
University of Oxford

Hilary Term 2013

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ABSTRACT

The physical properties of viral capsids are major determinants of vaccine efficacy for several picornaviruses which impact on human and animal health. Current picornavirus vaccines are frequently produced from inactivated virus. Inactivation often reduces the stability of the virus capsid, causing a problem for Foot and Mouth Disease Virus (FMDV) where certain serotypes fall apart into pentameric assemblies below pH 6.5 or at temperatures slightly above 37°C, destroying their effectiveness in eliciting a protective immune response. As a result, vaccines require a cold chain for storage and animals need to be frequently immunised.

FMDV is a member of the Aphthovirus genus of the *Picornaviridae*. Globally there are seven FMDV serotypes: O, A, Asia1, C and SAT-1, -2 and -3, contributing to a dynamic pool of antigenic variation. As part of collaboration between the Division of Structural Biology, Oxford University, The Pirbright Institute, Reading University and ARC, Ondespoort, South Africa we sought to rationally engineer thermo-stable FMDV capsids either as infectious copy virus or recombinant empty capsids with improved thermo-stability for improved vaccines. In this project, *in silico* molecular dynamics (MD) simulations, molecular modelling, free energy calculations, X-ray crystallography, electron microscopy and various biochemical/biophysical techniques were used to design and help characterise the capsids.

For the most unstable FMDV serotypes (O and SAT2), panels of stabilising mutants were characterised by MD. Promising candidates were then engineered and shown to confer increased thermo- and pH-stability. Thus, *in silico* predictions translate into marked stabilisation of both infectious and recombinant empty viral capsids. A novel *in situ* method was used to determine crystal structures for quality assessment and to verify that no unanticipated structural changes have occurred as a consequence of the modifications made. The structures of the wildtype and two of the stabilised mutants were solved and the antigenic surfaces shown to be unchanged.

Animal trials showed stabilised particles can generate a similar or improved neutralising antibody response compared to the traditional vaccines and may therefore lead to a new generation of stable and safe vaccines.

DECLARATION OF WORK

The work described in this thesis is my own, except where collaborators have contributed experimental results that are described in this thesis. These contributions are explicitly stated prior to the chapter and in the text, where appropriate.

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisors, Prof. David Stuart and Dr. Liz Fry for their constant support and guidance throughout my DPhil and for giving me the opportunity to study such a challenging and stimulating project. I would also like to thank Dr. Robert Esnouf for his support and guidance during the development of molecular dynamics simulations methods.

I would like to thank many people who helped me in several facets of this project. I am grateful to many of our collaborators for working very hard on production of stabilised FMDV particles to make this project a success. Many thanks go to Dr. Julian Seago and Dr. Bryan Charleston for working with us on production of stabilised infectious FMDV particles of O1M and SAT2 serotypes and Dr. Alison Burman and Dr. Claudine Porta for the production of stabilised FMDV recombinant empty capsids of O1M and A22 serotypes. I would also like to thank Dr. Katherine Scott and Dr. Francois Maree for their contribution with the work on stabilised infectious SAT2 FMDV particles and MSD animal health for performing animal trials.

I also express gratitude to everyone in STRUBI for helping me in the lab and making my time at Oxford very enjoyable. I would like to acknowledge Dr. Karl Harlos and Dr. Tom Walter for their help and invaluable guidance in setting up crystallisation trials, Dr. Ren Jinshan and beamline scientists at Diamond I24 station, for their help with diffraction data collection, Dr. Jun Dong and Dr. Jon Diprose for help with IT and Dr. Alistair Seibert for electron microscopy training. I would also like to thank all Stuart group members for helpful discussions and practical advice. In addition, I would like to thank Prof. Elspeth Garman for theoretical discussions and tutorials on x-ray crystallography.

I would also like to show my appreciation to the Wellcome Trust and Oxford University Press for funding my DPhil fellowship.

Finally, I would like to thank my wife Dimple Karia for her love and constant support throughout my DPhil at Oxford.

ABBREVIATIONS

Å	Angström
µg	Microgram
µl	Microlitre
µM	Micromolar
µm	Micrometre
AcMNPV	Autographa californica nucleopolyhedrovirus
AEI	N-acetyleneimine
AlOH ₃	Aluminium hydroxide gel
BEI	Binary ethyleneimine
BHK	Baby Hamster Kidney cells
CAR	Coxsackievirus-adenovirus receptor
CBV	Coxsackie B viruses
cDNA	Complementary DNA
CPE	Cytopathic effect
<i>cre</i>	cis-acting replication element
DAF	Decay accelerating factor
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EI	Ethylene imine
ELISA	Enzyme Linked Immunosorbent Assay
EM	Electron Microscopy
FMD	Foot and Mouth Disease
FMDV	Foot and Mouth Disease Virus
fs	Femosecond
GB	Generalised Born
HAV	Hepatitis A virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HS	Heparan sulphate
ICAM-1	Intracellular adhesion molecule type 1
ID ₅₀	Infectious dose where 50% cattle are infected
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IRES	Internal Ribosome Entry Site
K	Kelvin
kb	Kilo-base pair
kDa	Kilodalton
kV	Kilovolt
L	Litre
M	Molar
MD	Molecular Dynamics
min	Minutes
mg	Milligram
ml	Millilitre
MM	Molecular Mechanics
mM	Millimolar
MR	Molecular Replacement
mRNA	messenger RNA
NCS	Non-crystallographic symmetry

NDSB	Non Detergent Sulfofetaines
NMR	Nuclear Magnetic Resonance
ns	Nanosecond
OIE	Office of International des Epizooties
ORF	Open Reading Frame
PASTRY	Particle Stability Thermal Release Assay
PB	Poisson Boltzmann
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PD ₅₀	Protective dose where 50% cattle are protected
PEG	Polyethylene glycol
PME	Particle Mesh Ewald
ps	Picosecond
PVs	Polioviruses
PVR	Polioviruse Receptor
RNA	Ribonucleicacid
RVs	Rhinoviruses
ssRNA	Single stranded RNA
SAT	South African Territory
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
<i>sf</i>	<i>Spodoptera frugiperda</i>
SOP	Standard Operating Procedure
UTR	Untranslated region
VNT	Virus Neutralising Titres
VP	Virion Protein
VPg	Virion protein genome-linked
VV	vaccinia virus
v/v	volume/volume
w/v	weight/volume
W/O	water-in-oil
W/O/W	water-in-oil-in-water
WRL	World Reference Laboratory

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Chapter 1 : Introduction

1.1 Introduction

The existence of particles smaller than bacteria was first discovered in 1892 by Dmitri Ivanovsky who noted that the infectious agent could pass through the smallest available filter at that time (a Chamberland filter with a pore size smaller than a bacterial cell) while studying what is now known as tobacco mosaic virus. The infectious agent was later described as *contagium vivum fluidum*, meaning a contagious living liquid, in 1898 by Martinus Beijerinck which is now known by the term *virus*. In 1897 Loeffler and Frosch described the first filterable agent of an animal disease, foot-and-mouth disease virus followed by the first human virus, yellow fever virus (Brown 2004; Knip 2007; Lederberg 2000).

Viruses are genetically diverse microscopic obligate intracellular parasites that can only multiply inside the appropriate living cells. They are ubiquitous in nature and are known to infect all forms of life including prokaryotes, eukaryotes and archaea. A virus particle consists of genomic material which is protected from the external environment by a protein coat known as capsid. Some viruses have an extra envelope of lipids and polysaccharides surrounding (or sometimes within) the capsid. In contrast to their cellular counterparts which use double stranded DNA genomes, viruses have more varied genetic material and can be in the form of single or double stranded, linear, circular or segmented DNA or RNA. The single stranded genome of viruses may be positive sense (same polarity as mRNA), negative sense or ambisense, or a mixture of both (Cann 2005; Knip 2007).

1.2 Picornaviridae family

The picornavirus family consists of small, non-enveloped animal viruses. They carry a single-stranded, positive-sense RNA genome. The family was originally classified into four genera based on physical properties such as particle density, pH sensitivity and clinical diseases (Hyypia et al. 1997; Newman et al. 1973). However, this classification was later revised using phylogenetic analysis. At the time of writing these thesis, the family *Picornaviridae* was divided into twelve different genera which consists of Aphthovirus, Avihepatovirus, Cardiovirus, Enterovirus, Erbovirus, Hepatovirus, Kobuvirus, Parechovirus, Sapelovirus, Senecavirus, Teschovirus and Tremovirus (Hughes 2004; Knowles 2012; Palmenberg 1989). Each genus is further divided into several genetically diverse species and each species in turn contains several serotypes characterised by their distinct antigenic properties. Different genera and their natural hosts are listed in (Table 1-1).

Table 1-1: Classification of the *Picornaviridae* family and their natural host.

Genus	Species	Host
Aphthovirus	Foot-and-mouth disease virus	Livestock
	Equine Rhinitis A virus	Horses
Avihepatovirus	Duck hepatitis A virus	Ducks
Cardiovirus	Encephalomyocarditis virus	Mouse
Enterovirus	Human enterovirus A-D •Coxsackieviruses A1-A24 •Enteroviruses 68, 69, 70, 71 •Echoviruses 1-33 •Human Poliovirus 1-3	Human
	Bovine enteroviruses 1 and 2	Cattle
	Human Rhinoviruses A and B	Human
	Equine Rhinovirus	Horses
	Equine rhinitis B virus 1-3	Horses
Erbovirus	Hepatitis A virus	Human
Kobuvirus	Aichi virus A, Aichi virus B (<i>Bovine kobuvirus</i>), Aichi virus C (<i>Porcine kobuvirus</i>)	Human, Cattle, Pigs

Parechovirus	Human parechovirus, Ljungan virus	Human
Sapelovirus	Porcine Sapelovirus, Avian Sapelovirus and Simian Sapelovirus	Pigs, Ducks, Monkeys
Senecavirus	Seneca Valley virus	Human
Teschovirus	Porcine Teschovirus	Pigs
Tremovirus	Avian encephalomyelitis virus	Chickens

Picornaviruses are responsible for causing a wide range of mild to life threatening diseases affecting both humans and animals. The common cold (rhinoviruses; RVs), poliomyelitis (enterovirus C/polioviruses; PVs), hepatitis A (hepatitis A virus; HAV) and foot-and-mouth disease (foot-and-mouth disease viruses; FMDVs) are well known examples. Table 1-2 lists some of the clinical diseases caused by the members of this family. Five of the twelve genera are known to cause human diseases (Grist et al. 1978; Stanway 2002).

Table 1-2: Clinical diseases associated with picornavirus infection.

Genus	Clinical Diseases
Enterovirus	Poliomyelitis, myocarditis, paralysis, meningitis, hand-foot-and-mouth disease, common cold, pneumonia
Rhinovirus	Common cold
Cardiovirus	Encephalitis and myocarditis
Erbovirus	Respiratory diseases
Aphthovirus	Foot-and-mouth disease
Kobuvirus	Gastroenteritis
Teschovirus	Encephalomyelitis
Hepatpivirus	Hepatitis
Parechovirus	Respiratory diseases, Gastroenteritis

1.2.1 Viral life cycle

The life cycle of picornaviruses occurs in the cytoplasm of host cells. Like any other viruses, picornaviruses attach to their respective host cells via specific cell surface receptors and are then internalized. Receptor attachment may lead to conformational changes in the capsid, which in turn mediates the uncoating and release of viral genome into the host cytoplasm (Figure 1-1). In some cases uncoating may be triggered by acidification of endosomes (Belnap et al. 2000; J. Hogle et al. 1985; M. G. Rossmann et al. 2002). Following its release into the cytoplasm, the viral RNA is translated into a single polyprotein which is proteolytically cleaved to generate mature viral proteins and various intermediate products (Pelletier et al. 1988).

One third of the picornaviral polypeptide contains the structural proteins and the remaining two-thirds contain the non-structural proteins. The non-structural proteins are required for viral replication. Viral proteases are involved in polypeptide processing; they also inhibit host cell protein translation, by cleaving the cellular components required for cap-dependent translation. The viral polymerase and helicase facilitate genome replication by re-arranging the endoplasmic reticulum to form replication complexes, where viral RNA synthesis can occur. The newly synthesised RNA is then packaged into the viral capsid. The encapsidation of RNA leads to the final maturation event generating the infectious virus particles which are released into the intracellular environment following the lysis of the host cells (Jarvis and Kirkegaard 1992; M F Ypma-Wong and Semler 1987; M. F. Ypma-Wong et al. 1988).

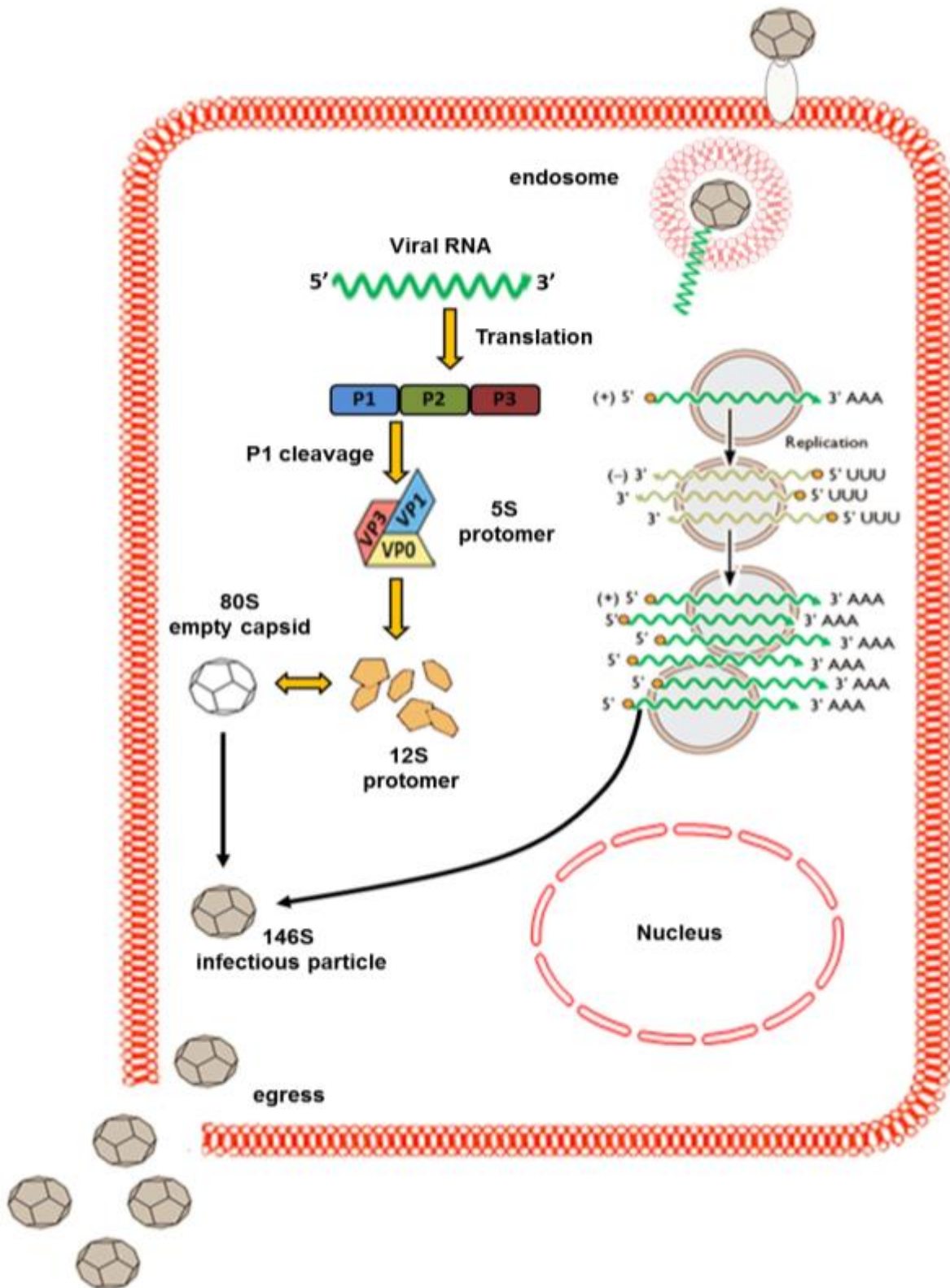


Figure 1-1: Picornavirus life cycle. Schematic diagram showing a typical lifecycle of picornaviruses in infected cells. The main steps of the lifecycle are described in the text. Figure adapted from J. M. Hogle (2002).

1.2.2 Picornavirus genome

Viruses within the picornavirus family share several common features; they all have a single-stranded, positive-sense RNA genome (Figure 1-2). Their RNA genomes vary in size from ~7000 to ~8500 nucleotide bases and are encapsidated within a protein coat. The genome is divided into three regions: the 5' untranslated region (5' UTR), the coding region and the 3' untranslated region (3' UTR). The UTR regions contain RNA secondary structures elements which are involved in the regulation of translation and viral RNA replication (Flanegan et al. 1977; Nomoto et al. 1977a).

The 5' end of picornaviral RNA contains a covalently linked, viral encoded VPg (virion protein genome-linked), a cis-acting replication element (*cre*) and an internal ribosome entry site (IRES). The VPg protein acts as a primer for RNA synthesis (Nomoto et al. 1977b), the *cre* element is involved in genome replication and the IRES assists in cap-independent translation. In addition, the 5' end also contains a clover leaf structure which is involved in RNA replication (Beales et al. 2003). In aphtho- and cardioviruses, the 5' UTR also contains an s-fragment (approximately 360 nucleotides) and a poly-C tract. The precise functions of these elements are unknown but they are believed to play a role in translation and replication of the genome (Serrano et al. 2006). The 3' UTR contains a poly-A tail which acts as a binding site for components of the replication complex and for the synthesis of negative strand RNA. In addition, the polyA tail also stabilises the viral RNA (Bergamini et al. 2000; Lin et al. 2009; Yogo and Wimmer 1972). The genome, being very small, encodes the minimal amount of proteins in a single open reading frame (ORF). The polyprotein undergoes a series of proteolytic processing events to generate the structural and non-structural proteins which are involved in viral RNA replication, capsid assembly and the release of the progeny virus particles from infected cells.

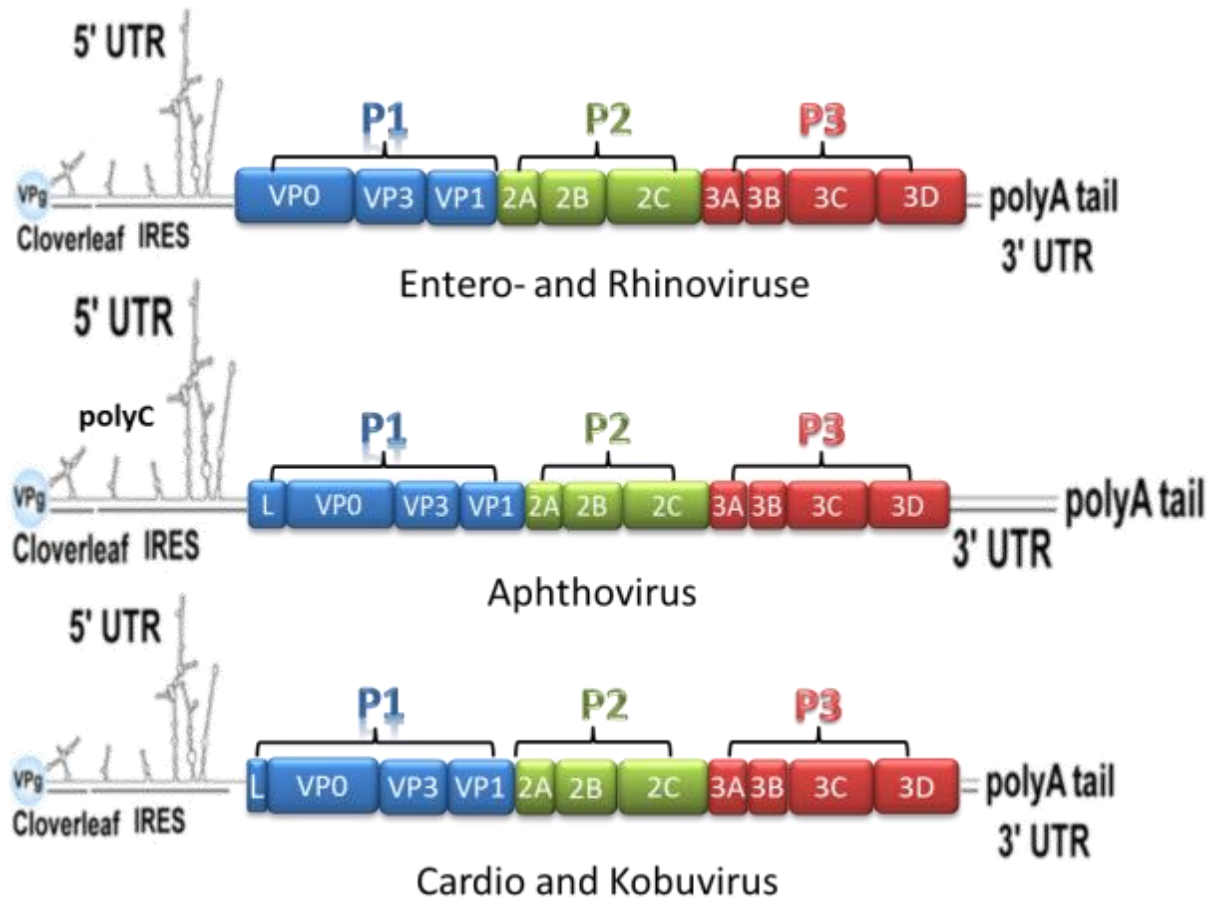


Figure 1-2: Genomic structure of picornavirus RNA. A diagrammatic representation of the picornavirus genome. VPg, viral protein genome linked; UTR, untranslated regions; IRES, internal ribosome entry site; P1-3, viral structural and non-structural precursor products. Figure adapted from Lin et al. (2009).

1.2.3 Translation

The picornavirus genome is translated into a single ~250kDa polyprotein. The process of picornaviral translation is quite different to the eukaryotic cap-dependent mechanism. Eukaryotic mRNA contains a methylated guanosine cap structure at the 5' end. This cap is recognised by elongation initiation factor 4E (eIF4E), a subunit of the eIF4F translation complex along with eIF4A and eIF4G. The interaction recruits the ribosomal 40S subunit, charged with eIF2-Met-tRNA-GTP, eIF3 and eIF1A, to the mRNA. The small ribosomal subunit scans the mRNA from the 5' UTR until the AUG is reached, at which point the 60S ribosomal subunit binds the complex, releases the initiation factors and elongation of translation occurs (Beales et al. 2003; Bedard and Semler 2004).

Picornavirus genomes do not contain a cap, instead the 5' UTR contains a region of highly structured RNA of 400 to 450 nucleotides in length, termed the internal ribosome entry site (IRES), which interacts directly with the 40S ribosomal subunit and allows assembly of translational machinery close to the initiation codon. This is advantageous for viral translation, because during picornaviral infections proteases cleave several cellular proteins including eIF4G, a scaffolding protein required for cap dependent translation, leading to the rapid shutoff of host cell translation (Krausslich et al. 1987; Pelletier et al. 1988).

1.2.4 Polyprotein processing

The polyprotein undergoes several co- and post-translational cleavages yielding a number of intermediate and mature products that facilitate replication and the generation of mature viruses (Figure 1-3). The polyprotein can be divided into three regions, termed P1, P2 and P3, based on early cleavage events. The P1 region (VP0, VP1 and VP3) encodes all the structural proteins that self-assemble to form viral capsids. The P2 (2A, 2B and 2C) and P3 (3A, 3B, 3C and 3D) regions encode the non-structural proteins such as proteases, helicases and RNA dependent RNA polymerases (Hanecak et al. 1982).

The proteolytic processing of the picornaviral polyprotein occurs in three steps driven by viral-encoded proteases: L (only found in aphtho- and cardioviruses), 2A (only found in entero- and rhinoviruses), 3C and 3CD (found in all picornaviruses). The functions of the proteases are summarised in Table 1-3. The primary cleavage occurs at the P1-P2 junction separating the structural protein precursor P1 from the non-structural protein precursor P2-P3. The process is catalysed by the 2A protease which cleaves tyrosine - glycine residues. The non-structural and capsid precursors are further processed by the 3C and 3CD proteases, which preferentially cleave at glutamine-glycine residues resulting in the generation of mature viral proteins. Slight variations in processing occur amongst the genera (Parsley et al. 1999; Toyoda et al. 1986).

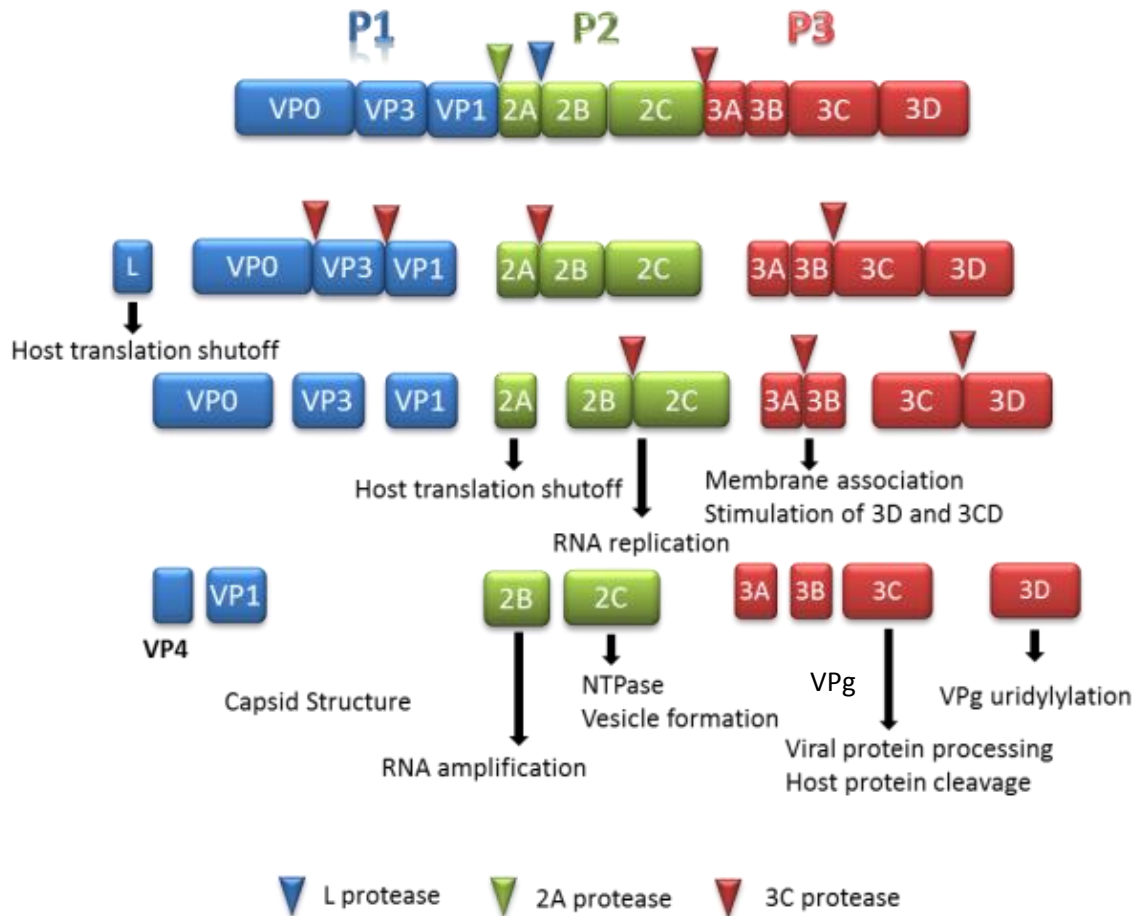


Figure 1-3: Picornavirus polypeptide processing. Schematics of polyprotein processing in picornaviruses showing the three major proteases, their cleavages sites, precursor products and mature proteins. The main biological functions of precursor products and mature proteins are highlighted. Figure adapted from Lin et al. (2009).

The situation with aphthoviruses is different from that of other picornaviruses, because they contain a leader (L) protease at their amino terminus. The L protease is a papain-like cysteine protease with auto-proteolytic activity, responsible for mediating the cleavage of its own C-terminus from the adjacent P1 precursor (L-P1 junction) and thus liberating L from the polyprotein. The L protease then cleaves eIF4G and induces host protein synthesis shut off. Cardioviruses also have an L protease which differs from that of aphthoviruses and does not possess protease activity, instead its C-terminus is processed by 3C protease (Borghese and Michiels 2011; Devaney et al. 1988; Medina et al. 1993).

Table 1-3: Summary of picornaviral proteases and their functions during infection.

Protease	Functions	Genus
L	- Primary autocatalytic cleavage between L-P1. - Host cell translational shut off.	FMDV
2A	- Primary autocatalytic cleavage between P1-P2. - Host cell translational shut off	Enteroviruses and Rhinoviruses
3C	- Release of L protein in cardioviruses. - Cleavage of all precursor proteins. - Cleavage of host cell proteins.	All picornaviruses

Further processing of the precursor protein is catalysed by 3C and 3CD proteases to generate three structural proteins VP0, VP3 and VP1 and ten non-structural proteins 2A, 2B, 2C, 2BC, 3A, 3B, 3C, 3CD, 3D. The 3D protein is an RNA-dependent RNA polymerase (3Dpol) which specifically replicates copies of positive strand RNA into complementary negative strand RNA in the presence of VPg primer (Baltimore et al. 1963). All picornaviruses have a cis-acting RNA element (cre) in the genome which aids 3Dpol in the synthesis of an uridylated form of VPg, VPg-pUpU. The 3Dpol-VPg-pUpU complex then moves to the 3' poly-A tail of the genome and commences negative strand viral RNA synthesis using pUpU as a primer.

The negative strand RNA in turn serves as a template for newly synthesised positive strand RNA. During the process of replication, VPg is removed from some of the newly synthesised RNA, which is then translated to amplify production of viral proteins (Pathak et al. 2007; Paul et al. 2000; Rieder et al. 2000).

1.2.5 Picornavirus Capsid Structure

The picornaviral RNA is protected from the external environment by an icosahedral protein capsid. The capsids are non-enveloped with a diameter of about 25 to 30nm. The atomic structures of several picornaviruses have been characterised to date and these show several common properties (Acharya et al. 1989; Filman et al. 1989; J. Hogle et al. 1985; Muckelbauer et al. 1995; M. G. Rossmann et al. 1985). The capsid is composed of 60 copies of each of the VP1, VP2, VP3 and VP4 proteins.

After proteolytic processing of the structural proteins; VP0, VP1 and VP3 self-assemble into a heteromeric protomer which has sedimentation co-efficient of 5S. Five of these protomers self-assemble into a 12S pentamer and twelve pentamers are subsequently assembled into a provirion with the RNA genome (Figure 1-4). In the final stage of maturation the viral RNA mediates autocatalytic cleavage of VP0, a process which in polio virus is thought to involve Ser 102 acting as a nucleophile and the RNA as a proton abstracting base, releasing VP4 and VP2 (Acharya et al. 1989; Curry et al. 1997; J. Hogle et al. 1985). During the process of infection empty particles are also produced with some picornaviruses, however, although these resemble the mature virus in structure and antigenicity they contain uncleaved VP0 and are inherently less stable which suggested the role of VP0 cleavage in stabilising the particles (Basavappa et al. 1994; Curry et al. 1997).

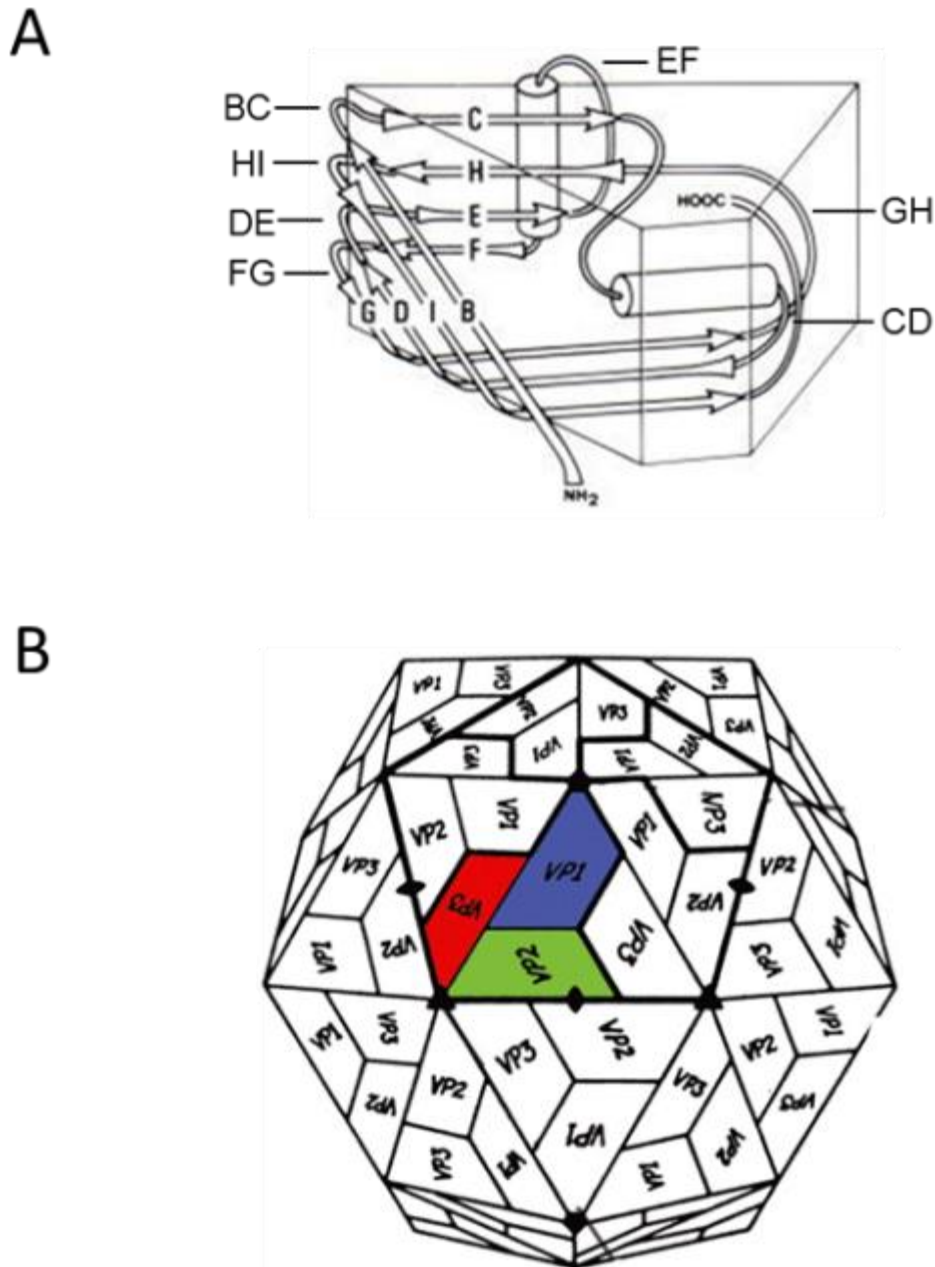


Figure 1-4: Schematic diagram showing the protein organisation and structure of picornavirus capsids. Picornavirus capsids are composed of 60 copies each of VP1-VP4 structural proteins. (A) A cartoon representation of the core β -barrel structure found in VP1-VP3 proteins showing the organisation of chains and connecting loops. (B) Schematic diagram of a picornavirus capsid showing the organisation of VP1 (blue), VP2 (green) and VP3 (white) proteins. VP4 is on the inside of the capsid and not shown in this figure. Figure adapted from Fry *et al.* (2005); J. M. Hogle (2002).

In FMDV, the process of final cleavage is not well understood. There is no Ser at the cleavage site, although Arnold *et al.*, 1987 (Arnold et al. 1987) have suggested the nearest Ser may perform the same function. Interestingly, the crystal structure of naturally occurring empty particles of A22 serotype showed VP0 had been cleaved into VP4 and VP2 (Curry et al. 1997).

The capsids are arranged in a pseudo T=3 icosahedral lattice in which VP1, VP2 and VP3 decorate the outside of the capsid and VP4 is buried inside. VP4 is believed to stabilise the inter-pentameric interfaces. Each protein (except VP4) contains an eight-stranded anti-parallel β -sheet forming a β -barrel core. The strands are labelled as CHEF and BIDG and are connected by loops that contain major antigenic sites and receptor recognition sequences (Figure 1-4).

The VP1 proteins are located at the five-fold axes, whereas VP2 and VP3 alternate around the two- and three-fold axes respectively. The crystal structures of enterovirus capsids have shown a characteristic canyon formation at the base of the five-fold axis which is proposed to contain the receptor binding sites. At the bottom of the canyon there is a hydrophobic pocket in VP1 which contains a pocket factor (lipid) of cellular origin. The pocket factor stabilises the capsid structure and prevents premature uncoating and RNA release (Filman et al. 1989; M. G. Rossmann 1989; M. Rossmann 1994; M. G. Rossmann et al. 2002).

1.2.6 Receptor Attachment

In order to initiate infection, viruses must manage to enter their host cells. In picornaviruses the first step to enter the cells is mediated by the viral capsid proteins by binding to a receptor on the host cell membrane. Picornaviruses can utilise a great variety of cell surface molecules (Table 1-4) including immunoglobulin-like molecules such as the poliovirus receptor (PVR also known as CD155) and adhesion proteins such as intracellular adhesion molecule type 1 (ICAM-1). Several enteroviruses, such as echoviruses and coxsackie B viruses (CBV), use as a cellular receptor decay accelerating factor (DAF also known as CD55). Some isolates of CBVs also bind to coxsackievirus-adenovirus receptor (CAR). Other picornaviruses such as human parechovirus, coxsackievirus A9 and FMDVs utilise integrin receptors (Freimuth et al. 2008; Hafenstein et al. 2007; T. Jackson et al. 2000; M. G. Rossmann et al. 2002; Xiao et al. 2001).

Table 1-4: Cell surface receptors used by picornaviruses.

Viruses	Receptors
Polioviruses	PVR
Coxsackie A viruses	ICAM-1, DAF
Coxsackie B viruses	CAR, DAF
Rhinoviruses	ICAM-1, LDLR
Hepatitis A	HAVcr-1
Foot and mouth disease viruses	Integrin, Heparan sulphate
Parechovirus 1	Integrin
Coxsackie A9	Integrin

PVR, poliovirus receptor; ICAM-1 intracellular adhesion molecule 1; DAF, decay accelerating factor; CAR, coxsackievirus and adenovirus receptor; LDLR, low-density-lipoprotein receptor; HACcr-1, hepatitis A virus cellular receptor-1

Most picornaviruses penetrate host cells by using the clathrin-mediated endocytosis pathway. Some enteroviruses such as echovirus-1 and coxsackievirus B3 enter cells via the caveolar pathway (Tuthill et al. 2010). Once inside, the viruses require appropriate conditions to initiate uncoating and release their genome. Binding to the receptor often induces conformational changes in the capsid structure. For example, in the case of enteroviruses and rhinoviruses binding the receptor results in the production of an intermediate particle called an A-particle with altered sedimentation behaviour. In the A-particle, the N-terminus of VP1 and the whole of VP4 are externalised, resulting in an altered sedimentation coefficient of 135S instead of 160S as for native virions. The intermediate particle is then internalised by endocytosis and the genomic RNA released (Fricks and Hogle 1990). Other picornaviruses require more than one receptor where the first receptor is required for binding and another for uncoating or entry, for example CAV21 requires both DAF and ICAM-1 for successful infection (Xiao et al. 2001).

1.3 Foot-and-Mouth Disease

Foot-and-mouth disease virus (FMDV) is the etiological agent of foot-and-mouth disease, one of the most contagious diseases affecting cattle, pigs, goats, sheep and other cloven hoofed animals. The disease is characterised by fever and lesions resembling blisters inside the mouth and on the feet of the infected animals. The lesions may rupture resulting in excessive salivation and lameness. Other symptoms include anorexia, depression and severe pain (Alexandersen et al. 2003). The disease is not fatal in adult animals; however mortality is high in new-borns due to myocarditis. Although most animals recover, it leaves them debilitated leading to severe loss in growth and productivity. The disease is easily transmissible by direct contact with animals, indirect contact by contaminated motor vehicles and farm equipment, clothes and skin of farmers, standing water and feed supplements

containing infected animal products. There is also a likelihood of airborne infection over long distances (Bachrach 1968).

The disease is wide spread around the world, being endemic in parts of Asia, Africa and South America, but North and Central America, New Zealand, Australia and Chile as well as some European countries are FMD free (Figure 1-5). However sporadic outbreaks have occurred within some of these countries, for example the 2001 outbreak in the United Kingdom lasted for almost seven months spreading all across the country, and even to Ireland, France and the Netherlands. Over 6 million animals were slaughtered and economic losses were estimated between 5.8 and 6.3 billion pounds (Bachrach 1968; Grubman and Baxt 2004). Due to its devastating impact on livestock FMDV is on the A list of infectious diseases of animals of the Office of International des Epizooties (OIE). Countries that are free from FMDV have introduced a number of very strict measures to retain this status, including restrictions on international trade in animals and animal products (Gibbens et al. 2001; Leforban 1999; Leforban and Gerbier 2002).

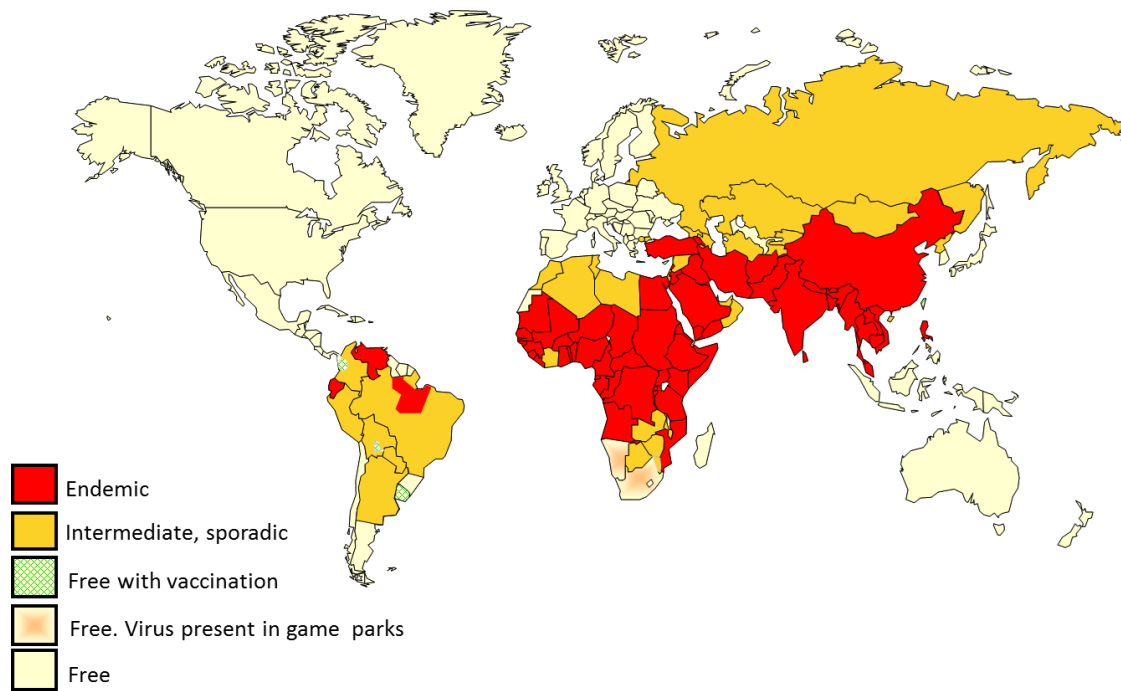


Figure 1-5: Global status of FMDV. FMD is currently endemic in many parts of the world including Middle East, Asia, Africa and some parts of South America. (data received from Dr. Bryan Charleston, The Pirbright Institute).

1.3.1 Foot and Mouth Disease Virus

The infectious agent, subsequently named foot-and-mouth disease virus (FMDV), was probably the first animal virus to be identified. It was discovered by Loeffler and Frosch in 1897 who showed FMDV was a filterable agent (Loeffler 1897). FMDV belongs to the aphthovirus genus of the family *Picornaviridae*. Similar to other picornaviruses, the FMDV genome is protected from extracellular conditions by a non-enveloped icosahedral capsid of about 30nm diameter.

During centuries of evolution, FMDV has had ample opportunity for genetic variations, which has led to the diversification of the virus into the seven different serotypes seen today (Figure 1-6) namely: O, A, C, Asia1, SAT 1, SAT 2 and SAT 3 (South African Territories). Serotypes are classified based on the antigenic structure of the viral capsids. Each serotype

contains several antigenically variable subtypes within them. Infection with any one serotype does not confer immunity against another. Also, because of the antigenic diversity within the serotype, there is very low to no cross-protection between subtypes within the same serotype (Brown 2003).

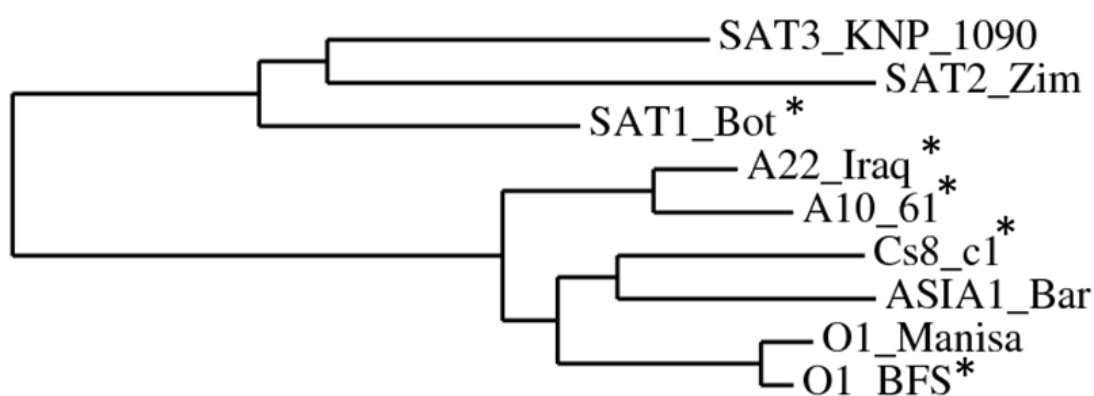


Figure 1-6 : Phylogenetic analysis. Phylogenetic tree showing isolates of different FMDV serotypes and subtypes. The tree is based on the analysis of amino acid sequence of P1 region (VP1-VP4 proteins). * indicates the serotypes/subtypes for which atomic structures are available.

1.3.2 FMDV Capsid Structure

Capsid structures of several representatives of 4 different serotypes of FMDV have been determined by crystallography, including O1BFS 1860 (1BBT), A22 Iraq (4GH4), Cs8-c1 (1FMD) and SAT1 (2WZR). The structure of FMDV is similar to that of other picornaviruses (Acharya et al. 1989; Curry et al. 1996; Lea et al. 1994). The capsid consists of 60 copies each of four structural proteins VP1 (32kDa), VP2 (28kDa), VP3 (27kDa) and VP4 (8kDa) arranged in an icosahedral lattice of 12 pentameric assemblies. VP1-VP3 proteins are surface exposed and form the majority of the capsid structure. These proteins also have similar eight-stranded beta-barrel folds to those found in other picornaviruses. VP4 has an extended

conformation and is buried within the capsid. The β barrel strands of VP1-3 are connected by surface exposed loops (Acharya et al. 1989; Terry Jackson et al. 2003). In common with other picornaviruses, the N-terminal residue of VP4 contains a covalently linked myristoyl group (Belsham et al. 1991).

During infection empty particles are also produced (natural empty particles) which sediment at 75S and resemble mature viruses in structure and antigenicity. The empty particles differ from the virus in their polypeptide composition and contain VP1, VP3 and uncleaved VP0 proteins. They are slightly more pH stable than mature virus, however they are very sensitive to temperature (Basavappa et al. 1994; Curry et al. 1995; Curry et al. 1997; Rweyemamu et al. 1979). Despite the overall similarities, the surface properties of FMDV are quite different to that of other picornaviruses. VP1-3 proteins are comparatively small compared to those of other picornaviruses and so are the loops connecting the β -strands. VP4 is largely extended with very little secondary structure and it is somewhat longer than the VP4 of other picornaviruses. As a result of having a shorter VP1, there is an exposed pore at the 5-fold axis which permits the entry of small molecules such as CsCl into the capsids, making the buoyant density of FMDV very high in CsCl gradients. The canyon structure, observed at the base of VP1 in entero- and cardioviruses, is absent in FMDV making the capsid very spherical (Acharya et al. 1989; Belnap et al. 2000; Belsham et al. 1991; J. Hogle et al. 1985).

1.3.3 Capsid Stability

Like other picornaviruses, the VP1 protein of FMDV decorates the 5-fold axis of the capsids and contributes to the stability of the pentamers whilst VP2 and VP3 mostly contribute to the stabilisation of inter-pentameric interfaces and hence capsid structure. The N-terminus of VP3 also contributes to the stabilisation of the pentameric assemblies. FMDV capsids are

very unstable and are held together by electrostatic interactions, hydrogen bonds and weak hydrophobic interactions between the inter-pentameric subunits (Acharya et al. 1989; Ellard et al. 1999). Unlike other picornaviruses, FMDV capsids dissociate into 12S pentamers below pH 7.0 (Brown and Cartwright 1961; J. M. Hogle 2002). The dissociated pentamers only contain VP1, VP2 and VP3 proteins and therefore differ from those of the assembly pathway which contain VP0 (VP4+VP2). The pH sensitivity of FMDV capsids is required for disassembly and release of the viral genome within infected cells (Carrillo et al. 1984; Carrillo et al. 1985).

The reason for such pH instability is proposed to be the protonation of a cluster of histidine residues at the inter-pentameric interface between VP2 and VP3 (Acharya et al. 1989). Curry et al. 1995 and Twomey *et. al.* 1995 proposed that His 142 and His 145 of VP3, and possibly His 65 and His 21 of VP2, become protonated at low pH and destabilise the interface by electrostatic repulsion. Curry et al. 1995 proposed that His 142, which is in close proximity to the positive end of the α -helix at the 2-fold axis, would destabilise the helix dipole when protonated. The role of His 142 was further verified by mutational analysis by Ellard et al. 1999. The mutation H142D formed pH stable capsids, however with significantly lower yield compared to native capsids. In addition mutation H142F resulted in aberrant complexes (Curry et al. 1995; Ellard et al. 1999; van Vlijmen et al. 1998).

In addition to pH instability, FMDV capsids are also very sensitive to temperature. Doel and Baccarini, 1981, characterised the thermal stabilities of mature (146S) and natural empty particles (75S) for each of the seven FMDV serotypes. The results from their experiments suggested that different strains of FMDV differ considerably with regards to thermo-stability. At 49°C live viruses of types A and C are more stable than types O and SAT1-3, with SAT

viruses being extremely sensitive to temperature. The 75S empty particles of all serotypes were more unstable. They also demonstrated the effects of AEI (N-acetyleneimine) inactivation, used for vaccine manufacturing, on thermal stability. AEI inactivated 146S particles were even more unstable than their native counterpart. The destabilising effect of AEI was also observed with the otherwise more stable A and C serotypes (Doel and Baccarini 1981).

1.3.4 FMDV receptor interaction

The virus attaches to cells using integrin receptors $\alpha_v\beta_3$, $\alpha_v\beta_6$ and $\alpha_v\beta_8$. The VP1 protein contains an unusually long and conformationally flexible loop (residues 136 to 157) identified as the VP1 GH loop. This loop has been shown to be the major antigenic site on the virion. It also contains an Arg-Gly-Asp (RGD) motif which is highly conserved in all FMDV serotypes and it is involved in receptor binding (Fox et al. 1989). Removing the loop by treatment with proteolytic enzymes, such as trypsin resulted in reduction of infectivity. The involvement of the RGD motif in cell binding was further implicated by competition experiments utilising RGD-containing synthetic peptides, although high concentrations of peptide were required to achieve inhibition of cell entry (Baxt and Becker 1990). Final confirmation of the essential role the RGD motif plays in receptor binding and cell entry came from Mason *et al*, 1994 who showed that mutation of RGD to RGE resulted in non-infectious particles. They also demonstrated that RGD is required only for entry, as mutated viruses were viable and can replicate after entering the cells by endocytosis as a virus-antibody complex (Mason et al. 1994).

In addition to integrins, tissue culture adapted variants of FMDV are also shown to bind heparan sulphate (HS). In type O FMDV, the ability to bind HS results from a single change in VP3 residue 56. In the field isolate this residue is a histidine which mutates to an arginine in cell culture, resulting in a high affinity HS binding phenotype. Once bound the capsids are internalised by the endocytotic pathway. Unlike other picornaviruses, uncoating of the FMDV genome is triggered by acidification of endosomes (Fry et al. 2005; Ruiz-Sáenz et al. 2009).

1.3.5 Immune Response induced by FMDV

FMDV induces a rapid humoral immune response upon infection, which is considered to confer protection against reinfections of the same or antigenically related viruses. High levels of neutralising antibodies can be detected soon after FMDV infection or vaccination with inactivated FMDV. Serum immunoglobulin M (IgM) is the first neutralising antibody, being detected 3-4 days post-infection or vaccination and reaching a peak between 10 to 14 days after infection before declining. Serum IgGs appear between 4 to 7 days post-infection and become the major neutralising antibodies by two weeks post-immunisation (McCullough et al. 1992; Salt 1993; Windsor et al. 2011). In general, the IgG1 response is greater than that of IgG2. Early upon infection or vaccination, there is a detectable antibody response in secretions of the upper respiratory and gastrointestinal tracts; these initially contain IgM and are followed by IgA and IgG. However, very little is known about the contribution of innate immune responses in infected animals. A typical duration of immunity from vaccination is short lived and lasts for about 6 months in cattle, sheep and pigs in comparison with several years in naturally infected animals (Alexandersen et al. 2003; Salt 1993).

Protection against FMDV has often been correlated with induction of high levels of neutralising antibodies in serum. This has led to the establishment and acceptance of *in vitro* assays such as virus neutralising antibody titres and protection against challenge. This response does not ensure clinical protection and exceptions have been reported in which animals with low serum neutralising titres have been protected whereas animals with acceptable titres were susceptible to infection (McCullough et al. 1992). However, due to the involvement of antibodies in protection against FMDV, as is evident from early protective responses, most immunological studies have mainly focused on humoral responses. More recent studies have suggested that T cell responses also contribute to the immunity to this virus (reviewed in Doel (1996); Sobrino et al. (2001)). T cells have been reported to recognise a number of epitopes located on both structural proteins and non-structural proteins. In cattle and pigs, B-cell activation and antibody production are associated with a lympho-proliferative response mediated by T helper cells CD4+. These helper T cells are likely to be required for long term protective immunity against FMDV. Recent studies have also shown that FMDV infection results in a rapid reduction of MHC class I expression in susceptible cells. Such reduction could impair the presentation of viral peptides to the cytotoxic T lymphocytes by FMDV infected cells. As a result virus can escape from this particular antiviral response route (Sáiz et al. 2002; Sobrino et al. 2001). Therefore, approaches to enhance the T-cell responses in vaccination strategies to produce stronger and long lasting protection against the disease are of increasing interest (McCullough et al. 1992).

1.4 Control of FMD

FMD is one of the most economically important diseases of livestock. It is endemic in many parts of the world, limiting trade of animals and animal products. Controlling the disease is an immensely complex problem due to several factors including high transmissibility, wide host range, antigenic variability and the existence of carrier populations (found mostly in water buffalo population in Africa). Since the various serotypes and subtypes contribute to a large pool of antigenic variants there is very limited or no cross protection between and within serotypes. Another major problem in dealing with the disease is the relatively short lived immunity in vaccinated animals. Several measures have been established to control and eradicate the disease such as the stamping out or slaughter method practiced in the United States and several European countries. This method is adopted by developed countries with a FMD free status. In developing countries and other endemic countries this method is not cost effective; instead the outbreaks are controlled by quarantine and vaccination methods (Bachrach 1968; Parida 2009).

1.4.1 FMD Vaccines

FMD vaccines were among the first animal vaccines to be developed at beginning of the 20th century (Lombard et al. 2007). Before the discovery of vaccines, rational approaches such as deliberately infecting livestock with infectious material, was used to reduce the impact of the disease. Inoculation of healthy animals with the hyperimmune serum was also used. During the same period attenuated live virus vaccines were also used (Sutmoller et al. 2003).

The first inactivated FMDV vaccines were produced in Germany shortly before the Second World War. They comprised virus, obtained from infected cattle, adsorbed onto aluminium hydroxide and inactivated with formaldehyde (Fogedby et al. 1962). The inactivated virus

effectively protected against the disease caused by some serotypes. Although these vaccines helped to reduce the outbreaks, the production capacity was severely limited as large numbers of cattle were required to produce the virus (Bachrach 1968). The industrial productions of inactivated FMD vaccine only began in the 1950s and used the Frankel method, which was based on *in vitro* cultivation of virus in bovine tongue epithelium that had been collected from healthy slaughtered animals and grown in culture medium. The cultivated viruses were inactivated using formaldehyde and used for immunisation. This technique did improve production; however, the capacity still remained insufficient to vaccinate animals on a large scale. In addition, formaldehyde inactivation was not always successful and infectious viruses have been detected for several weeks after inactivation periods of up to 48hrs. Indeed there have been several outbreaks reported which are associated with poorly inactivated vaccines that used formaldehyde inactivation (Barteling and Woortmeyer 1984; Brown 1993; Suttmoller et al. 2003; Wild and Brown 1968). Wild and Brown, 1968 have also shown that formaldehyde affects the immunogenic properties of the capsid and produces a reduced immunogenic response in guinea pigs.

It was not until cell suspension culture technologies were developed in the 1960s that FMDV cultivation started at full industrial scale using Baby hamster kidney (BHK) cells. During this period formaldehyde inactivation was replaced with inactivation using organic compounds based on aziridine (ethylene imine (EI)) such as N-acetyleneimine (AEI) and binary ethylenimine (BEI). Use of AEI containing oil adjuvants as an inactivating agent was found considerably more efficient than the standard formaldehyde method. As AEI is not stable at room temperature, it was later replaced with BEI for routine inactivation. The vaccines prepared using BEI were comparable to AEI and with some strains were much better in generating an immunogenic response compared to the formaldehyde method. BEI is

produced by cyclisation of chloro- or bromoethylamine under alkaline conditions and is mixed with the live virus culture and incubated once or twice at 37°C for 24hrs. BEI inactivation is now a mandatory requirement and used in current commercial vaccine manufacturing (Bahnemann 1975; Rodriguez and Grubman 2009).

In addition to inactivation, purification procedures were also refined. Initially, virus concentration was achieved by adsorption to aluminium hydroxide or by polyethylene glycol precipitations (PEG) which are now replaced by industrial ultra-filtration and chromatographic techniques that produce almost pure antigen by removing not only cellular debris but also non-structural viral proteins. Purified vaccines thus prevent unwanted allergic responses and help in the differentiation of infected versus vaccinated animals. Current vaccines also contain more than one serotype, typically trivalent vaccines are used which contain a mixture of three different serotypes (Rodriguez and Grubman 2009; Uttenthal et al. 2010).

1.4.2 Limitations of Current FMD Vaccines

Chemically inactivated vaccines have been very useful in managing and reducing the impact of FMD outbreaks in many countries. However, despite all the successes, current inactivated vaccines still have many limitations. Not all strains of FMDV respond well to inactivation, especially some SAT2 serotype viruses. Ferris *et al.* 1984 showed that there is marked variation in the quantities of inactivated intact 146S particles, in particular inactivation of SAT2 and Asia-I Turkey with AEI leads to significant reduction of 146S particles (Ferris et al. 1984). In addition, the inactivated vaccines are very sensitive to temperature. The capsids dissociate into pentamers at little above 37°C. This has a major consequence, since one of the most important factors in the potency of inactivated virus vaccines is the integrity of virus

capsids. Several studies have shown that neither the 12S pentamers nor the antigenic peptides are sufficient to generate a strong neutralising antibody response (Brown 1988 ; Doel et al. 1988; Francis et al. 1987). In addition, conventional FMD vaccines require high containment manufacturing facilities which are expensive to maintain, despite which there is a potential for the escape of live virus from these facilities; such an incident led to the 2007 outbreak in the UK in which live virus was leaked from the drainage pipework at the Pirbright facility (Anderson 2008).

1.4.3 Alternative approach to conventional FMD vaccines

A number of strategies have been employed as an alternative approach to conventional vaccines. These have included the use of highly immunodominant protein sites located on the surface of FMDV such as the antigenic VP1 GH loop. Experimental vaccines containing purified VP1 protein from the virus, recombinant VP1, peptides containing VP1 GH loop epitope or chemically synthesised antigenic VP1 peptides have been widely used (Bachrach et al. 1975; Brown 1988). In addition, live vectors expressing VP1 fusion peptides and transgenic plants expressing VP1 have been tested as a vaccine candidate. However, all these approaches have so far proved to only confer partial protection against challenge for livestock (for review see Grubman (2005)). The immunogenicity produced by VP1 protein or synthetic peptides is several orders of magnitude lower than that obtained with conventional vaccines. The lower immunogenicity might be related to incorrect folding adopted by the VP1 protein and synthetic GH loop peptides in solution, reducing the exposure of epitopes to the host immune system. Furthermore, attempts to make attenuated FMD vaccines by serial passaging of FMDV in non-susceptible hosts was also unsuccessful due to the reversion of attenuated virus to virulent forms (reviewed in Parida (2009); Sáiz et al. (2002); Sobrino et al. (2001)).

Another approach has been to use empty virus like particles as vaccines. Empty virus capsids contain all immunogenic sites as virion particles but lack the infectious nucleic acid. They are produced naturally in infected cells and retain most of the immunogenic and antigenic property of native virus but are not infectious. Such empty capsids have been also produced recombinantly using vaccinia and baculovirus expression systems (Grubman et al. 1993; Li et al. 2011). FMDV empty capsids were also expressed in a live replication defective human adenovirus (hAdV5) vector. Inoculation of pigs with this vaccine induced neutralising antibody responses within 7 days post-vaccination and protected animals against challenge (Moraes et al. 2002). However, hAdV5 based vaccines require large doses of virus presumably due to severe instability of the empty capsids. As discussed in section 1.2.4 empty capsids are even more sensitive to temperature and readily dissociate into pentamers. In addition, since the adenovirus infections is widespread in cattle, the efficacy of such hAdV5 derived vaccines may be limited by pre-existing immunity and such approach requires further experiments to validate the use and efficacy of such vaccines. More recently FMDV empty capsids were expressed using baculovirus and silkworm larvae to express FMDV P1 and 2A. The empty capsid vaccines prepared in this manner for two FMDV serotype, Asia-1 and O, produced immunogenic responses in mice and conferred protection in cattle (Li et al. 2011). Similarly, O serotype empty capsids were also assembled using the baculovirus insect cell expression system and were shown to confer protection against challenge in cattle experiments (Mohana Subramanian et al. 2012). However, recombinant expression yielded very limited amounts of intact capsids not suitable for large scale commercial vaccine manufacturing.

Despite intensive research in the field, only intact particles have been shown to produce strong immune responses. However, as a result of extreme sensitivity to temperature and pH, vaccines based on either inactivated or empty FMDV particles have a very limited shelf life, especially in climates with elevated temperatures where it is difficult to maintain cold chains. This is a major limiting factor in vaccine manufacturing and storage. In addition current FMD vaccines do not produce long term protection and require frequent immunisation of animals to maintain a good level of immunity. Furthermore, there is a need for novel vaccines which can provide protection against emerging FMDVs. Overall, despite the availability of vaccines against the most common serotypes these various problems have meant that, to date, it has proved impossible to effectively control the disease on a global scale.

1.5 An Overview of This Thesis

The work in this thesis focuses on developing *in silico* methods and combining them with experimental methods to increase the stability of the FMDV capsids without affecting the antigenic properties of the virus. We used molecular dynamics simulations, molecular modelling, free energy calculations, biochemical and biophysical techniques and X-ray crystallography to design stabilised FMDV capsids, and also to validate the effect of stability and demonstrate that the antigenic properties remain unchanged. The ultimate goal is to be able to produce, relatively cheaply, completely safe, engineered FMDV particles that will form the basis for a new generation of vaccines sufficiently effective to provide hope that the disease might be brought under control.

Chapter 2 showcases the methods designed for the preparation of models used for simulation and describes a simulation protocol for a portion of virus structure. Novel restraints were developed to reduce the noise in simulations containing a very large interface. A list of

stabilised mutants was ranked based on the changes in the binding free energy of the interface. Preparation of these mutants and stability validation is described in chapters 3 and 6 along with the methods for cloning, expression and purification of native and recombinant virus particles of several FMDV serotypes.

In chapters 4 and 5, the crystal structures of recombinant empty wildtype and stabilised mutants are presented along with the methods used for solving such large virus structures. In chapter 4 a novel method of *in-situ* data collection at room temperature and from multiple crystals are described. Phasing and real space non-crystallographic averaging methods are described in chapter 5. The crystal structures showed the mutation to be in the same position as predicted.

In chapter 6, methods used for verifying the stability of mutants are described. We have successfully demonstrated the mutants to be more stable than wild type in the case of both inactivated and novel recombinant empty particles. The stabilised mutants hold the potential to be used as the next generation of vaccines with improved properties.

Chapter 7 describes the formulation of vaccines with the stabilised FMDV particles and its usage as a vaccine in guinea pig and cattle trials followed by conclusion and future work described in chapter 8. We have for the first time reported stabilised vaccines for foot and mouth disease virus which generate a strong and long lasting immunogenic response.

Chapter 2 : Molecular dynamics simulations and capsid stabilisation.

2.1 Introduction

One of the major factors affecting the potency of FMDV vaccines is the intactness of the viral capsids. Attempts to produce alternative vaccines have shown that intact virus particles stimulate the best immune response (Grubman and Baxt 2004). The seven different serotypes of FMDV differ significantly in their capsid stability. The capsids of serotypes A and Asia-1 are relatively more stable compared to other serotypes, with O and SAT (SAT-1, -2 and -3) capsids being extremely sensitive to heat and pH (Doel and Baccarini 1981). The chemical inactivation used for vaccine preparation makes them even more unstable. This has a major impact on current vaccines as it requires cold-chains in order to maintain a shelf life of 6 months or more. This is especially difficult to maintain in the tropical and developing countries in which FMD is often endemic. Even then, serotype O vaccines have a very limited biological half-life, typically only a few months. The situation is worse for the three SAT serotypes which yield vaccines with very low protective capacity, even when administered multiple times per year (Grubman and Baxt 2004).

This chapter provides details on the strategies used for performing molecular dynamics simulations on inter-pentameric interfaces of the FMDV capsid. These simulations facilitated the calculation of the changes in binding free energy (ΔG) for the interactions between interfacial residues, involved in maintaining the integrity of the FMDV capsid. For two of the least stable FMDV serotypes, O1M and SAT2, mutations were modelled at the inter-pentameric interface based on existing three-dimensional FMDV structures. Molecular dynamics simulations and ΔG calculations on the wild type and mutant structures were used to assess the *in silico* stability of the interface. A stable A22 serotype was also used in the simulations. The chapter begins with a general introduction to the methodologies used for molecular dynamics simulations and the calculation of binding free energies, followed by the

specific variations in construction of FMDV models for simulations. Finally, the results from the simulations are discussed.

2.2 Introduction to molecular dynamics

Proteins are dynamic biological macromolecules which experience local and global movements on different time scales. This dynamic behaviour plays a crucial role in regulating their functions. The conformation and dynamics of proteins are encoded in their three dimensional (3D) structures. With the advent of X-ray crystallography and NMR spectroscopy, many 3D structures of proteins have been determined; however, the dynamics of individual molecules as a function of time is more difficult to probe experimentally.

Molecular dynamics (MD) is a computerised simulation technique to model the dynamic behaviour of complex biological macromolecules at the atomic level. It enables the exploration of the conformational energy landscape accessible to protein molecules and provides a link between the static 3D structures and their dynamic properties. The application of MD methodology was first described by Alder and Wainwright in the late 1950s while studying interactions of hard spheres (Alder and Wainwright 1957). In 1974, Stillinger and Rahman showed the first simulation of a realistic system, liquid water (Stillinger and Rahman 1974). The first protein simulations appeared in 1977 when McCammon *et al.* published the dynamics of a folded globular protein, bovine pancreatic trypsin inhibitor (BPTI) by solving the Newtonian equations of motion for the atoms with an empirical potential energy function (McCammon *et al.* 1977). In the last three decades, MD simulations have contributed significantly to the understanding of the structure and functions of biological macromolecules including studies on solvated proteins, protein-protein and protein-nucleic acid interactions, protein folding and conformational changes, protein and

ligand binding, structure based drug design, the computation of binding free energies, membrane protein interactions and enzyme actions (Cheatham and Kollman 2000; Gilson and Zhou 2007; Perez et al. 2008; Rastelli et al. 2010; Taft et al. 2008). Restrained MD simulations also aid in the refinement of structures obtained by X-ray crystallography and NMR (Brunger and Adams 2002).

In MD simulations, the dynamic properties of biological macromolecules are studied in full atomic detail by describing the movement of each atom as a function of time. This is achieved by solving Newton's equations of motion iteratively for each atom.

$$F_i = m_i a_i = m_i \frac{d^2 r_i}{dt^2} \quad (1)$$

Where F_i is the force exerted on particle i ; m_i is the mass of the particle and a_i is the acceleration of the particle. r_i is the position of the particle and t is the time. The force on each atom can also be expressed as the gradient of the potential energy with respect to the position of the atom.

$$F_i = -\frac{dV}{dr_i} \quad (2)$$

Combining these two equations yields,

$$-\frac{dV}{dr_i} = m_i \frac{d^2 r_i}{dt^2} \quad (3)$$

Where V is the potential energy function of the system.

Thus, Newton's equations of motion can now relate the derivative of the potential energy to the change in position of an atom as a function of time. The reliability of simulations depends on the correct use of a potential energy function in the above equations. The potential energy function is a semi-empirical function which contains several parameters for representing bonded and non-bonded interactions, all embedded into an ensemble called the force field and typically has the following form (Ponder and Case 2003):

$$V = V_{bonded} + V_{non-bonded} \quad (4)$$

Where

$$V_{bonded} = V_{bond} + V_{angles} + V_{dihedrals} \quad (5)$$

and

$$V_{non-bonded} = V_{LJ} + V_{Coulomb} \quad (6)$$

The bonded interactions include covalent bonds (V_{bond}), bond angles (V_{angles}) and dihedral energies ($V_{dihedrals}$; torsion energies), whilst the non-bonded interactions occur between atoms which are not linked by covalent bonds.

The non-bonded interactions can be categorised into two classes: (1) Short range van der Waals interactions which are generated by the attractive forces between two atoms as a result of the dipole movement of their electrons, in combination with the repulsive forces induced when the atoms come too close and their electron clouds overlap, resulting in a distance

dependent repulsion. The attractive and repulsive forces can be approximated using the Lennard Jones potential (V_{LJ}). (2) Long range interactions are generated by the electrostatic interactions, defined by the Coulomb potential ($V_{Coulomb}$), between two charged atoms and are believed to be significant over distances of up to 15Å. Long range interactions are represented by the following equation:

$$F = \frac{q_1 q_2}{Dr^2} \quad (7)$$

Where F is the force, q_1 and q_2 are the charges between two atoms, D is the dielectric constant and r is the distance between two atoms.

Non-bonded interactions are much more computationally expensive to calculate because a typical atom interacts with every other atom in the system. As a result, a cut-off is used to speed up the calculation, enabling atom pairs whose distances are outside the cut-off radius to have non-bonded interaction energies of 0. All these parameterisations are employed in several different force fields such as AMBER, GROMOS and CHARMM (Ponder and Case 2003).

Therefore, to calculate the trajectory, MD simulations require initial atomic coordinates, and distribution of velocities and acceleration of atoms, which is determined by the potential energy function. The protein coordinates can be obtained from X-ray crystallography, NMR spectroscopy or computational structure homology modelling. Initial velocities are randomly assigned to each atom based on the Maxwell-Boltzmann distribution corresponding to the temperature at which the MD simulation is performed.

MD simulation has become a very useful tool in structure and functional biology to understand the dynamic properties of biological macromolecules. However, like X-ray crystallography and NMR, it suffers several serious limitations. The time scale is one of the main limitations of this method. Computationally, MD is a very expensive method, and depending on the size of the molecule the simulation times can be limited from hundreds of nanoseconds to a few microseconds. For example, the protein interface discussed later in this chapter takes about 2 days to compute a 1ns trajectory using 8 cores, AMD Opteron 6174 at 2.2 GHz. This is too short to study events such as conformational changes in proteins and protein folding which occur over time scales ranging from milliseconds to seconds. In addition, MD methods are very difficult to apply to quantum-mechanical phenomena such as electron transfer, bond formation and bond breaking, as the method is based on classical descriptions of the particles. Although a few methods exist which combine both quantum mechanics and molecular mechanics (QM/MM) to treat phenomena such as electron transfer, they are computationally very demanding and restricted to a few picoseconds (Senn and Thiel 2009).

2.3 MD method

MD simulations describe the time dependent behaviour of the biological macromolecule based on Newton's equations of motion and the potential energy function, i.e. force field. The result of such a simulation is a trajectory showing a time dependent conformational change in a macromolecule. The simulation begins with the choice of initial coordinates of the system, generally taken from an X-ray crystal structure or an NMR structure. The steps leading to the simulation and analysis of trajectories are described in Figure 2-1.

Prior to starting an MD simulation, hydrogen atoms are added to the model and surface charge is neutralised by adding the counter ions. The system is then solvated in a periodic box of water and minimised. The initial velocity is assigned by a process called heating and the system is equilibrated. This is followed by the final production simulation. The trajectory from the production simulation can be used for biophysical analyses of the system such as its thermodynamics.

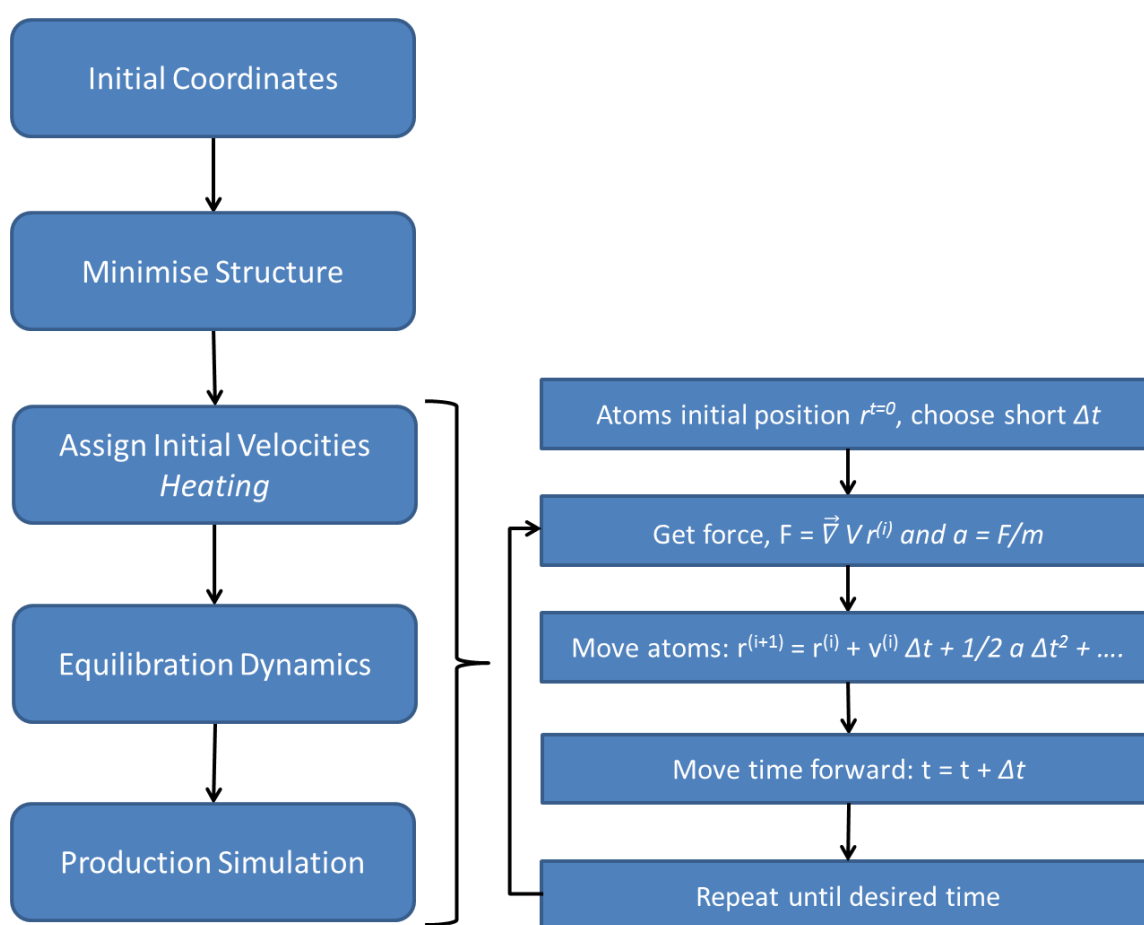


Figure 2-1: MD simulation work flow. MD simulations describe the time dependent conformational change of the macromolecule. The process begins with a set of initial coordinates to which the velocities are applied and the forces are calculated, based on iterative solving of the Newton's equations of motion and force field function, leading to a MD trajectory.

2.3.1 Periodic boundary condition

The earliest system used proteins and other molecules as single, isolated entities and simulations were performed essentially in a vacuum (Karplus and McCammon 2002). Nowadays, with the increase in computing power and to make the simulations realistic, an explicit solvent is used in the simulation. The model (solute) is immersed in a bulk solvent system, a buffer of water in this case. Such a system requires a very large number of solvent molecules to be included with the solute and becomes computationally expensive. One approach would be to put the solute in a small box of water such that all atoms of the solute are fully covered with solvent. However, simply putting a solute in a box of water is not sufficient because the bulk of the solvent will be at the edge of the box which is surrounded by vacuum. This leads to the boundary effect where the outer solvent molecules start boiling off into the surrounding space. To prevent such a boiling effect and to produce the bulk solvent-like effect using relatively few solvent molecules, periodic boundary conditions are employed. This implies that a central simulation box is repeated infinitely in all directions. The atoms that are outside the central box are simply the images of the atoms that are simulated. In such a system, when an atom drifts outside the simulation box, another atom will appear on the opposite side of the box (Figure 2-2).

In such a system, the minimum-image convention makes sure that the interactions between atoms are not duplicated. Only one pairwise interaction for each pair of atoms is counted. Although such a system would appear to be very computationally intensive, it is possible to calculate the infinite electrostatics using the Particle Mesh Ewald (PME) summation method where the calculation is divided into a real space component and a reciprocal space component and specified by VDW cutoff (Adcock and McCammon 2006; Allen 2004).

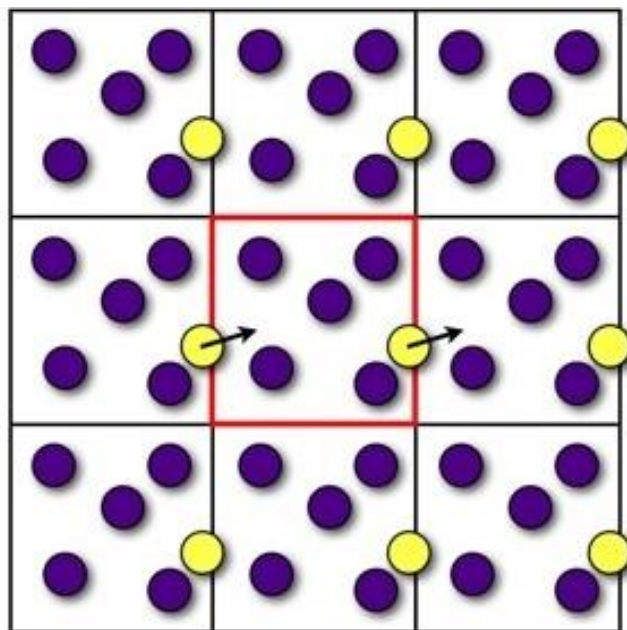


Figure 2-2: Periodic boundary condition. In a periodic boundary system, as a solvent molecule moves out of the central simulation box, an image of the molecule moves into replace it. Figure adapted from Allen (2004).

2.3.2 Energy minimisation

Before running the MD simulation, the model needs to be energy minimised. The geometry of the model does not always match well to the actual force field in use. In addition, there may be overlaps with solvent, steric clashes due to addition of hydrogen atoms and strong VDW interactions. Energy minimisation removes any such strong interactions and clashes and allows the solvent molecules to re-adjust around the protein molecule. If the system is not properly energy minimised, it can lead to local distortion and can result in an unstable simulation.

2.3.3 Heating the system

In MD simulations, initial velocities are assigned to each atom before Newton's equations of motion are integrated. The initial velocities are assigned at low temperature and the simulation started. With each time step, the new velocities are scaled to a slightly higher temperature. The process is repeated until the desired temperature is reached. Several algorithms such as the Berendsen method, Brownian dynamics and Langevin dynamics can be used to heat the system (Hünenberger 2005; Wu and Brooks 2003). Discussing all these methods is out of the scope of this thesis; however, for the work described in this thesis we used Langevin dynamics to regulate the thermostat. The equation for Langevin dynamics is similar to Newtonian dynamics; however, it contains two additional artificial terms, the frictional term and the random force term and takes the following form:

$$m_i \frac{d^2 r_i}{dt^2} = F_i - \zeta_i \frac{dr}{dt} + R_i(t) \quad (8)$$

Where ζ is a frictional constant and $R_i(t)$ is the random force term.

The Langevin dynamics method is significantly more efficient than other methods at equilibrating the system temperature and offers improved conformational sampling by keeping the temperature constant throughout the system (Adcock and McCammon 2006; Wu and Brooks 2003).

2.3.4 Equilibration

After the system is heated, the simulation is continued for about 500ps to 1ns to allow the protein, counter ions and water molecules to settle at the desired temperature. During this stage several properties such as temperature, potential energy, kinetic energy, and total energy and RMS deviation of the system from the starting model (crystallographic structure) are monitored. If there are any unexpected changes, such as a large fluctuation in total energy or a sudden increase or decrease in temperature, the velocities can be rescaled. The point of performing an equilibration run is to make sure that all the properties in the simulation become stable.

2.3.5 Production simulation

The final simulation step is called the production phase. In the production phase the system is allowed to evolve until the desired length of time is reached. The length of time can be from several hundred picoseconds to several nanoseconds. It is from the production phase of the simulation that biophysical properties such as the binding free energy can be calculated.

2.4 Binding free energy calculations

The key features of biological processes are the ability of macromolecules to bind to each other and to different ligands and small molecules to form macromolecular complexes. These features depend on the structure and energetics of the molecular complexes and are largely governed by the binding free energy of the complexes. The binding free energy is the amount of energy required to separate a protein or ligand from a solvated complex made from protein-protein or protein-ligand interactions into two separate solvated components. A complex has a lower potential energy than its constituent parts which is what keeps the complex together. In thermodynamic terms, this means energy is released upon the formation of a complex and hence corresponds to the negative binding free energy.

The free energy calculations are based on estimating the relative free energy differences (ΔG) between two states. In thermodynamics, the free energy is represented as Gibbs free energy in the following form:

$$\Delta G = \Delta H - T\Delta S \quad (9)$$

Where ΔG is change in Gibbs free energy; ΔH is enthalpy change (internal energy of the system), T is absolute temperature and ΔS is entropy change (measure of disorder in the system).

Therefore, ΔG is a measure of net change in the total energy between two states of the system (bound vs. unbound). If the ΔG of complexation is negative the binding is favoured and if the ΔG is positive, the complex tends to disassemble. At equilibrium ΔG is equal to 0.

Numerous methods have been developed for computing the binding free energies of protein-protein and protein-ligand interactions. Some of the most popular methods include free-energy perturbation (FEP), thermodynamic integration (TI), umbrella sampling and MM-PBSA. Discussing all these methods are beyond the scope of this thesis. However, as we used the MM-PBSA method to calculate relative binding free energy within the AMBER package, an introduction to the MM-PBSA method is discussed (Kollman et al. 2000; Meirovitch 2007; van Gunsteren and Berendsen 1987; Zwanzig 1954).

The MM-PBSA method is the most widely used method for estimating the relative binding free energy of protein-protein or protein-ligand complexes. In this method, the bonded and non-bonded interactions are expressed by molecular mechanics (MM), the polar solvation free energy (i.e. the interaction of molecules with solvent) is calculated using the Poisson Boltzmann (PB) or Generalised Born (GB) model and the non-polar part using the solvent accessible surface area (SASA) method (Kollman et al. 2000; Srinivasan et al. 1998; J. Wang et al. 2006). The binding free energy between two solvated molecules is calculated using the following equations:

$$\Delta G = \Delta H - T\Delta S \cong \Delta E_{MM} + \Delta G_{sol} - T\Delta S \quad (10)$$

Where

$$\Delta E_{MM} = \Delta E_{internal} + \Delta E_{electrostatics} + \Delta E_{vdw} \quad (11)$$

And

$$\Delta G_{sol} = \Delta G_{polar} + \Delta G_{non-polar} \quad (12)$$

ΔE_{MM} is the internal energy of the system and it is calculated using the gas phase energies from MM, ΔG_{sol} is the solvation free energy and $-T\Delta S$ represents the entropy change upon binding. ΔE_{MM} is the sum of bonded energies ($\Delta E_{internal}$) which includes energies from bond-stretching, angle-bending and torsion energies; other components include electrostatic interactions ($\Delta E_{electrostatics}$) from charged atoms described by the Coulomb potential as shown

in equation 7 above and energies from van der Waals (vdw) interactions (ΔE_{vdw}). ΔG_{sol} is the sum of polar (ΔG_{polar}) electrostatics and non-polar ($\Delta G_{non-polar}$) contribution to the solvation free energy. The polar contribution is calculated using the PB/GB method and the non-polar contribution is estimated by SASA. The entropy change $-T\Delta S$ is calculated using normal mode analysis. All these calculations are iteratively performed on a set of snapshots taken from MD trajectories to get the statistically significant ΔG_{bind} and an estimate of the error (Gohlke et al. 2003; Hou et al. 2011).

The free energy of binding can then be calculated by subtracting the ensemble average of the free energy of free proteins or ligands from the free energy of the complex using the following equation:

$$\Delta G_{bind} = \langle \Delta G_{complex} \rangle - \langle \Delta G_{protein-1} \rangle - \langle \Delta G_{protein-2} \rangle \quad (13)$$

The ensemble averages, $\langle \rangle$ are computed from the snapshots taken from the MD trajectories.

2.5 Models for simulation

Model preparation and visual analysis used COOT (Emsley and Cowtan 2004) and PyMOL (Schrodinger 2010) along with some custom scripts for generating symmetry-related molecules and detecting bad clashes. The creation of trimmed structures and dummy atoms for restraints used the program SELECT3 (Esnouf, unpublished). All simulations were performed using the AMBER (Assisted Model Building with Energy Refinement) suite of programs.

2.6 Preparation of reduced models for simulation

As picornavirus capsids contain a very large number of atoms, it would have been computationally very expensive to simulate the whole capsid. A program SELECT3 was developed by Robert Esnouf to prepare trimmed models with a set of dummy atoms placed at the centre along the interface of interest. The program reads in two PDB files describing the molecules on either side of the inter-pentameric interface and a third file describing crystallographic waters. First, SELECT3 detects all inter-pentamer interactions (inter-atomic distances across the interface of $< 4\text{\AA}$) and creates a list of “dummy” atoms that are placed at the midpoint of the interface. A reduced model is then generated which contains all atoms that are within the radius of $13\text{\AA}$ from the interface. Isolated amino acids are filtered out since AMBER does not have a definition for a single amino-acid force field and the remaining selected residues are written out in AMBER compatible PDB format (with appropriate N- and C-terminal modifications and TER cards) ready for simulations. The water atom list is similarly trimmed and written out in AMBER PDB format.

2.7 Design and construction of candidate mutants

For each truncated FMDV model, the amino acids contributing to the inter-pentameric interface (around the capsid 2-fold symmetry axis) were identified using the EBI-PISA web server. Visual analysis of the interactions made by these residues (using COOT and PyMOL) facilitated the rational design of a panel of putative stabilizing interactions that might improve hydrophobic and/or electrostatic interactions across the interface. These mutations were incorporated into the trimmed models using COOT by simple mutation and minimization of the mutant residues. Any clashing water molecules were simply deleted.

2.8 Defining restraints using dummy atoms

A set of dummy atoms placed along the centre of the interface was used to define positional restraints on the atoms around the inter-pentameric interface during simulation. Dummy atoms (in residue “DUM”, of name “DU” and of type “Du”) were defined based on the existing definitions for neon atoms. These atoms are heavy (atomic weight 2000), form no bonds and make no non-bonded interactions with other atoms. The definitions were included into AMBER readable format in files, *dummy.lib* and *frcmod.dum*, and listed in Appendix-I.

2.9 Simulation protocol

The truncated models for wild type FMDV serotypes and the mutants were simulated using the AMBER 10 package. The program TLEAP was used to add hydrogen atoms and counter ions. The system was then solvated into a rectangular box of TIP3P (Price and Brooks 2004) water molecules such that all atoms in the starting model are no less 10Å from the edge of the water box. AMBER specific topology and parameter files were then created based on the AMBER Parm99SB force field (Case et al. 2005; Curry et al. 1995). Before the production simulation, a stepwise minimisation was performed. This was followed by a gradual heating of the system from 0K to 310K over 50ps. Minimizations, heating and simulations were all performed using the SANDER (Simulated Annealing with NMR-Derived Energy Restraints) module. Dynamic simulations were performed at constant volume (NVT) ensemble with an integration time-step of 2fs. The temperature was controlled by Langevin dynamics with a collision frequency, γ , of 2.0ps^{-1} . PTRAJ was used for plotting the trajectory and monitoring simulations. The protocol consisted of five stages and typical command lines and control scripts are shown in Appendix-I.

Step 1: Minimization of the solvent keeping all non-water atoms effectively fixed (restraint weight 500 kcal.mol⁻¹). Up to 3500 steps of steepest descent followed by 1500 steps of conjugate gradient minimization are performed.

Step 2: A further minimization of the solvent and counter ions keeping all protein (and dummy) atoms effectively fixed (restraint weight 500 kcal.mol⁻¹). Up to 2500 steps of steepest descent followed by 2500 steps of conjugate gradient minimization are performed.

Step 3: A final minimization of all atoms (except dummy atoms) under the influence of the dummy-atom derived restraints (restraint weight 50 kcal.mol⁻¹ for atoms >10Å from any dummy atom). Up to 10000 steps of steepest descent minimization are performed. The output coordinate set from this minimization was used as the reference coordinate set for the restraints that were applied to the dynamic stages.

Step 4: The simulation model is heated gradually to 310K over a period of 50ps (25000 simulation steps of 2fs) with restraints derived from the output of Step 3 (restraint weight 50 kcal.mol⁻¹ for atoms >10Å from any dummy atom). The use of dummy atoms allowed efficient definition of restraints in AMBER syntax:

$$\text{restraintmask} = ' (:DUM >@10.0) | :DUM ' \quad (14)$$

Step 5: The equilibration and production runs comprised a total of 1.55ns (775000 simulation steps of 2fs) with restraints derived from the output of Step 3 (restraint weight 50 kcal.mol⁻¹ for atoms >10Å from any dummy atom) and the temperature restrained to 310K. The SHAKE algorithm was used to constrain the lengths of bonds involving hydrogen atoms.

2.10 Binding free energy

Calculation of the binding free energy, ΔG_{bind} , between two protomers (on either side of the inter-pentameric interface) to form a dimer was performed using the Molecular Mechanics Poisson Boltzmann/Generalized Born Surface Area (MM-PB(GB)SA) approach embedded in the MM-PBSA module of AMBER. A snapshot of the trajectory was taken at every 10ps during the final 1ns of each simulation and the binding free energy was calculated as the mean of these 100 snapshots to give an improved value and allow an estimate of the error. Finally, the difference in binding free energy, $\Delta\Delta G$, between the respective candidate mutant truncated model and the parent wild-type reduced model was calculated to assess the stability of each mutant.

2.11 Results and Discussion

As discussed in Chapter 1, the FMDV capsid dissociates into pentameric subunits at pH < 7.0 and elevated temperatures. Therefore, the simplest approach to stabilise the capsids would be to stabilise the inter-pentameric interfaces by promoting electrostatic and/or hydrophobic interactions. Designing, making and evaluating mutant viruses experimentally is time consuming, expensive and difficult. As a result, we devised an *in silico* MD simulation protocol for the rapid evaluation of potential mutant viruses which has been shown to correlate well with subsequent experimental evaluation (discussed later).

2.11.1 FMDV models for simulation

Several X-ray crystallographic structures of FMDV capsids have been determined and are available from the RCSB database. Wild-type models were constructed for FMDV strains O1BFS (PDB ID: 1BBT, 2.60 Å), A22 (4GH4, 3.0 Å), SAT1 (PDB ID: 2WZR, 3.0 Å) and CGer (PDB ID: 1FMD) by expanding the crystallographic and non-crystallographic

symmetry to generate a dimer interface. Currently, there are no crystallographic structures for either the O1M or SAT2 serotypes. Therefore, O1BFS was used as a representative model for the O1M serotype (the O field strain predominantly used in current vaccines), and the SAT1 structure for SAT2. The amino acid sequence identity between VP2 proteins of O1BFS and O1M is 98% and between SAT1 and SAT2 is 78% respectively. The crystallographic structures of O1BFS and SAT1 were determined some time ago and/or were only of moderate resolution; as a result careful preparation was required to make suitable starting models. The models were corrected for missing side chain atoms and symmetry clashes between subunits and water molecules. For the SAT1 serotype, 12 N-terminal residues of VP2 were poorly resolved in the structure and were simply removed as these were distant from the interface.

The usual approach to simulations is to perform large biologically relevant simulations of intact proteins. However, this was not computationally feasible because of the very large number of atoms in the FMDV capsids. Simulating a pair of pentamers was also time consuming, even simulating two protomers that are directly contributing to the interface took about one week on an 8 core machine (Figure 2-3). In addition, simulating such a large complex would generate significant artefacts, making it very difficult to observe the small changes in binding free energy of the interface.

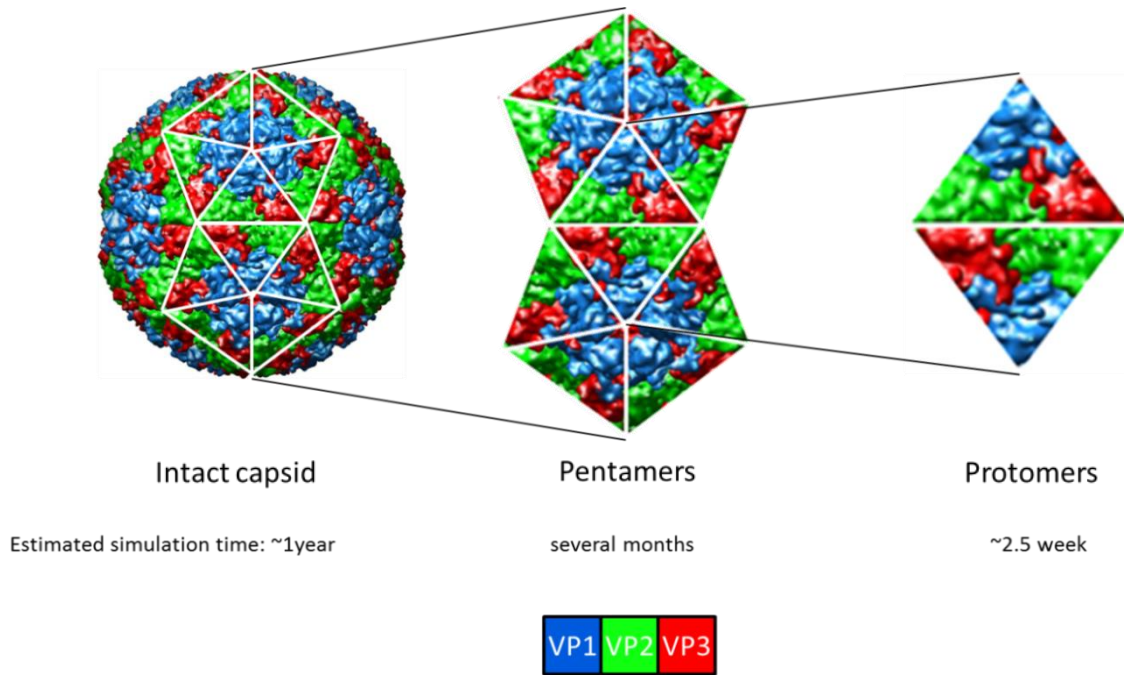


Figure 2-3: Schematics of FMDV capsid structure. FMDV capsid structure and its components showing the association of two pentamers and the main dimer interaction by two protomers of the inter-pentameric interface. In a simplest approach, stabilising the dimer interface on the right should ultimately lead to increase in the stability of virus capsids.

Looking at the structure of the FMDV capsid, it is clear that the VP2 and VP3 proteins contribute directly at the inter-pentameric interface. VP1 is distal and decorates the 5-fold axis. Studies by Curry et al. (1995); Twomey et al. (1995); van Vlijmen et al. (1998) have shown that only residues within a 15Å radius of the interface contribute to the acid lability of the FMDV interface. Therefore, to reduce the time taken to perform simulations, the protomers were further trimmed producing a reduced model (Figure 2-4).

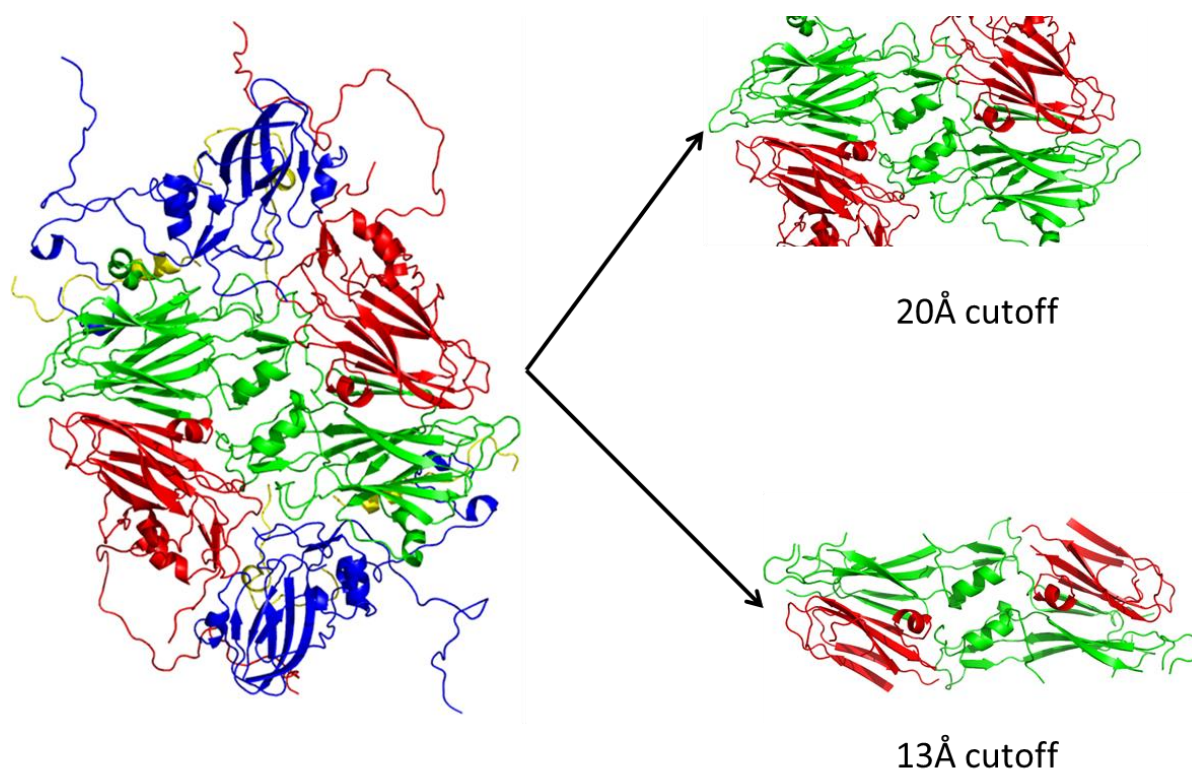


Figure 2-4: Truncated models for simulation. Simulating intact capsids would be computationally very demanding. In addition, they would generate a very low signal to noise ratio due to the large number of atoms associated with the FMDV capsid. To increase the efficiency and reduce the simulation times, hypothetical reduced models were generated by trimming the dimer that forms a 2-fold interface. The respective models that include atoms within a 20Å and a 13Å radius from the interface, generated using the SELECT3 program, are shown above.

The truncated models were generated using a custom script, SELECT3, which takes two protomers on either side of the interface and crystallographic waters. The output from SELECT3 is an AMBER-style PDB file containing amino acids with a desired cut-off from the centre of the interface. Initially models used three different cut-offs, 20Å, 15Å and 13Å. To perform the complete process of minimisation, heating, equilibration and production simulation of 1ns took 56hr, 42hr and 29hr respectively at 310K on an 8 core computer using these three types of model. These models differ significantly from the biological structure

unit and assume that only those residues directly at the interface contribute to the stability of the capsids.

To further improve the efficiency and increase the signal to noise ratio for binding energy calculation, a novel protocol was devised in which the 13Å model was simulated with extensive restraints. This protocol was based on “dummy” atoms being placed at the midpoint of inter-protomeric interactions around the capsid 2-fold axis. A set of restraints were then defined such that the atoms within a 10Å radius of the dummy atoms were allowed to move freely and the atoms outside this radius, and the dummy atoms themselves, were fixed.

The advantages of using such a protocol are multiple and include (a) a further reduction in the time taken to perform a simulation and (b) a substantial reduction in the effect of simulation artefacts on the calculations. Moreover, the system also better mimics the biological dimer interface, as the effect from the missing residues is compensated to some extent by restraints. As it can be seen from Figure 2-5, RMS deviation, from the starting structure, of the non-restraint 1ns simulation are large compared to the restraint simulations.

A complete simulation including minimisation, heating, equilibration and 1ns production simulation used to calculate binding free energy took 25hr and 21hr on a 8 core (AMD Opteron 6174 at 2.2GHz) and 12 core (Intel Xeon X5675 at 3.07GHz) machines, respectively.

Such a restraint system however has its own limitations. The mutants can only be sampled around the 2-fold axis. Attempts were made to extend the sampling to the 3-fold region; however, we were not successful in reproducing the results. The reason for the failure can be attributed to the fact that the loops at the 3-fold axis were disordered and not clearly refined in the crystal structures. Nevertheless, (X. Wang et al. 2012) have recently showed that the helix at the 2-fold axis expands significantly to create a hole at the 2-fold axis, confirming that this portion of the interface is likely to be critical to the stability.

There is also a growing body of evidence from electron microscopic studies that the 2-fold region may be important for genome release in enteroviruses (Bostina et al. 2011). It is still not clear if the mechanism is the same in FMDV and/or other picornaviruses, but it is likely to be similar.

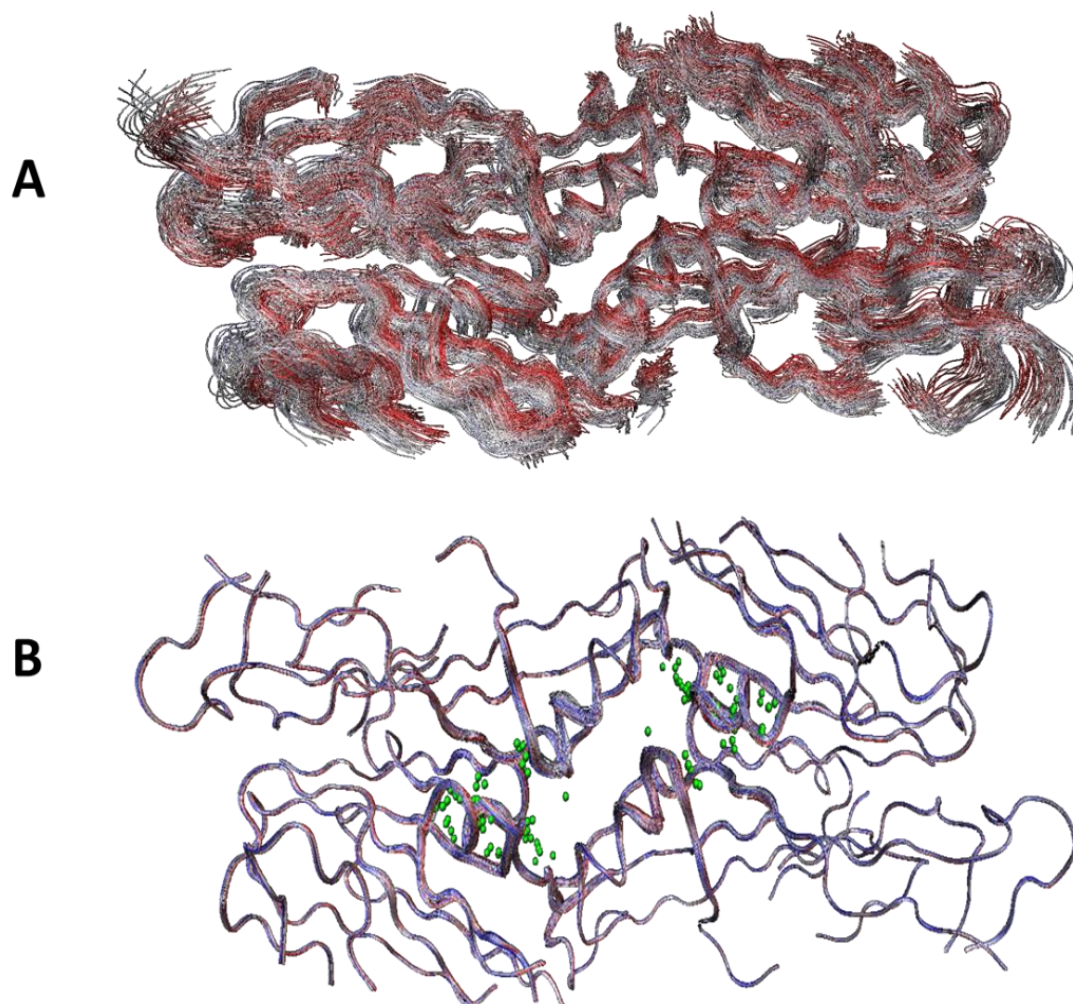


Figure 2-5: 1ns trajectory of non-restraint and restraint simulations on O-serotype model, O1BFS. (A) Simulation performed on a non-restraint truncated model showed a very large RMS deviation from the starting structure especially at the termini from where the models are trimmed. This can generate simulation artefacts leading to increasing inaccuracies in measurements. (B) The artefacts can be reduced by using the procedure developed by us, involving the use of restraints defined by a set of dummy atoms (green) as reference points; The dummy atoms are placed at the midpoint of the inter-protomeric interface around the 2-fold axis to define the restraints. Starting models are shown in red and the deviations in grey.

2.11.2 MD simulations

All simulations in this thesis were performed using the AMBER10 package. There are essentially two methods to treat the solvent in AMBER. The first is an implicit solvent model which uses an approximation to account for solvent effect. Instead of using a solvent box, the solvent effect is included within the force field equation.

The second is a more reliable method which uses an explicit solvent model in which the contribution from each water molecule is used in the calculation. All simulations in this thesis were performed using the explicit solvent model. The hydrogen atoms were added using a TLEAP program, as crystallographic structures often lack hydrogen atoms because the hydrogens scatter X-rays very weakly. TLEAP was also used to add the counter ions (in this case 18 Na⁺ ions) to neutralise the surface charge. The model was then immersed into a rectangular box of TIP3P water molecules extending to 10Å beyond the nearest protein atom using the Ambertool, Solvate program (Figure 2-6).

The Solvate program superimposes a box of water of the desired dimensions on the model (solute) and then subtracts the overlapping waters. This creates a vdW void at the solute and solvent interface. As a result, extensive minimisation and equilibration is required before performing the production simulation used to calculate the binding free energy. It is the interaction of solute with the bulk solvent that makes the computation very demanding.

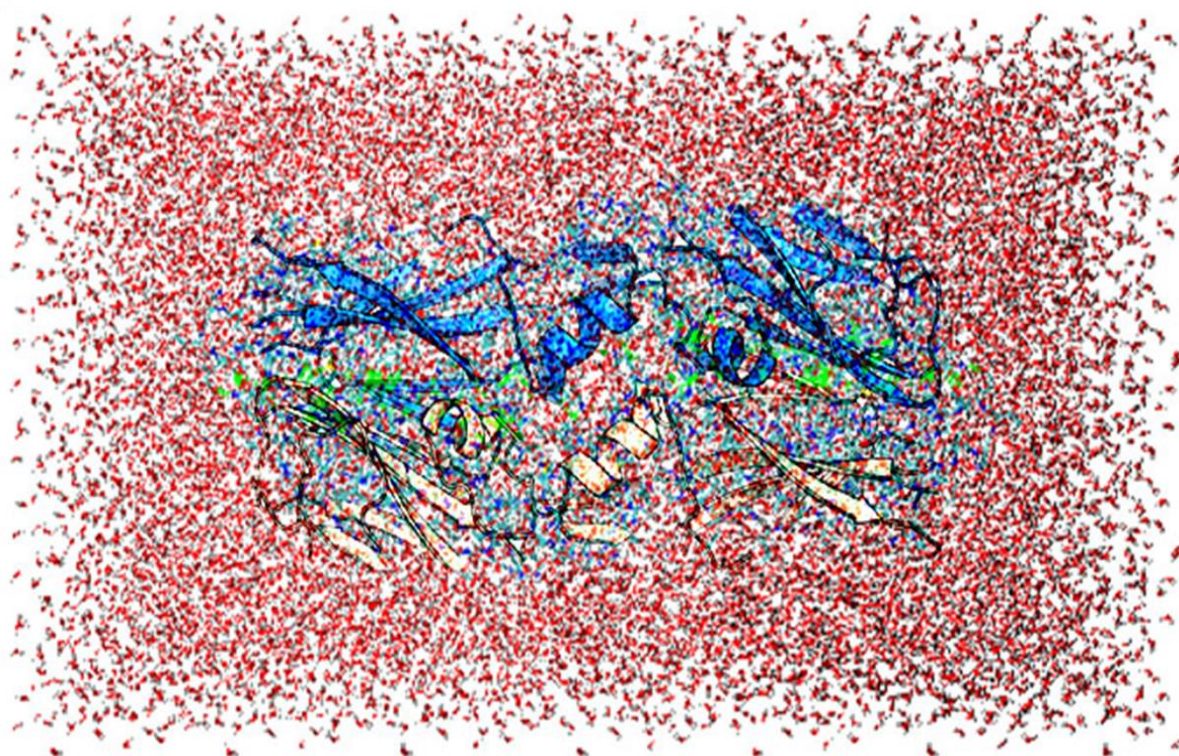


Figure 2-6: FMDV model solvated in a 10Å rectangular box of water. All simulations in this thesis were performed using the explicit solvent method. The FMDV model was immersed in a 10Å rectangular box of TIP3P water molecules.

Before starting simulations the models containing hydrogen atoms and water molecules were energy minimised. Energy minimisation is used to compute the equilibrium configuration of the molecules and to move the atoms based on the geometry specified by the force field, reducing the potential energy of the system. All the steps from minimisation to the production simulation were performed using the SANDER program.

The SANDER program supports a number of different algorithms for energy minimisation. Here we have followed the most commonly used approach of combining two different algorithms, the steepest decent and conjugate gradient methods. The steepest decent method is numerically very stable and good at efficiently removing the largest strains, such as strong vdw interactions or side chain clashes; however, it converges very slowly. In comparison, the conjugate gradient method is efficient but potentially unstable with potential energy functions. Thus, a combination of both methods was used.

The system was minimised in three stages as described in the methods section. First, the water molecules were minimised, keeping the proteins and ions heavily restrained. This was followed by further minimisation of the water molecules and ions, keeping the protein atoms restrained. Lastly, all atoms except for the dummy atoms were minimized.

The system was heated to 310K (37°C) over 50ps and equilibrated for 550ps at constant temperature molecular dynamics. Lastly, a production simulation was performed for 1ns. The trajectory for the entire process was analysed. Parameters such as temperature, RMS deviation from the starting structure and changes in energy were found to be stable (Figure 2-7). The process was repeated for all mutants.

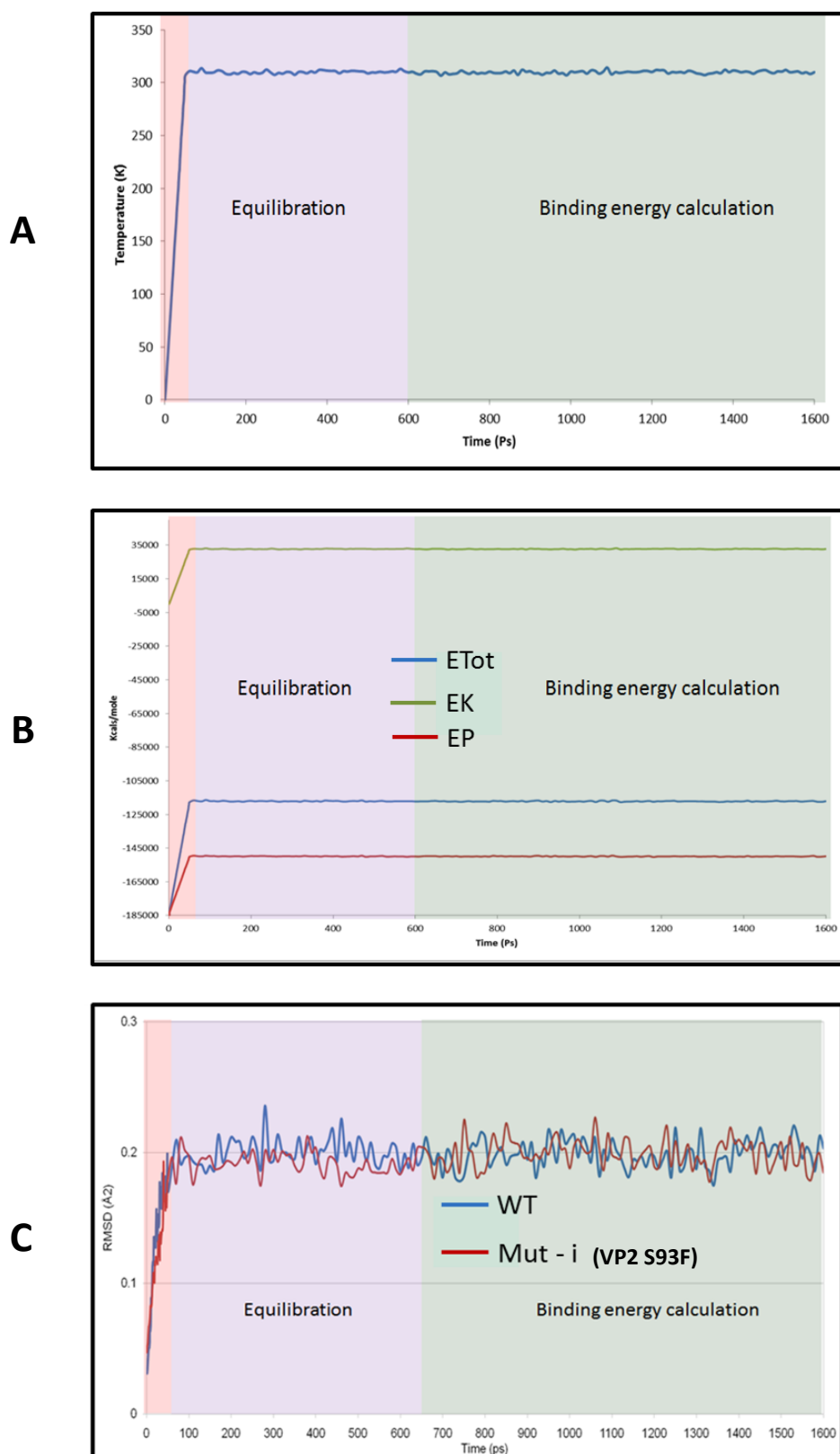
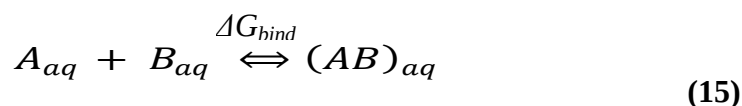


Figure 2-7: Simulation stability. Several simulation parameters were monitored from the trajectory (A) Temperature, (B) Energies and (C) RMS deviation from the starting model. The behaviour of wildtype (WT) and a stable mutant (Mut – i: VP2 S93F) models for O-serotype, O1BFS, are shown. All parameters were found to be stable.

2.11.3 Binding free energy

The binding free energy (ΔG) was calculated using the MM-PBSA module of the AMBER package. Preferably, we would have liked to calculate the binding free energy directly from the following equation:



However, such a simulation of a solvated system would have the majority of the energy contribution coming from solvent-solvent interactions, which would have significantly affected the ΔG calculations between two protomers. In the MM-PBSA method, the binding free energy of a complex is calculated using the following thermodynamic cycle:

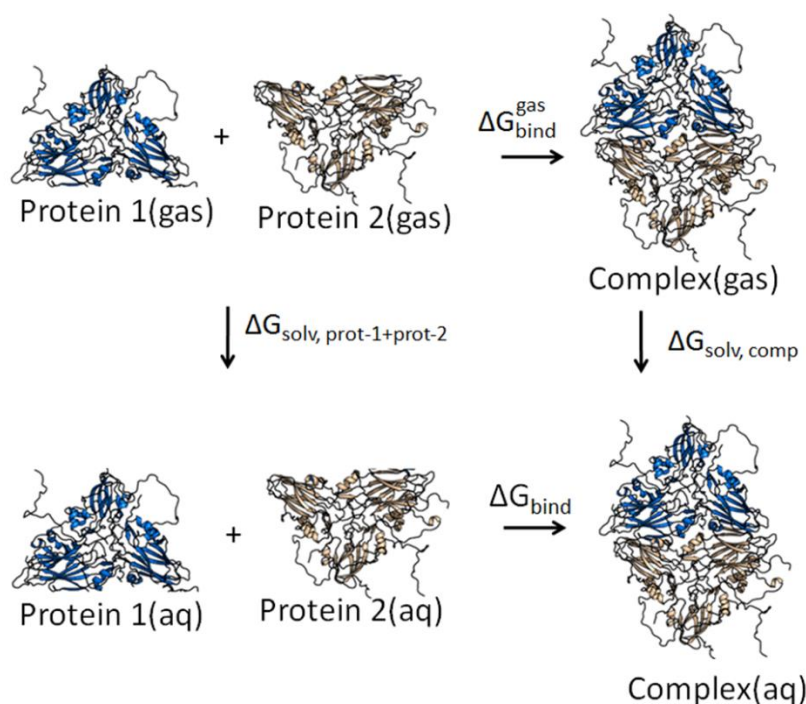


Figure 2-8: Thermodynamic cycle used to calculate the binding free energy. In the MM-PBSA method the binding free energy is calculated by dividing the energy terms into gas phase and solvation energies. The method not only reduces the required computational times, but also significantly reduces noise leading to more accurate estimations of ΔG .

From the thermodynamic cycle shown above, the binding free energy is decomposed into the gas phase binding energy plus the difference between the free energy change of solvation of the complex and the respective protomers as shown below:

$$\Delta G_{bind} = \Delta G_{bind}^{gas} + \Delta G_{solv, comp} - \Delta G_{solv, prot1, prot2} \quad (16)$$

The internal energy and the solvation free energy were calculated using equations 11 and 12. Structures of FMDV O1BFS, SAT1, A22 and CGer were simulated for 1ns and a snapshot of the trajectory was taken every 10ps. The binding free energy was calculated from each snapshot using the above equations and then averaged. In MM-PBSA program averages can be calculated using two methods. In the first method the averages are calculated from three different simulations, one for each component (complex and individual proteins). However, to further reduce the noise only the complex was simulated and the snapshots for all three species were obtained from a single trajectory. This method is based on the assumption that no significant conformational changes occur upon binding and has been commonly used for such calculations (Foloppe and Hubbard 2006). A structure of type 1 poliovirus was also used in simulation. Poliovirus and FMDV A22 are naturally more stable and hence they were used as positive controls for these simulations.

In addition to binding free energy, the surface electrostatics for each model was calculated using the COOT program. The results for the binding free energy and the surface electrostatic properties are shown in Figure 2-9.

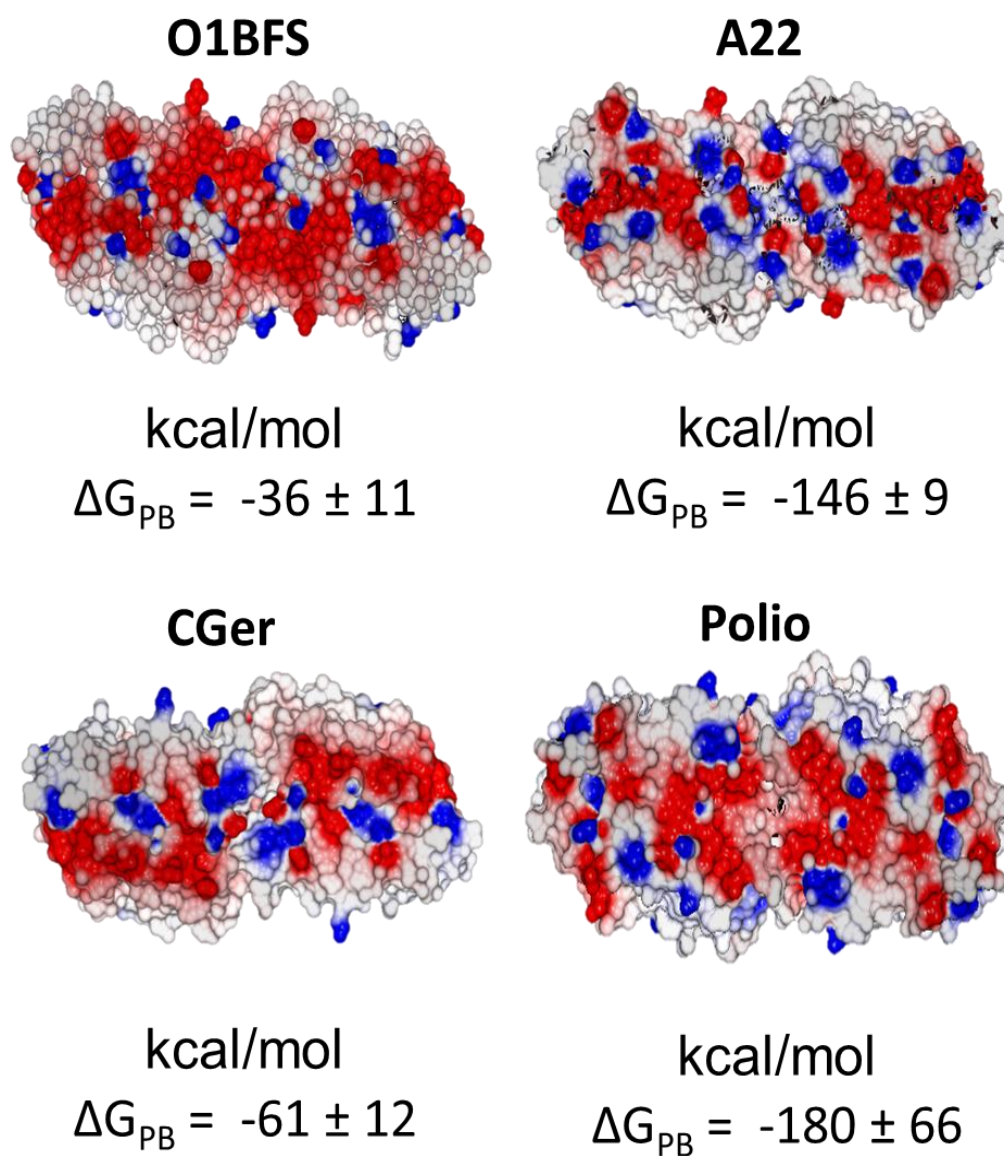


Figure 2-9: Electrostatic surface and binding free energy. Modelling the surface electrostatics showed that the O serotype is very negatively charged at the interface, barely maintaining capsid integrity. In A22, CGer and Polio, the charges are uniformly distributed across the interface. These findings are also reflected in the estimated binding free energies derived from simulations.

It was immediately clear that the interface of O1BFS was very negatively charged whereas with the A22, CGer and Polio interfaces, the charges were uniformly distributed. This was also reflected in the respective binding free energies, ΔG_{PB} . O1BFS at -36 kcal/mol is less stable than other serotypes (the negative value represents favourable binding). However, it should be mentioned that ΔG values may not be directly comparable between structures, as the simulations and ΔG calculations heavily rely on features of the models and the models are significantly different from each other. Nevertheless, it is clear that the charge distributions at the interface play important roles in maintaining the integrity of the capsids. In addition, it was noticed that A22, and especially Polio virus, contained hydrophobic residues at the interface, whereas O1BFS was filled with polar charged residues. In general, the interfaces of FMDV serotypes were found to contain more polar charged residues in comparison to other picornaviruses.

Calculated binding free energies are between protein-protein complexes in pure water, but one has to keep in mind that these results do not equate to the real binding free energies, since we are not using the entropic contributions to the binding. However, entropic contributions can be neglected because they cancel out when comparing small changes between the same structures.

2.11.4 Candidate mutants

Stabilisation can be achieved by two different methods; a covalent modification at the 2-fold such as disulphide bonds across the interface, or by promoting non-covalent interactions such as hydrophobic or electrostatic interactions. Covalent modifications will be discussed later.

Three different strategies were used to rationally design the putative stabilising mutations. First, the amino acid residues contributing directly to the interface were identified using the EBI-PISA webserver (Figure 2-10).

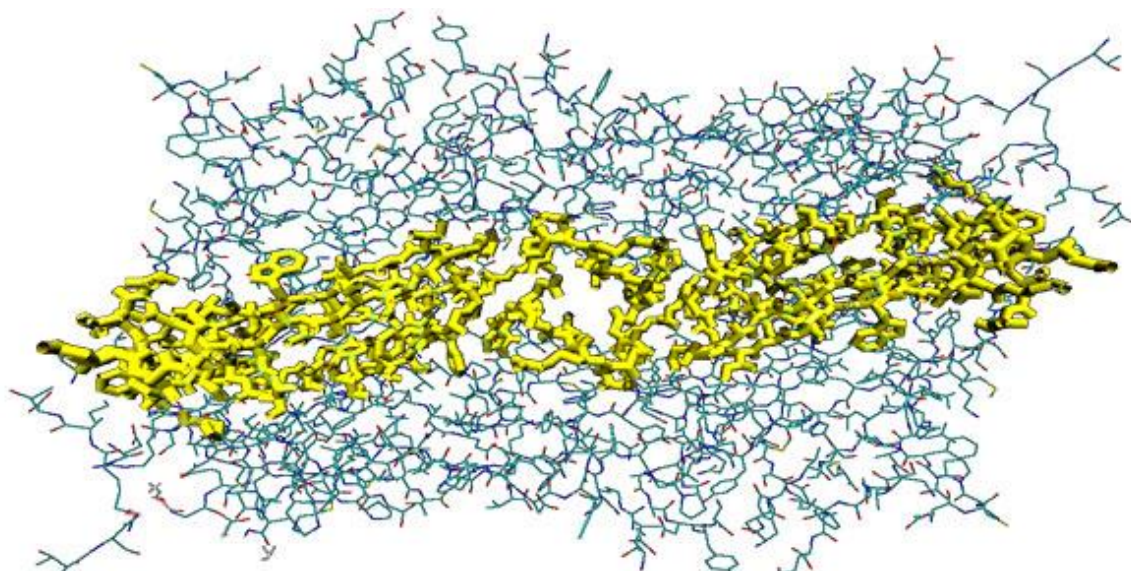


Figure 2-10: Amino acid residues contributing directly at the interface. The interfacial residues of FMDV contributing directly at the interface were mapped using the EBI-PISA program and are marked in yellow above.

The results (Appendix-II) obtained using the PISA program helped narrow down the list of amino acid residues to 85. A major impact on the interface comes from the VP2 protein, which contributes 57 residues directly to the interface. The remaining 28 residues are from VP3. A sequence alignment between representatives of all serotypes was also performed. VP4 has the highest sequence identity between all serotypes, followed by VP2 and then VP3. In comparison, the amino acids in VP1 were less conserved. Figure 2-11 shows the sequence alignment of amino acid residues in VP2. The α -helix adjacent to the 2-fold axis is shown in red. A complete sequence alignment between all viral proteins of representative serotypes is presented in Appendix-III.

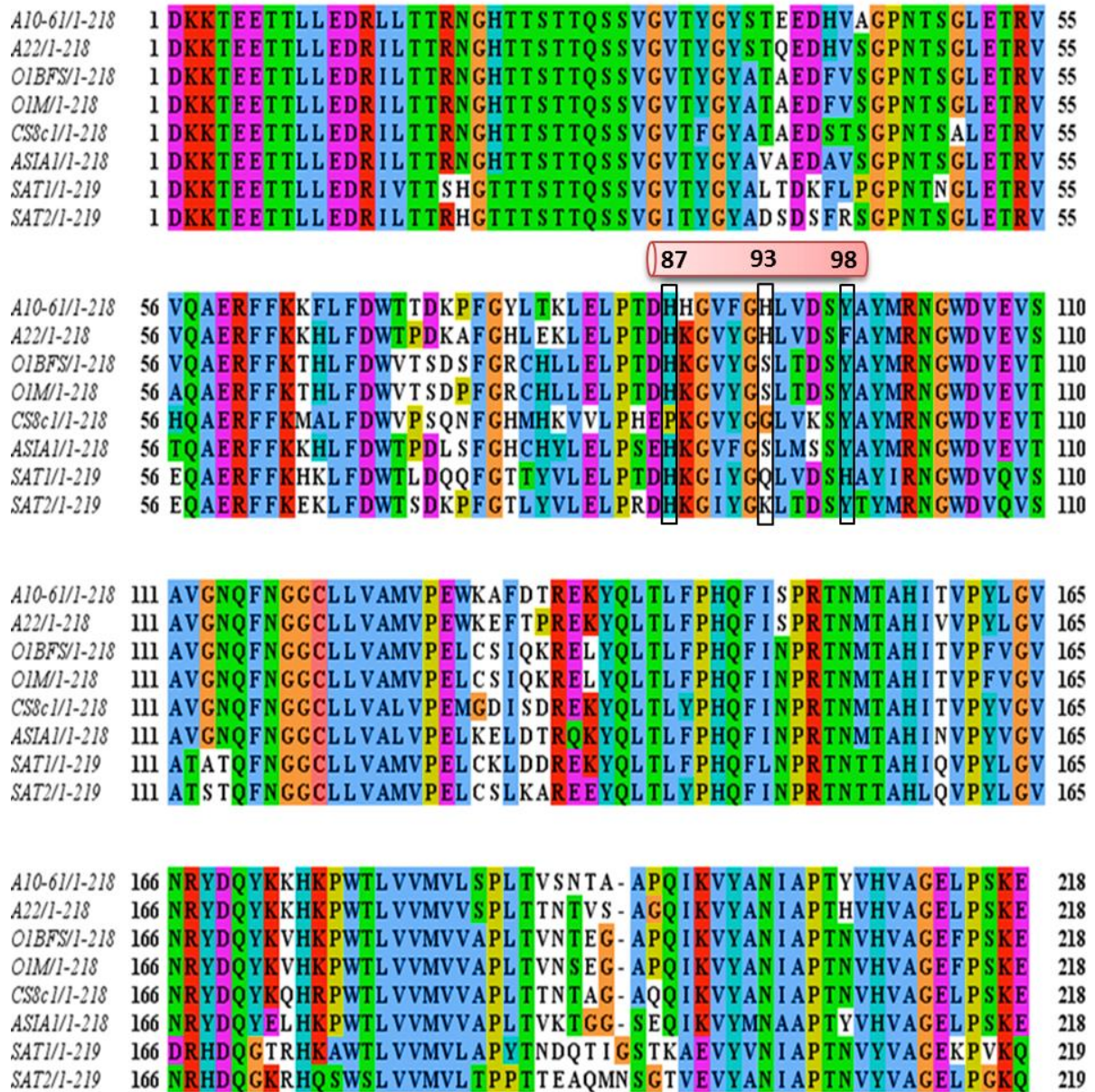


Figure 2-11: Sequence alignment of FMDV VP2. VP2 protein shares 80 to 92% sequence identity between all FMDV serotypes and dominates the interactions at the interface. The position of the α -helix found in all picornaviruses at the 2-fold symmetry axis (residues 87-98) is shown as red cylinder and the positions 87, 93 and 98 are highlighted by black lines.

As the simulation protocol was restricted to the region around the 2-fold axis, the α -helix in this region was chosen to sample the effect of mutations. A panel of putative stabilizing mutants were rationally designed by visually inspecting the structures of FMDV and Polio viruses. The mutations were incorporated using a simple mutate command in the COOT program and then energy minimised.

The mutant structures were analysed using the simulations and ΔG calculations described above. The stability of the mutant structures for each serotype was assessed by comparing their respective binding free energies ($\Delta\Delta G$) with that of the wild type complex using:

$$\Delta\Delta G = \Delta G_{mut} - \Delta G_{wt} \quad (17)$$

For the ease of classification the mutations were divided into three generations. The stability of each subsequent generation was enhanced by using the data derived from the preceding generation.

2.11.5 First generation mutants

First generation mutants were single amino acid substitutions at or near the 2-fold axis on the α -helix of the inter-pentameric interface. In particular, amino acid residues at position 90, 93 and 97 that are directly pointing at the interface were selected (Figure 2-12). These mutations were designed by visual inspection of the O1BFS, SAT1 and A22 structures.

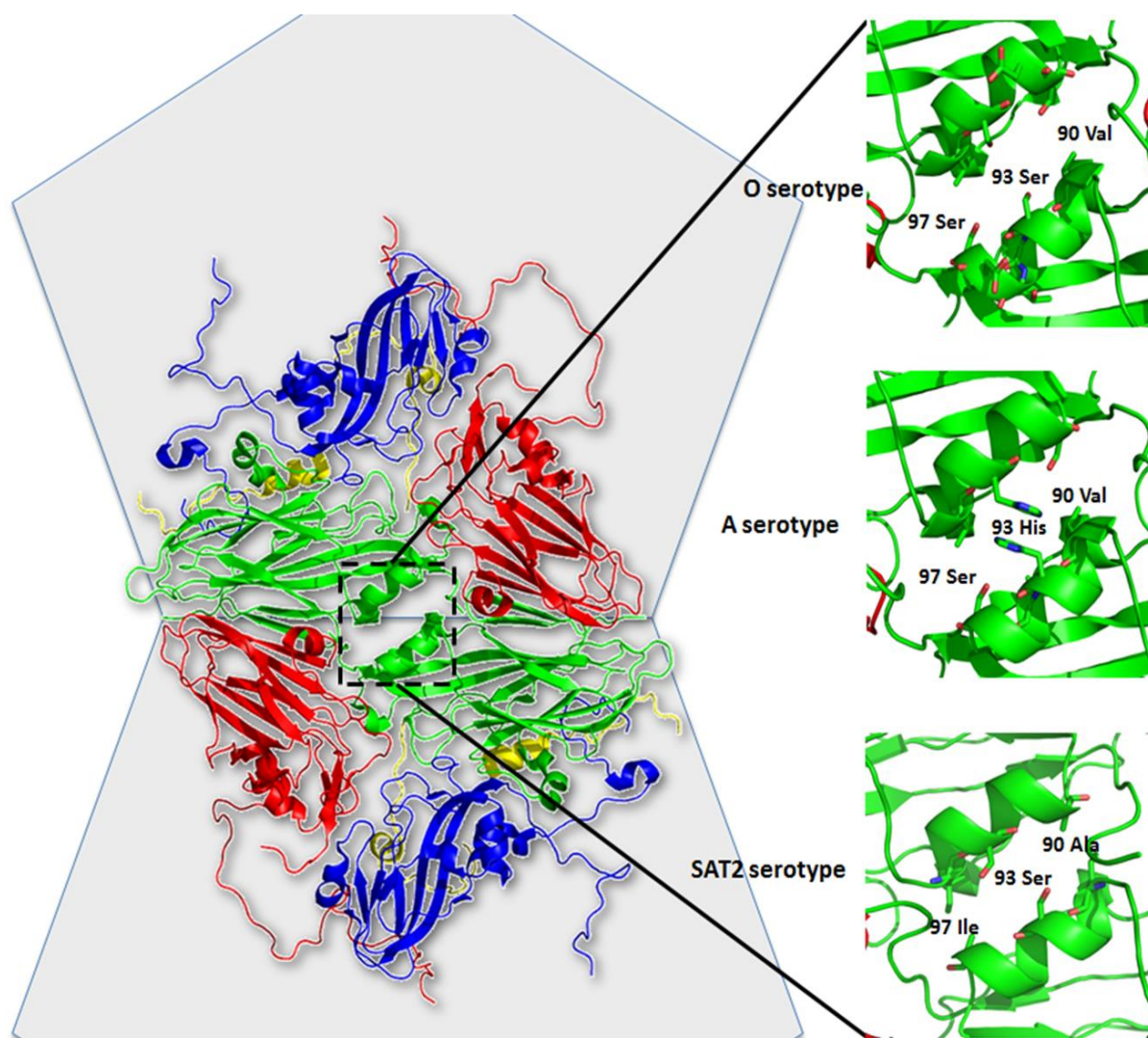


Figure 2-12: Residues at the 2-fold symmetry related α -helix. Target residues were selected from the amino acids on the α -helix at the 2-fold symmetry axis of the inter-pentameric interface. Amino acids at position 90, 93 and 97 were chosen for substitution.

The mutant models were energy minimised and any clashing waters were manually removed using the COOT program. The system was heated to 310K and simulated for 1ns as above. The change in binding free energy was calculated using equation 17. The list of mutants and the corresponding $\Delta\Delta G$ values for O and SAT serotypes are listed in Table 2-1.

Table 2-1: First generation FMDV capsid stabilising mutants for O and SAT serotypes

O Serotype			
No.	Name	Mutation	$\Delta\Delta G$ kcal/mol
1	l	VP2 S93H	- 7.7
2	m	VP2 S93Y	- 11.8
3	i	VP2 S93F	- 13.8
4	q	VP2 S93W	- 9.5
5	h1	VP2 S93V	-7.1
6	h2	VP2 S93L	-1.9
7	h3	VP2 S93I	-7.2
8	h4	VP2 S93M	-2.8
9	p1	VP2 S97Q	- 5.5
10	p2	VP2 S97I	- 6.8
11	p3	VP2 S97M	+ 1.9
12	o	VP2 V90N	- 3.5
SAT Serotype			
1	l	VP2 S93H	-5.3
2	m	VP2 S93Y	-12.2
3	i	VP2 S93F	-13.3
4	s	VP2 S98F	-5.6
5	q	VP2 S93W	-21.2

The advantageous effect of such a substitution on the α -helix is that this provides hydrophobic and/or electrostatic stabilisation to the protomer interface of the FMDV capsid. These mutations enhance the intermolecular interaction between the neighbouring proteins at the 2-fold symmetry axis of the FMDV capsids.

Stabilisation imparted on the FMDV capsid by the mutants can be further divided into two categories, (i) hydrophobic stabilisation (H, Y, F, W, I and M) such as hydrophobic stacking and (ii) electrostatic stabilisation (Q and N) such as hydrogen bonding interactions.

2.11.5.1 Hydrophobic and electrostatic stabilisation

Hydrophobic interactions are believed to be playing a central role in stabilising the native structure of the proteins in aqueous environments. Several theoretical and experimental studies have been performed to show the changes in binding free energies between wild-type and mutant proteins that favour protein folding, protein-protein and protein-ligand interactions (Chothia 1974; Glaser et al. 2001; Prevost et al. 1991). Studies have also shown that protein interfaces have a higher concentration of hydrophobic residues (Tsai et al. 1997; Vallone et al. 1998). Tsai et al. (1997) and Glaser et al. (2001) studied sets of more than 350 protein interfaces and found that hydrophobic residues were abundant in large interfaces whereas polar residues were more abundant in small interfaces. Glaser et al. (2001) also showed that common residue pairing in such interfaces involved stacking interactions between large aromatic residues such as hydrophobic interactions between Trp-Tyr or Trp-Leu.

In addition to hydrophobic interactions, electrostatic interactions such as hydrogen bonding and salt bridges also play a crucial role in protein binding. Several studies have shown that protein active sites often contain an electrostatic complementarity to the charge distribution of binding substrates. The binding is facilitated by a series of H-bonding and salt bridges (Lee and Tidor 2001; Novotny and Sharp 1992; Xu et al. 1997).

Inter-protomeric interfaces of viral capsids are comparatively very large, for example, the surface area of the FMDV interface is 3052 Å². The capsids are held together mostly by H-bonding and salt bridges across the interface. As previously mentioned the protomeric interfaces of FMDV are generally hydrophilic compared to other picornaviruses. We found that substituting polar residues on the 2-fold symmetry-related α -helix with large hydrophobic side chains has the highest stabilising effect on the binding free energy of the interface. The results were reproducible in *in-vitro* characterisation of FMDV capsids (discussed in Chapter 6).

2.11.5.1.1 Mutations at position 93

The amino acids at position 93 of the 2-fold symmetry-related α -helix are not well conserved between different serotypes of FMDV. However, within serotypes it is largely conserved. The polymorphism of amino acid residues at this position can be seen in Figure 2-11.

In stable A-serotypes there is a histidine residue at this position. The imidazole ring of the histidine is making a stacking interaction with the 2-fold symmetry related protomer, as shown in Figure 2-12. Simulation results showed that analogous mutations in O and SAT serotypes, leading to VP2 S93H, also showed a greater stability with $\Delta\Delta G$ of -7.7 and -5.3 kcal/mol, respectively. The degree of stabilisation was further improved when tyrosine was used instead, $\Delta\Delta G$ of -11.8 and -12.2 kcal/mol. In addition to hydrophobic stacking, tyrosine can also form a hydrogen bond with the backbone of VP2 89 (Figure 2-13). The effect was even greater when phenylalanine was used with a $\Delta\Delta G$ of -13.8 and -13.3 kcal/mol respectively. The $\Delta\Delta G$ values correlated remarkably well with the experimental stability data of the mutant FMDV capsids (discussed in Chapter 6). Tryptophan at this position (VP2 93) also has a similar stabilising effect with a $\Delta\Delta G$ of -9.5.

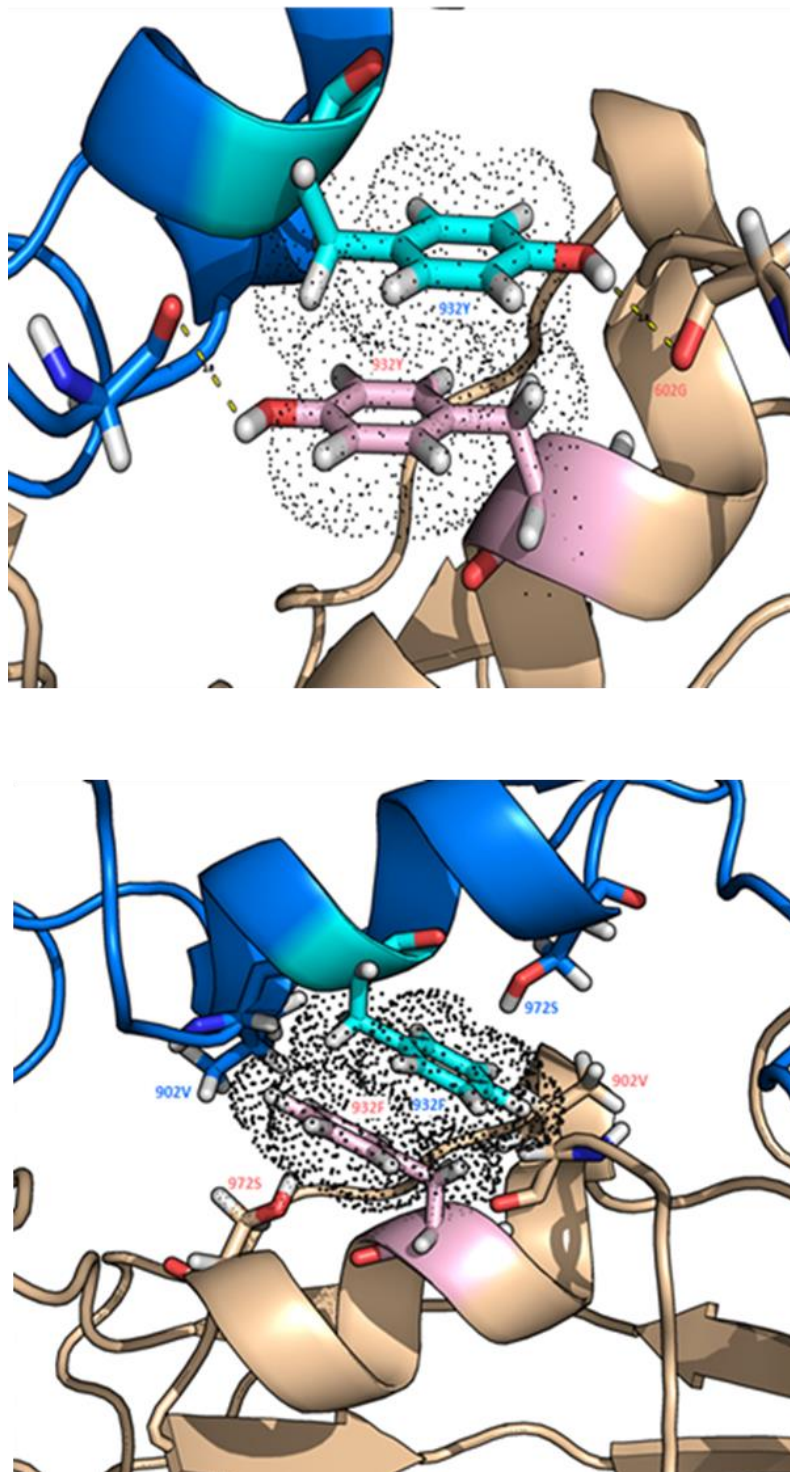


Figure 2-13 Hydrophobic stacking at the 2-fold symmetry axis. Aromatic residues like histidine, tyrosine, phenylalanine and tryptophan can make stacking interactions at the 2-fold axis. These interactions are known to stabilise protein-protein interfaces.

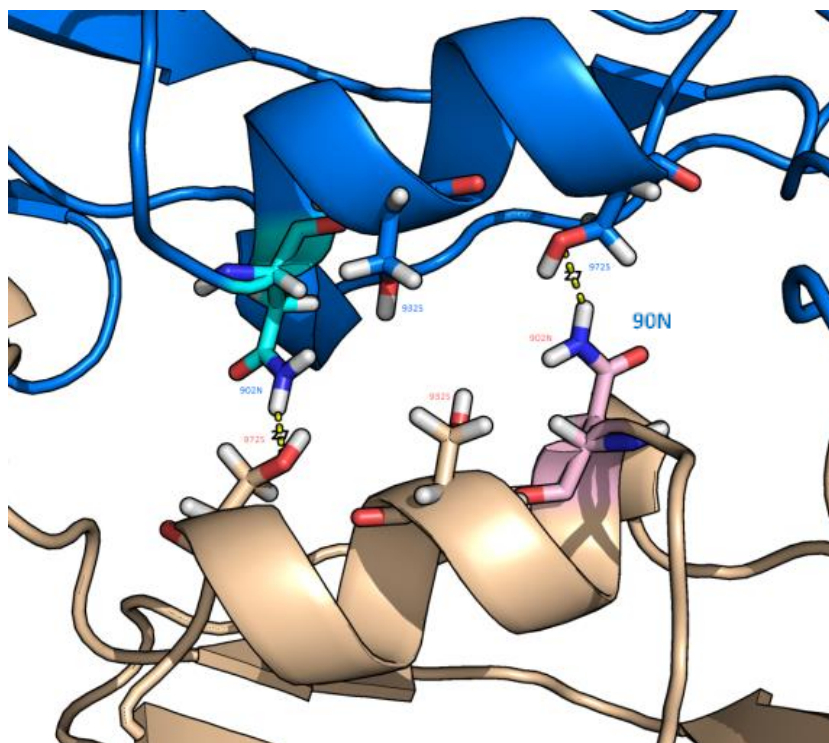
Additionally, other non-aromatic hydrophobic amino acids (V, L, I and M) were also tested at this position. However, stabilisation was greater with hydrophobic stacking of aromatic side chains, especially with phenylalanine. Similar results were obtained when a 2-fold histidine residue of an A-serotype, A22 Iraq, was substituted with phenylalanine, VP2 H93F, with a $\Delta\Delta G$ of -10.3 kcal/mol.

2.11.5.1.2 Mutations at positions 90 and 97

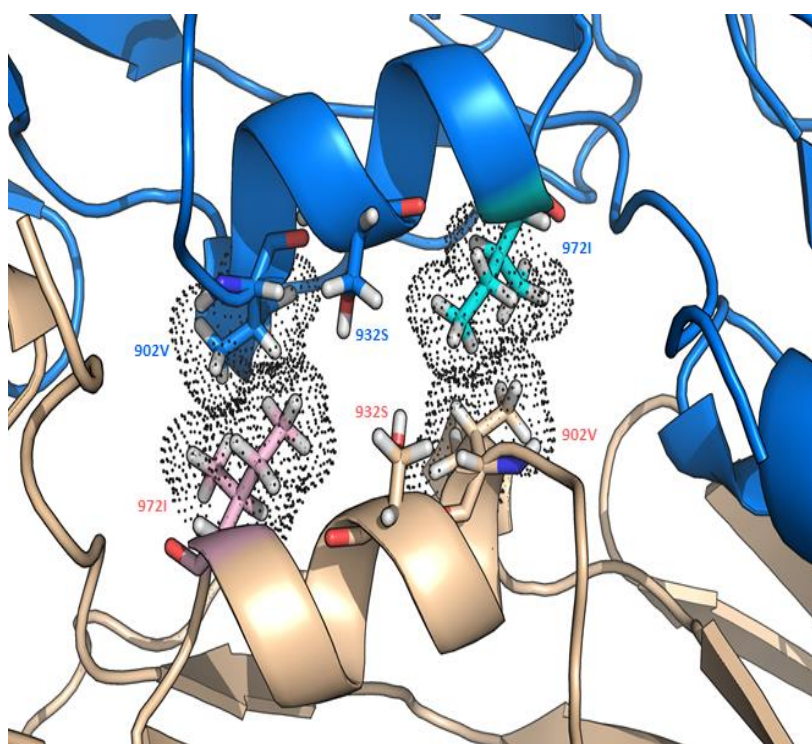
At position 90 of VP2 there is a conserved valine, except for in SAT serotypes which contain isoleucine. Both of these residues are considered hydrophobic. However, on the symmetry related α -helix there is a conserved hydrophilic serine at position 97 which does not contribute to the stability of the interface. Mutation VP2 V90N has a potential to form an H-bond with 97S (Figure 2-14). This results in a small increase in the stability of the interface which is reflected in $\Delta\Delta G$ of -3.5 kcal/mol.

Alternatively, 97S can be substituted with a hydrophobic residue such as S97I ($\Delta\Delta G$ = -6.8 kcal/mol) or S97M, ($\Delta\Delta G$ = +1.9 kcal/mol) to match the opposing hydrophobic residue. However, 97M was found to be less stable, possibly due to steric hindrance between its methionine side chain and the side chain of valine at position 90 on the opposite helix. Furthermore, glutamine was also tested leading to a VP2 S97Q mutation ($\Delta\Delta G$ = -5.5 kcal/mol). Glutamine has a potential of forming H-bonds with serine at position 93 on the opposite helix (Figure 2-14).

Although the hydrophilic residues were found to stabilize the interface, hydrophobic interactions had a stronger effect on capsid stability. An extensive list on all simulated mutants and their corresponding $\Delta\Delta G$ values are presented in Appendix IV.



VP2 S90N



VP2 S97I

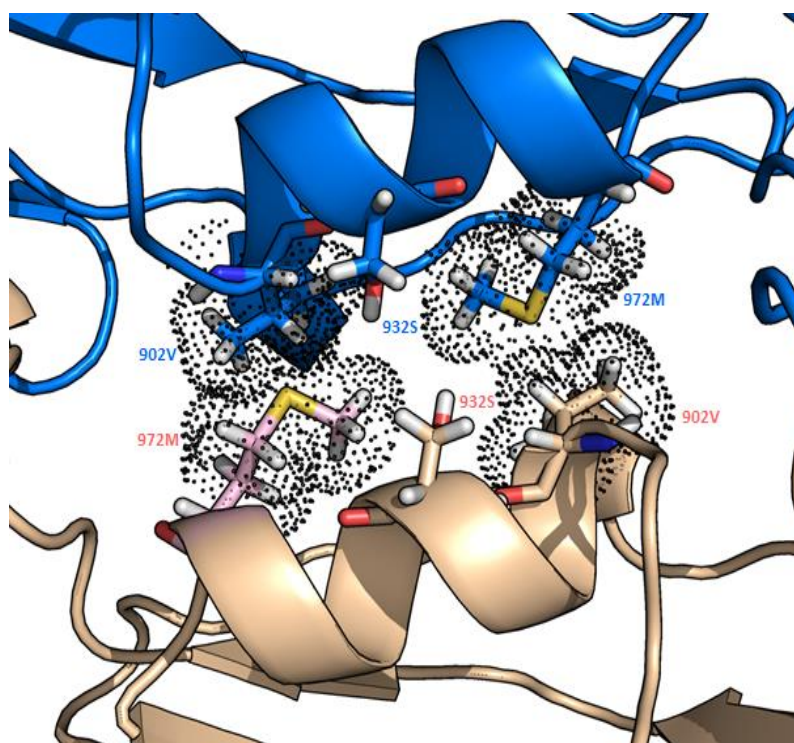
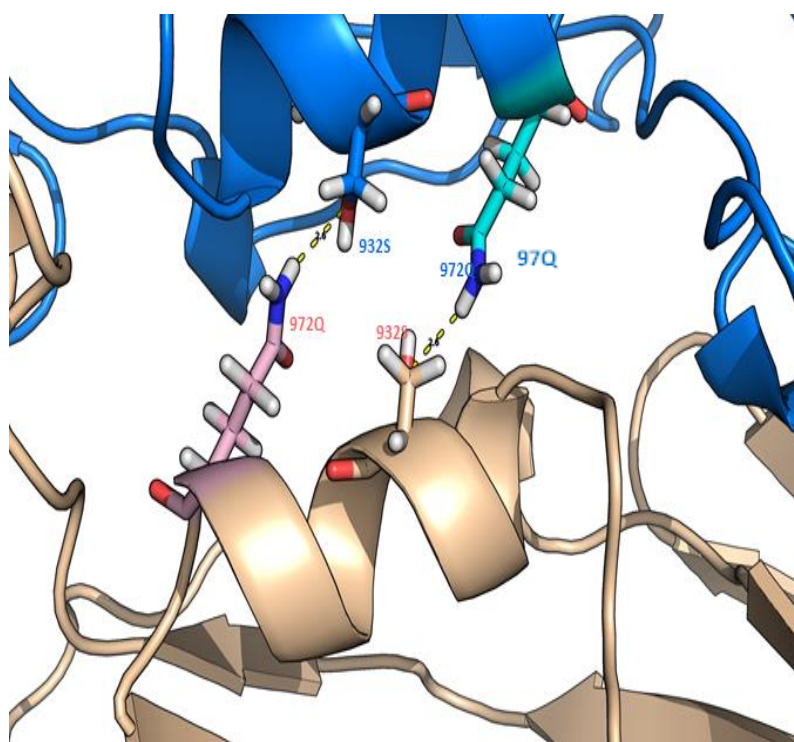
**VP2 S97M****VP2 S97Q**

Figure 2-14: Mutations near the 2-fold symmetry axis. Several amino acids were substituted at positions 90 and 97. In general, hydrophobic residues showed greater stability in simulations.

A set of negative controls were also tested to validate the simulation protocol. In one simulation VP2 arginine at position 60, that forms the salt bridge with the VP2 glutamate 212 on the opposite protomer, was substituted with either glycine or leucine. As expected, in both cases the interface was destabilised. Similarly, when glutamine at position 57, that makes a network of H-bonds across the interface, was substituted with glutamate or leucine an unstable interface was generated (Table 2-2).

Table 2-2: Negative controls used to validate the simulation protocol.

No.	Mutation	$\Delta\Delta G$ kcal/mol
1	VP2 R60G	+ 33.7
2	VP2 R60L	+ 24.0
3	VP2 Q57E	+ 17.7
4	VP2 Q57L	+ 12.3

2.11.6 Second and third generation mutants

The mutations for the second generation were designed by stabilising the side chain dynamics and combining the first generation mutants, creating double mutants with higher stability. Some of these mutations were also designed by comparing FMDV structures with the structure of stable enteroviruses like Polio virus, Human Rhinovirus and Enterovirus 71.

In case the desired stability could not be achieved with the first two generations of mutants, a third generation of mutants were also designed and simulated. These were derived from the first two generations of most stable mutants and contained three or four mutations at the 2-fold interface. A detailed list of second and third generation mutants is presented in Appendix IV.

2.11.7 Covalent stabilisation

An alternate approach is covalent stabilisation by mutating the amino acid residues to cysteine across the 2-fold interface. Cysteine residues can cross-link to form disulphide bridges in proteins. These disulphide bridges play a crucial role in protein stability and are very common in extra cellular proteins (Petersen et al. 1999). Several studies have shown that rationally engineered disulphide linkages have improved thermal and chemical stability of target proteins (Fass 2012; Kim et al. 2012). Mateo et al. (2008) modelled a series of disulphide bonds near the 3-fold symmetry axis of FMDV. However, the viral capsids either did not assemble or aggregated and precipitated. King et al. (2002) attempted to stabilise the empty capsids of FMDV A10 serotype by introducing a disulphide bridge at a 2-fold axis at position 93. VP2 H93C formed a stable disulphide bond with the symmetry related cysteine on the adjoining promoter. In our approach, we predicted several positions across the 2-fold interface that can be used to form energetically favourable disulphide bonds.

Because of the limitations of MD simulations, the stabilisation of interfaces with disulphide linkages cannot be estimated using binding free energy changes. Nevertheless, structural studies on proteins containing disulphide bonds reported the pair-wise $C\alpha_i-C\alpha_j$ atomic distance for a favourable disulphide bond is around 5-6Å (Dombkowski 2003; Petersen et al. 1999; Sowdhamini et al. 1989). Based on these results, the pair-wise $C\alpha_i-C\alpha_j$ and the corresponding $C\beta_i-C\beta_j$ distances were measured using COOT. This allowed us to pick the same three target residues at positions 90, 93 and 97 on the 2-fold α -helix used for non-covalent mutations (Table 2-3). Although the distances are larger than reported (Figure 2-15), the 2-fold helices are flexible enough to accommodate stereo-chemically stable disulphide bonds (X. Wang et al. 2012).

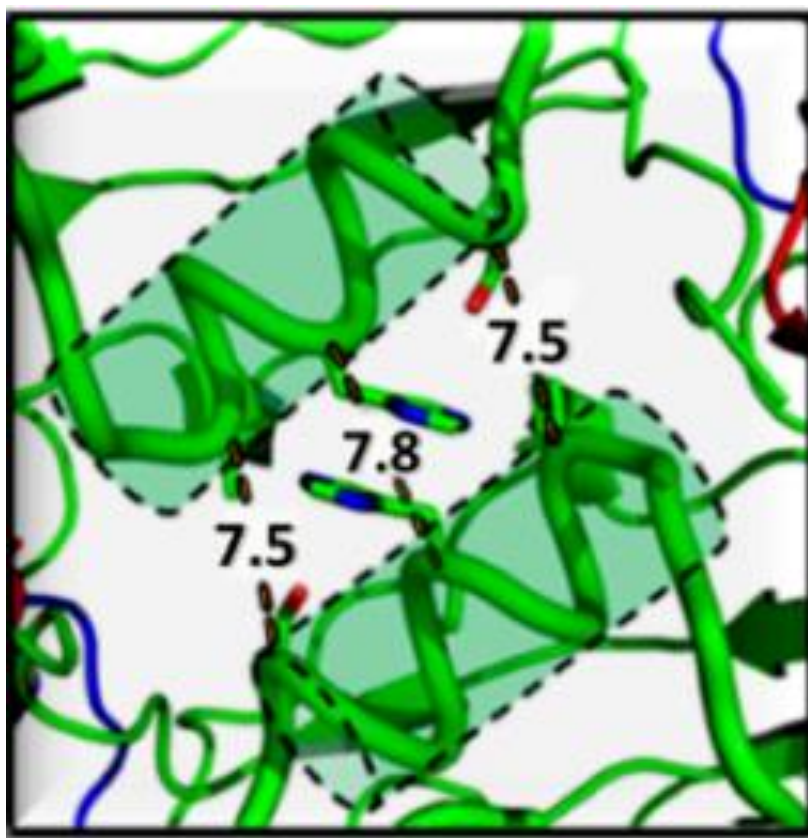


Figure 2-15: Pair-wise atomic distances at the 2-fold axis. $C\alpha_i-C\alpha_j$ and the corresponding $C\beta_i-C\beta_j$ distances were analysed manually using the COOT program. VP2 residues at positions 90, 93 and 97 on the 2-fold α -helix were predicted to form a covalent cross-linked disulphide bridge between the pentamers.

Table 2-3: Predicated mutations that can promote disulphide linkage across the 2-fold interface.

Mutant	
a	VP2 S93C
b	VP2 V90C, S97C
c	VP2 V90C, S93C, S97C

On the 2-fold helix three mutants were proposed, VP2 S93C (H93C in A22), which has been shown to produce capsids in the A10 serotype. The backbone is identical between all FMDV serotypes at this position. Additionally, a double disulphide can be introduced (VP2 V90C, S97C), clamping the ends of the α -helix. The distance between these residues is appropriate and although they are both mostly conserved they do not appear to be involved in significant interactions. Furthermore, a triple disulphide can also be made by combining the above two, leading to VP2 V90C, VP2 S93C, S97C (Figure 2-16). If successful, these mutations could produce very stable empty capsids.

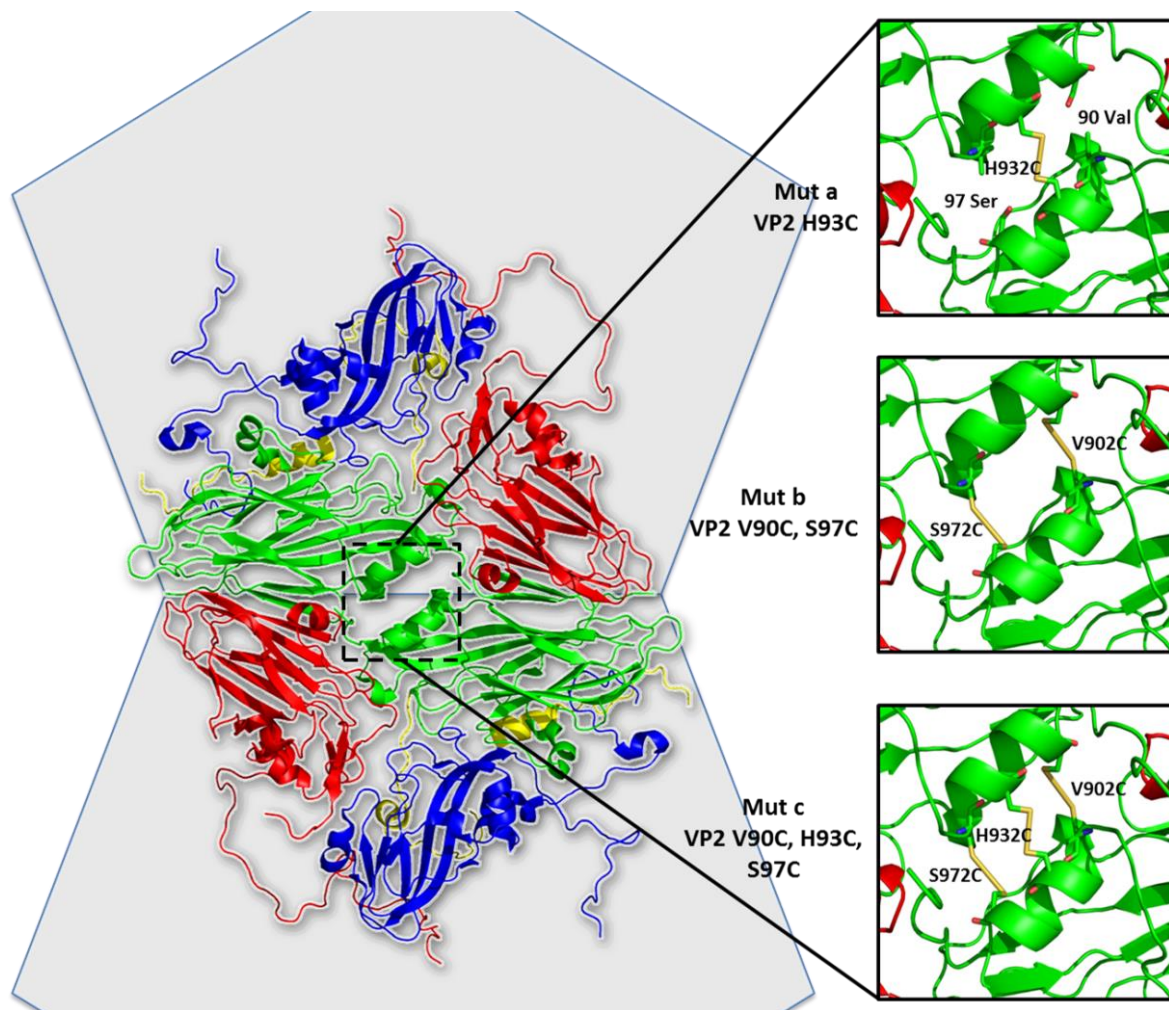


Figure 2-16: Covalent stabilisation at the 2-fold axis in A-serotype, A22 Iraq. Three cysteine mutants were proposed at the 2-fold helix that can form a disulphide linkage between the adjoining pentamers. Mutant-a forms a single disulphide bond at position 93 at the 2-fold axis. Mutant-b is a double disulphide bond which clamps the ends of the α -helix at positions 90 and 97, and Mutant-c is a combination of mutant-a and -b leading to triple disulphide bonds at the 2-fold axis.

The covalent approach is only applicable for the production of stabilised recombinant empty capsids and not for the infectious virions. Such disulphide linked capsids will generally produce permanently fixed capsids that can no longer uncoat upon infection of the host cell, essentially producing a dead virus.

2.12 Conclusion

Despite the relative simplicity of FMDV capsid dissociation, very few attempts have been made to rationally engineer the stabilised capsids and current vaccines are increasingly unstable. Realistic modelling of the stable interactions that occur at the FMDV interface and an accurate prediction of putative stabilising mutations is of increasing importance for making vaccines with improved properties.

The MD simulation and binding free energy calculation method that we have devised can be easily implemented to validate the effect of mutations on the stability of the FMDV capsid. The mutations showing a predicted increase in stability of the interface were introduced into infectious copy viruses. In a parallel project, the same mutations have also been used for stabilizing recombinantly derived empty capsids. Stabilised empty capsids could facilitate a next generation of infection risk free FMDV vaccines. The production of non-covalent and covalent mutations and their effect on capsid stability will be discussed in chapters 3 and 4.

This achievement also offers the future opportunity to use MD methods to design vaccines for other animal and human viruses, for example, the approach of MD simulation has been already applied on Polio and EV71 viruses.

Chapter 3 : Expression and purification of inactivated and recombinant capsids

3.1 Introduction

A major difficulty in the production of FMDV vaccines is obtaining enough viral stock for vaccine production. Many approaches have been used to express FMDV capsids to produce vaccines. The first approach to produce live FMDV on an industrial scale was pioneered in the 1950s by a Dutch scientist, Frenkel, who used epithelial tissue fragments from cattle tongues to grow virulent FMDV particles (Frenkel 1954). A major step in the progression of FMDV antigen production came with the development of cell culture technology in monolayers and in suspension cultures. Capstick and Telling developed the BHK-21 (baby hamster kidney cells) clone 13 cell line which can grow in suspension culture (Capstick et al. 1965). The culture can be infected with virulent FMDV and after overnight incubation, or when the cells show extensive cytopathic effect (CPE), the virus is harvested. Cells in suspension culture multiply rapidly and cultures can be easily scaled up for industrial production of FMDV. This system has become the most widely used method for production of inactivated FMD vaccines (Barteling and Vreeswijk 1991; Lombard et al. 2007).

In addition to producing live FMDV by infecting mammalian cells, many groups have also tried to produce empty viral capsids by expressing recombinant structural proteins in different expression systems. Ansardi et al. (1991) reported the production of poliovirus empty capsids using co-infection of two vaccinia viruses (VV), one containing the P1 (VP0, VP3, VP1) region and the other the 3CD protease. Similarly, Abrams et al. (1995) successfully produced FMDV empty capsids by using VV containing P1-2A and 3C protease.

Several studies have also reported the expression of recombinant FMDV empty capsids in transgenic plants, bacterial expression systems and baculovirus insect cell expression systems (Yimei Cao et al. 2009; Y. Cao et al. 2010; Mohana S et al. 2012; Pan et al. 2008). Another

significant difficulty is the purification of intact inactivated or recombinant empty capsids, as the inactivated and empty capsids are very unstable and readily dissociate into pentameric subunits.

This chapter describes the methods and materials used for growth and purification of infectious and recombinant empty capsids, followed by the analysis of capsids using sucrose density gradients and electron microscopy. The production of infectious and recombinant empty capsids was done in collaboration with four different institutes:

- i). The Pirbright Institute, UK; for production of infectious viruses using mammalian cells and recombinant empty capsids using the vaccinia expression system.
- ii). The Agriculture Research Centre, Onderstepoort Veterinary Institute (ARC-OVI), South Africa; for production of SAT2 infectious viruses.
- iii). Reading University, UK; for production of recombinant empty capsids in an insect cell expression system.
- iv). The Division of Structural Biology (STRUBI) University of Oxford, UK; for production of recombinant empty capsids in vaccinia and an insect cell expression system.

All purification, analysis and structural studies were performed at STRUBI unless otherwise stated.

The infectious O1M, SAT2 and ASIA1 viruses (native and mutants) were propagated in baby hamster kidney cell line (BHK-21) at The Pirbright Institute by Julian Seago. SAT2 viruses were originally produced at ARC-OVI and infectious viruses were delivered to The Pirbright Institute for large-scale virus production, biophysical and structural studies (in collaboration with STRUBI).

Recombinant empty capsids of O1M were produced by Alison Burman (The Pirbright Institute) using a vaccinia expression system and recombinant A22 by Claudine Porta (STRUBI) using an insect cell expression system. Recombinant O1M, A22 and SAT2 mutants were produced using an insect expression system by Ian Jones (Reading University).

The purification of inactivated capsids of O1M, SAT2 and ASIA1 was performed at STRUBI by myself and Julian Seago. Purification of recombinant capsids of A22, O1M and SAT2 was performed by myself and Claudine Porta.

3.2 Infectious virus growth and inactivation

This work was done by Julian Seago at The Pirbright Institute under strict disease security regulations (SAPO-4).

3.2.1 FMDV constructs

Briefly, Infectious copy FMDV O1K/O1Manisa (O1M) chimeric clones were constructed using O1K as a backbone. cDNA encoding the VP2, VP3, VP1 and 2A proteins was removed from an O1K infectious clone and replaced with the corresponding O1M cDNA (Seago et al. 2012). Infectious copy clones encoding targeted mutations in their VP2 capsid proteins were

derived by PCR mutagenesis (QuikChange Lightning Mutagenesis Kit, Agilent technologies, UK).

3.2.2 Competent cells, electroporation and preparation of virus stocks

Two different cell lines were used to propagate the infectious virus. BHK-21 cells were used to grow heparan sulfate positive (HS+) viruses and goat epithelium cells for HS- viruses. The cells were grown until they were confluent in Glasgow Minimum Essential Medium (GMEM; Sigma) supplemented with 10% Fetal Calf Serum (FCS, Autogenbioclear), L-glutamine and appropriate antibiotics in T175 flat bottom flasks (Greiner bio-one). The cells were harvested by trypsin treatment and pelleted by centrifugation at 2000xg for 4 minutes at 4°C. The pellet was washed once with 10 ml electroporation buffer (Bio Rad) and then resuspended in another 10 ml of fresh electroporation buffer. 800µl aliquots were mixed with 1µg of the appropriate FMDV RNA and electroporated using a Biorad Gene PulsarTM. The recombinant cells were incubated overnight at 37°C and the virus (P1) was harvested by freeze thawing. Virus stock was serially passaged to generate P2, P3 and higher stocks. After each infection, cells were incubated for 48 hours at 37°C or until CPE was clearly visible. For structural studies and biophysical analysis, 1.8 to 2 litres of cultures were prepared using 20 to 30 1700cm² roller bottles (Corning).

3.2.3 Virus Inactivation

The virus was chemically inactivated before transferring to STRUBI. Specific standard operating procedures (SOPs) were used that comply with the disease security regulations at The Pirbright Institute. Upon CPE, the cell culture media, containing the respective virus, was harvested. The crude lysate was clarified by centrifugation at 2095 x g for 30 minutes at 4°C. Clarified supernatant containing the virus stock was inactivated by 2 consecutive

incubations with BEI (binary ethyleneimine). The BEI treatment was performed by the addition of 10 ml of 0.1M BEI solution to every 1000 ml of virus harvested (to give a final BEI concentration of 0.001 M) followed by incubation for 24 hours at 37°C. During the incubations, the virus/BEI solutions were mixed by periodic inversion.

Inactivated viruses were deemed non-infectious according to inocuity tests performed by the world reference laboratory (WRL) at The Pirbright Institute. The inocuity test relies on the use of confluent monolayers of primary calf thyroid cells (BTY cells) in combination with ELISA to detect the presence of any remaining live (infectious) virus. The inactivated crude virus preparation was transported to STRUBI and stored at -20°C before further purification and analysis (discussed below).

3.3 Recombinant empty capsid production

The recombinant empty capsids were expressed using two different expression systems. The vaccinia expression system was used by Alison Burman at the Pirbright Institute and Claudine Porta at STRUBI. Claudine Porta also used the Baculovirus/insect cell expression system for A22 serotype. O1M and SAT2 empty capsids were produced in the Baculovirus/insect cell expression system by Ian Jones at Reading University.

3.3.1 Expression of recombinant FMDV using a vaccinia expression system

Expression cassettes containing synthetic cDNA encoding the VP0, VP3 and VP1 structural proteins and the 2A and 3C non-structural proteins of wild type FMDV serotypes were *de novo* synthesized (Geneart) and cloned into a vaccinia transfer vector, pBG200. The recombinant viruses were made by transfecting the pBG200 vector into Simian kidney derived CV-1 cells infected with vaccinia virus (VV). Recombinant VV were selected by

plaque assay and verified by PCR using FMDV specific primers. FMDV empty capsids were amplified in RK13 cells. At STRUBI, HEK293 cells were used for expression. All mammalian cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS, L-Glutamine and appropriate antibiotics at 37°C.

3.3.2 Expression of recombinant FMDV using an insect cell expression system

The pTri-EX-derived plasmid pOPINE (Berrow et al. 2007) was used to generate recombinant Baculoviruses encoding the FMDV proteins discussed in 3.3.1; the FMDV proteins were then overexpressed following infection of Sf9 insect cells (*Spodoptera frugiperda*) with these baculoviruses. The FMDV coding sequence was cloned using In-FusionTM cloning (Clontech). All mutants were made using a PCR mutagenesis kit (QuikChange Lightning Mutagenesis Kit, Agilent technologies, UK) according to the manufacturer's instructions.

Sf9 cells were grown in Insect-XPRESS (Lonza) supplemented with 2% FCS and appropriate antibiotics at 27.5°C. The pOPINE vector containing the FMDV coding sequence, and the AcMNPV (*Autographa californica* nucleopolyhedrovirus) bacmid, were transfected into Sf9 cells using Fugene transfection reagent. The recombinant AcMNPV was harvested from the supernatant 5 days post infection (P0 virus stock). Recombinant baculovirus stocks were amplified by infecting Sf9 cells at a confluency of 70% with 200 µl recombinant virus per 175 cm² flask and harvested from the supernatants after 5 days (P1 virus stock). Recombinant empty capsids were expressed by infecting SF9 cells ($1-2 \times 10^6$ /ml) with 1/10 volume of the P1 baculovirus stock.

3.4 Purification of inactivated viruses and Recombinant empty capsids

The crude lysate containing either inactivated virus or empty capsids was received from the Pirbright Institute or Reading University, respectively. Pellets of Sf9 cells expressing SAT2 and A22 mutants were also received from Reading University.

3.4.1 Purification of inactivated viruses

Frozen lysates containing inactivated viruses were thawed on ice and clarified by centrifugation at 3500xg for 30 minutes at 4°C. Virus was then precipitated by the addition of either 30% w/v ammonium sulfate or 8% w/v polyethylene glycol 6000 (PEG6000) by incubation at 4°C overnight. Precipitated virus was harvested by centrifugation at 3500xg for 1 hour at 4°C and re-suspended in 50mM phosphate buffer, pH 7.4 containing 150mM NaCl and 0.5% v/v NP40. The samples were treated with 0.1% RNase-A for 30 minutes on ice and again clarified by centrifugation at 3500xg for 1 hour at 4°C to remove any insoluble particulates. The inactivated capsids were purified using the sucrose density gradient method.

The capsids were first pelleted over a 3ml, 30% sucrose (in 50mM phosphate buffer, pH 7.6 and 150mM NaCl) cushion at 105,000xg for 2 hours at 12°C. The virus pellets were re-suspended in phosphate buffer containing 0.5% (v/v) NP40 overnight at 4°C, after which insoluble material was clarified by centrifugation using a benchtop centrifuge (16000xg for 10 minutes at 4°C). A linearised gradient of 15-45% sucrose solution in phosphate buffer without detergent was prepared using a gradient master (Biocomp). The gradient was layered with the clarified supernatant containing capsids and fractionated by centrifugation at 105,000xg for 3 hours at 12°C. Fractions were analysed by SDS-PAGE followed by Coomassie staining.

3.4.2 Purification of recombinant empty capsids

Frozen baculovirus infected Sf9 cell pellets containing over-expressed empty capsids were thawed and re-suspended in 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 8.0, 200mM NaCl and 1% NP40. Cell lysis was achieved by incubating the re-suspended pellets with detergent for 30 minutes on ice. Lysates were clarified at 48,000xg for 30 min at 4°C and capsids pelleted over a 3ml, 30% sucrose cushion by centrifugation at 105,000xg for 2 hours at 12°C. The recombinant capsids were then purified as described in 3.4.1 and analysed by SDS-PAGE followed by Coomassie staining.

Excess sucrose was removed using a desalting spin column (Zeba, Pierce) according to manufacturer's instructions. The samples were concentrated to 2mg/ml by ultrafiltration through a spin column with a 100 kDa molecular weight cutoff membrane (Millipore).

3.5 Electron Microscopy

Purified FMDV capsids were examined by electron microscopy. Aliquots of purified samples were diluted to 0.2 mg/ml and deposited onto glow-discharged, Formvar carbon-coated copper grids. The capsids were allowed to adhere to the grids for 30 seconds. Excess sample was blotted and the grids washed twice with deionized water. The samples were stained with 1% (w/v) uranyl acetate solution for 45 seconds and the excess stain was removed by blotting. The grids were examined on a Technai T12 transmission electron microscope operating at 80kV.

3.6 Results and Discussion

Purified proteins are required for the characterisation of structural and functional properties. Purification can be achieved by several different methods and requires a series of processes that facilitate isolation of the individual protein of interest from the bulk. Proteins can be separated by means of their size, charge, binding affinity, biological activity and/or by their physico-chemical properties such as density. In this thesis, all FMDV capsids were purified using the classical sucrose density gradient method.

3.6.1 Purification and analysis of inactivated virus

The inactivated viruses of serotype O (subtype O1M; wildtype and stabilized mutants) were isolated from the bulk of protein mixtures by using ammonium sulfate precipitation. Ammonium sulfate at 30% (w/v) was added to the crude lysate and incubated overnight at 4°C. In another experiment, 8% (w/v) PEG6000 was used as a precipitant. Precipitated capsids were pelleted by centrifugation at 3500xg for 1hr at 4°C. The results were similar with both precipitants; however, precipitation by PEG produced cleaner and more transparent pellets.

Supernatants were then discarded and the pellets resuspended in 50mM phosphate buffer, pH 7.6 containing 0.5% NP40 v/v and incubated on ice for 30 minutes. The detergent was used to solubilize cell membranes and aggregated proteins. Any insoluble material was removed by centrifugation at 3500xg for 1 hour at 4°C.

After precipitation of the viruses, they were purified in two further steps. The first step involved the removal of less dense material, such as plasma membranes, receptor proteins and other small proteins, by pelleting the denser components, including virus, through a 3ml, 30% sucrose solution using ultracentrifugation at 105000xg for 5hrs at 10°C. The supernatant was then discarded and the pellet was solubilized by the addition of a small amount of buffer (50mM phosphate buffer pH 7.6, containing 0.5% NP40) and incubation overnight at 4°C. At this point, the re-suspended pellets were treated with 0.1% RNase-A, for 20 minutes on ice followed by clarification at 16000xg for 10 minutes at 4°C using a benchtop centrifuge. The second step involved using two consecutive sucrose gradient purifications to separate remaining contaminants from intact virus particles.

The clarified crude virus preparations were layered onto 15-45% sucrose gradients and were then sedimented by ultracentrifugation at 105000xg for 3 hours at 12°C. Very faint and diffuse bands were observed in the bottom half of the gradient upon illumination (Figure 3-1).

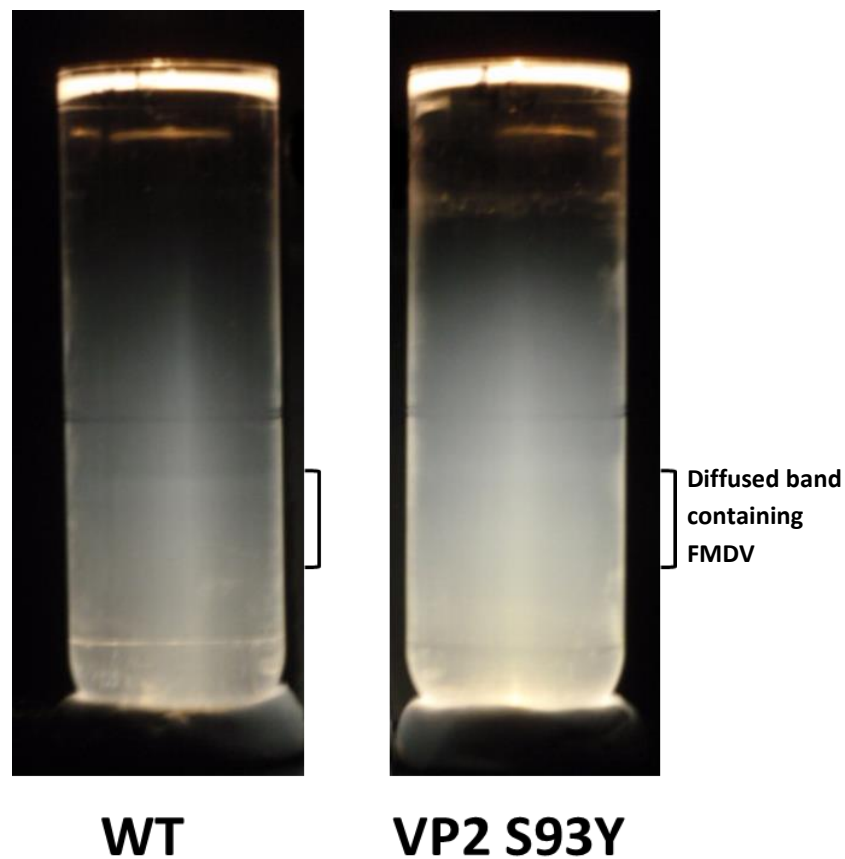


Figure 3-1: Sucrose gradient purification of inactivated FMDV O1M. WT and stabilized mutant (VP2 S93Y) FMDV, were purified using a 15-45% sucrose gradient. Both viruses were detected in a faint diffused band in the bottom half of their respective gradient. Bands were similar for both WT and mutant.

The supernatant from the top of the gradient was pooled (15ml) and the bottom half of the gradient was fractionated in 1ml aliquots. A small sample of each fraction was analysed by SDS PAGE and Coomassie staining (Figure 3-2).

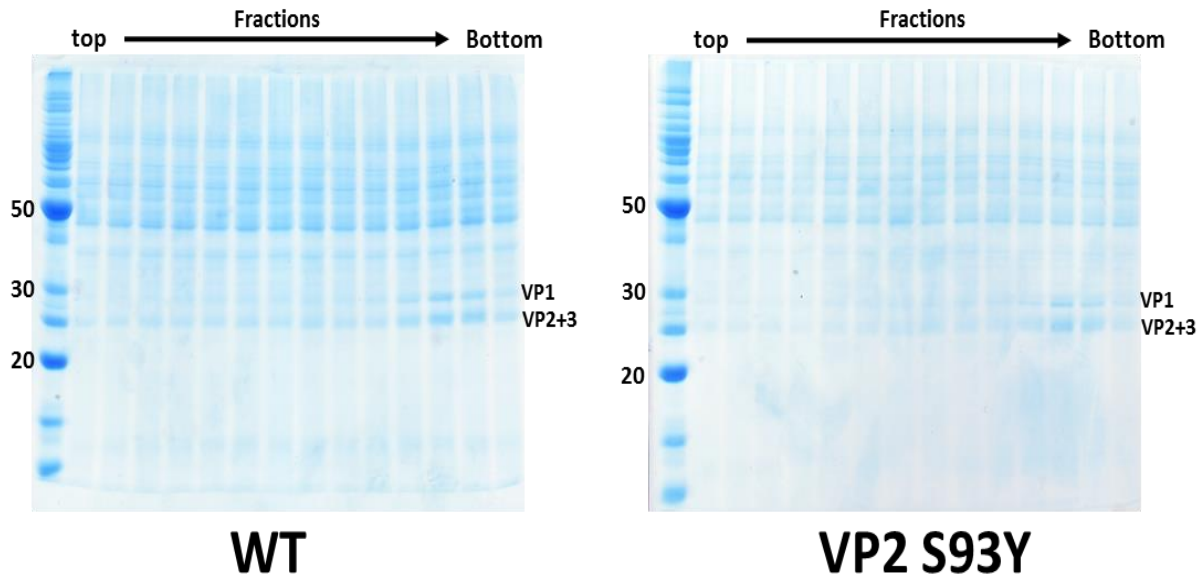


Figure 3-2: Detection of FMDV O1M inactivated capsid proteins. Fractions from sucrose gradient purification were analysed by SDS PAGE analysis and Coomassie staining. FMDV proteins VP1-3 were detected at the bottom half of the gradient.

Capsid proteins were visible in the samples from the lower fractions of the gradient; however, several contaminants were also detected. To further purify the capsids, fractions containing capsid proteins were pooled together, the sucrose removed and capsids concentrated to 2ml. The concentrated samples were then sedimented by ultracentrifugation through new 10-50% sucrose gradients.

This time, strong bands were observed in the middle of the gradient upon illumination. SDS PAGE analysis and Coomassie staining verified the corresponding fractions contained FMDV capsid proteins and showed the purity of the viruses had improved after the second gradient.

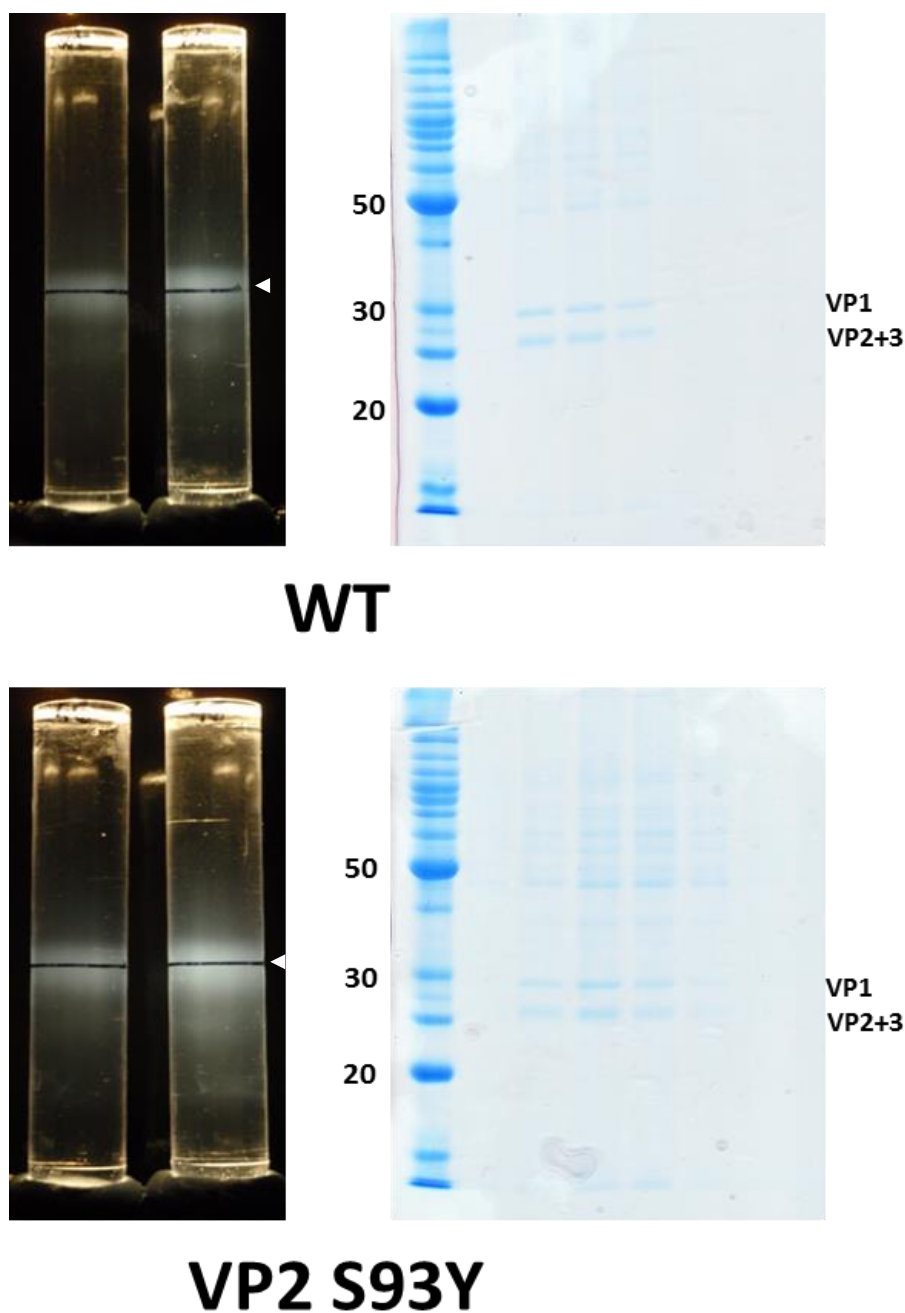


Figure 3-3: Further purification of inactivated FMDV O1M using sucrose gradients, Further purification was achieved by sedimenting the capsids through a second sucrose gradient. Strong bands were observed at the middle of the gradient upon their illumination. Fractions containing capsids were verified by SDS PAGE analysis and Coomassie staining. The second sucrose gradient increased the purity of the sample.

The yields were 0.08mg/ml for wildtype and 0.06mg/ml for VP2 S93Y inactivated particles. The purity and the intactness of the chemically inactivated capsids were further verified using electron microscopy (EM). A sample was loaded onto a Formvar coated EM grids and stained with 1% uranyl acetate. The capsids were examined under T20 transmission electron microscope running at 80kV.

The difference between FMDV particles containing the WT VP2 capsid protein and those containing the stabilized VP2 (S93Y) was remarkable (Figure 3-4). Most of the WT inactivated capsids were either dissociated or aggregated; only about 30% capsids were observed intact.

On the contrary, essentially all mutant capsids were intact. The results suggest that the mutant capsids are indeed more stable than WT. The thermo-stability results with other O1M mutants (discussed in chapter 6) agreed with the EM analysis and there was no noticeable change in the yields of WT and mutant O1M particles. A detailed analysis of the relative capsid stabilities and their correlation to the predicted $\Delta\Delta G$ derived from the simulations is presented in chapter 6.

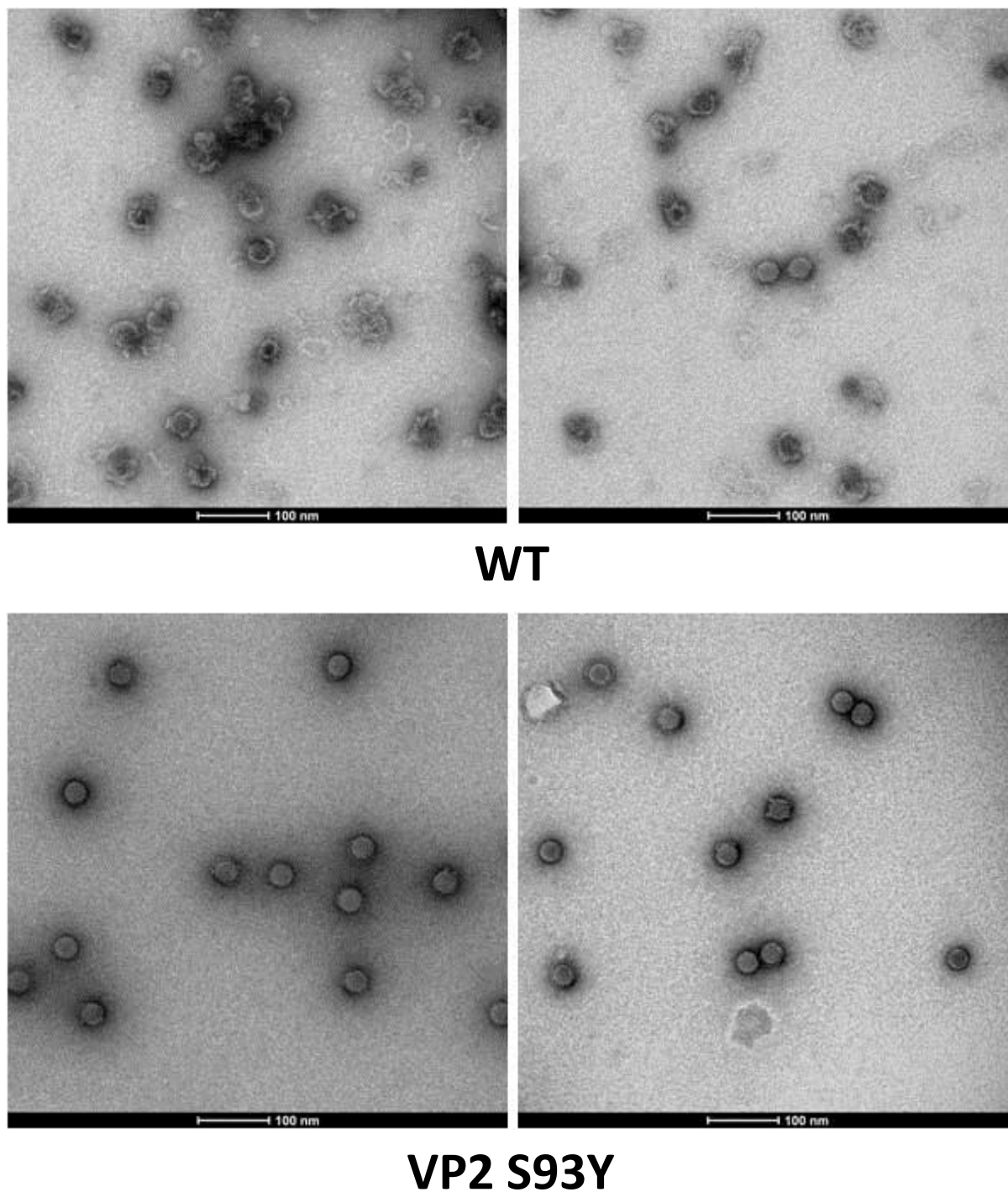


Figure 3-4: EM analysis of O1M WT and stabilized mutant capsids. When the purified inactivated capsids were examined by electron microscopy, WT capsids were found mostly dissociated or aggregated. In comparison, mutant capsids appeared intact, suggesting an increase in capsid stability.

3.6.2 Purification and analysis of FMDV ASIA1 serotypes

Inactivated serotype ASIA1 (subtype Bahrain 2003) was also purified using similar techniques. However, no band was observed after illumination of the first sucrose gradient used to sediment the virus. Samples of the gradient fractions were analysed by SDS PAGE and Coomassie staining.

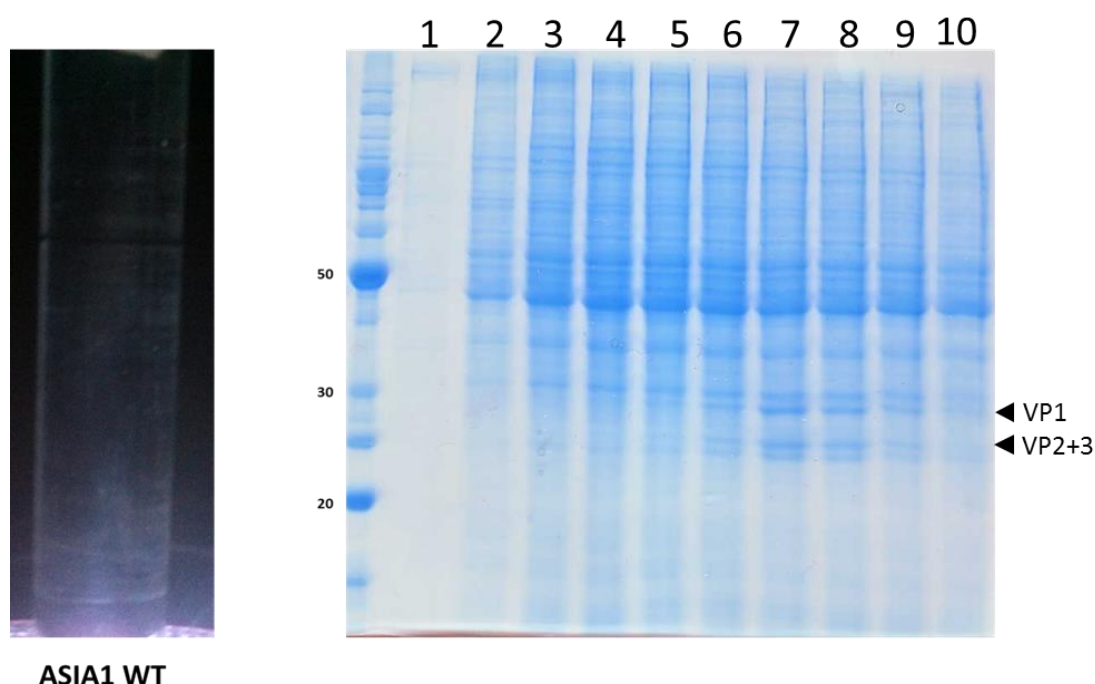


Figure 3-5: Purification of ASIA1 WT, no band was detected with wild type ASIA1 serotype, however, the viral proteins were detected in the bottom half of the gradient.

Although ASIA1 did not appear to band on a sucrose gradient, capsid proteins were observed on SDS PAGE at the bottom half of the gradient. The fractions containing virus were pooled together, sucrose removed and the sample concentrated. The concentrated sample was then sedimented on a second 15-45% sucrose gradient. Once again, no bands were observed on the illuminated sucrose gradient; however SDS PAGE analysis and Coomassie blue staining revealed the presence of capsid proteins in all samples corresponding to the lower fractions of the sucrose gradient (Figure 3-6).

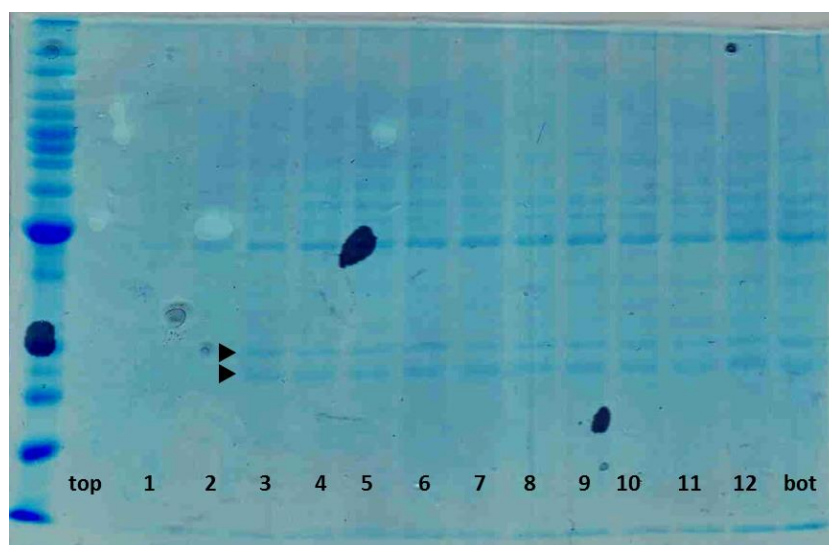


Figure 3-6: SDS PAGE analysis of ASIA1 WT. ASIA1 did not form a characteristic band after sedimentation through two consecutive sucrose gradients. However, capsid proteins were detected in the lower fractions of the second gradient when samples were analysed by SDS PAGE and Coomassie staining.

The non-banding behaviour of ASIA1 after sucrose gradient sedimentation and the presence of capsid proteins in all fractions of the gradients suggested aggregation of the inactivated viruses. With this in mind, fractions 4 to 7 and 8 to 12 were separately pooled, the sucrose removed and the intactness of the capsids was examined by EM. Intact capsids were observed in both samples and as expected, the capsids were aggregated, explaining the sedimentation behaviour of the viruses on the sucrose gradients. The aggregation may have been due to surface properties such as charge or hydrophobicity (Figure 3-7).

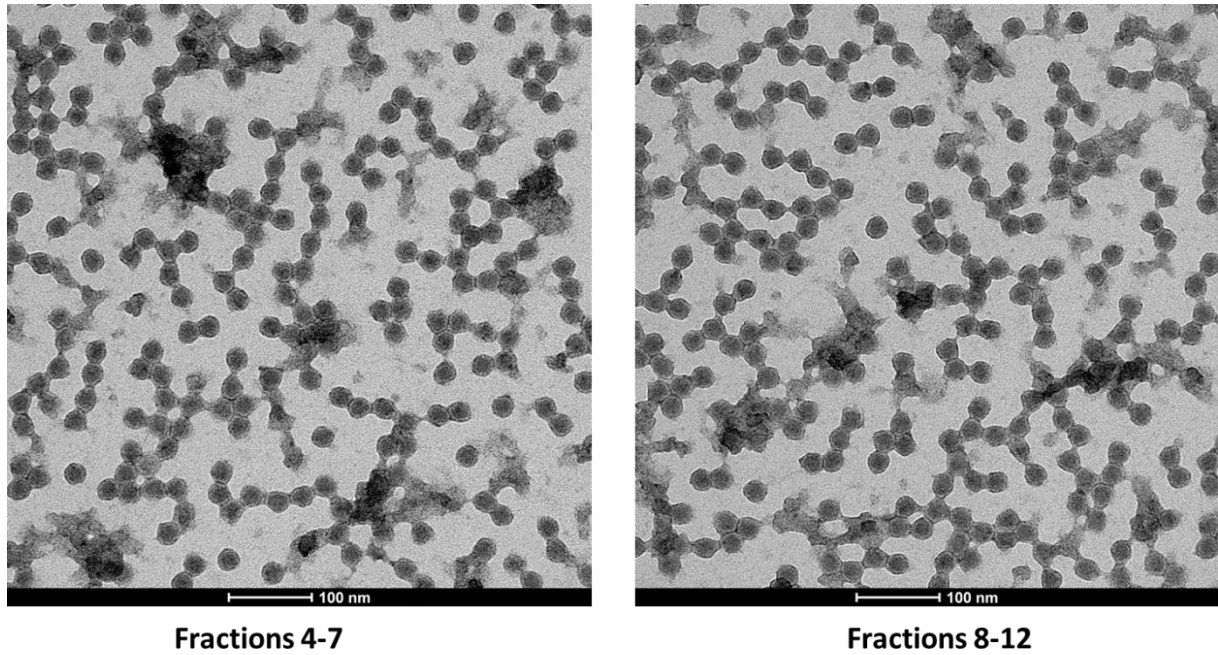
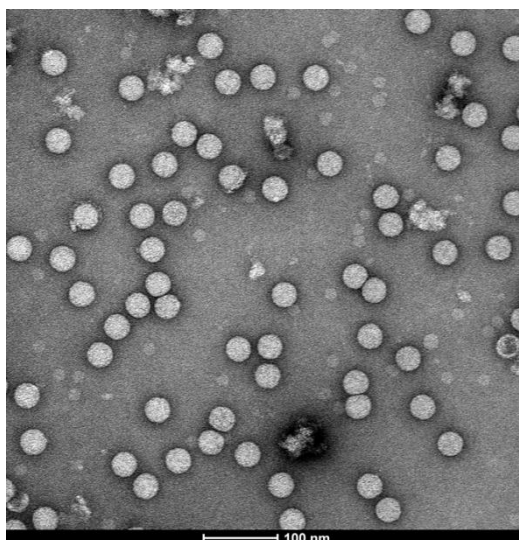


Figure 3-7: EM analysis of inactivated FMDV ASIA1. The ASIA1 capsids were intact but aggregated. The aggregation may have been caused by the surface properties of the capsids.

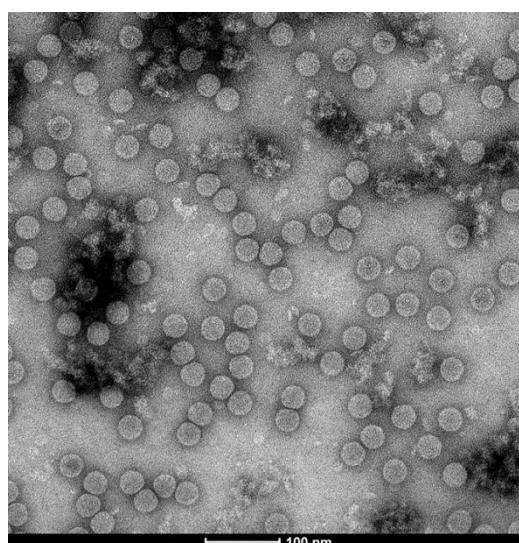
To stop the capsids from aggregation, the samples were incubated for 10 minutes, with a panel of additives including 0.5M NaCl, 5mM Dithiothreitol (DTT) or 50mM NDSB 256 (Non Detergent Sulfofetaines) and analysed by EM (Figure 3-8).

Addition of 0.5M NaCl or 5mM DTT to the sample removed aggregation and the capsids were found isolated. The change in pH to 9.0 also had the same effect, whereas NDSB had no effect on aggregation suggesting the inactivated ASIA1 particles were aggregating because of the surface charge of their capsids.

(A)



(B)



(C)

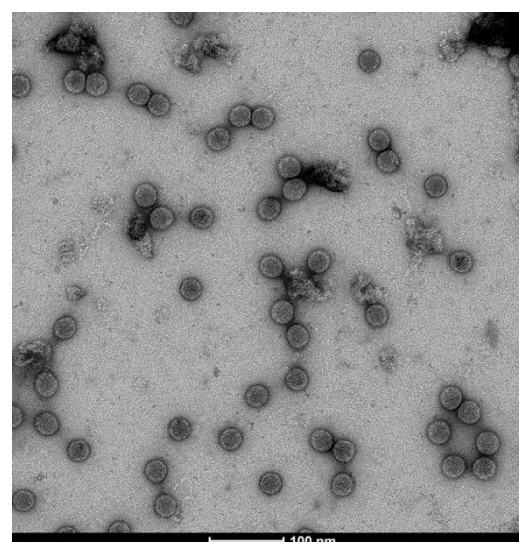


Figure 3-8: Optimization of inactivated ASIA1 capsid purification. Aggregation of ASIA1 capsids was prevented by addition of 0.5M NaCl (A) or DTT (B). Incubating the capsids at pH 9 (C) also prevented capsid aggregation suggesting the surface of the capsid is very charged.

3.6.3 Purification and analysis of FMDV SAT2 serotype

A sample of formulated vaccine of serotype SAT2, sub-type ZIM/7/83 was received from MSD (Merck) Animal Health. The purification used the same protocol as before, but instead of phosphate buffer, 20mM HEPES pH 7.4 and 150mM KCl was used as a buffer (according to MSD's instructions). Several bands were observed on the gradient. The gradient was fractionated and all fractions were analysed on the SDS PAGE however, no FMDV proteins were observed (Figure 3-9).

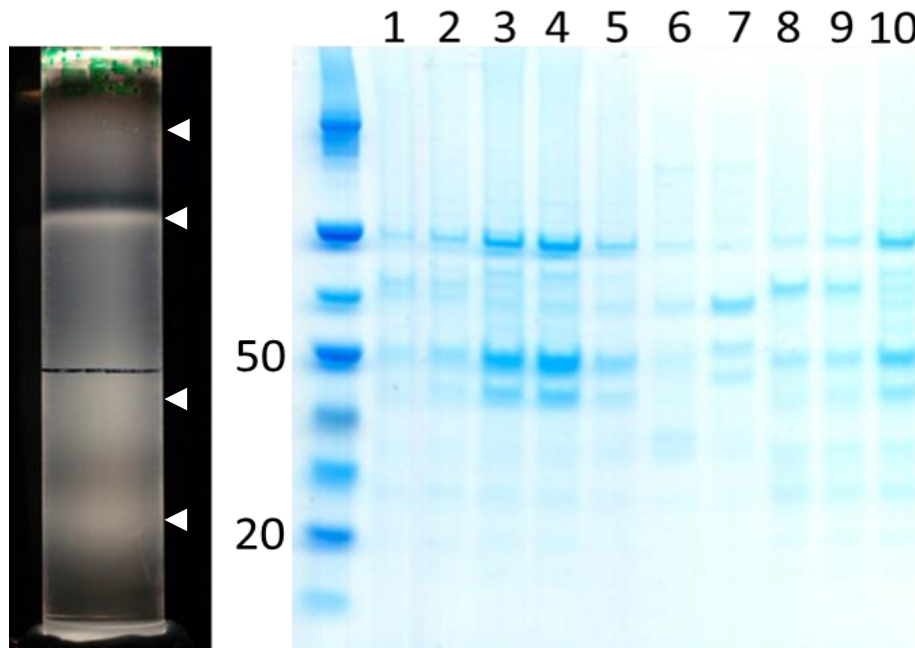


Figure 3-9: Purification of wildtype SAT2 from formulated vaccine. Inactivated SAT2 vaccine received from MSD Animal Health, showed several bands on a sucrose gradient; however, on SDS PAGE analysis no FMDV capsids proteins were detected.

New inactivated capsid samples were received from the Pirbright Institute containing SAT2 (Zim/7/83) wildtype and VP2 S93Y mutant. These samples were prepared from infectious virus received from ARC-OVI, and were inactivated twice with BEI treatment as before. The inactivated viruses were precipitated using 8% PEG6000 and then re-suspended in HEPES buffer (50mM HEPES pH 8.0, 200mM NaCl containing 1% NP40) and pelleted over a 3ml, 30% sucrose cushion by centrifugation at 105,000xg for 5 hours at 10°C. The pellets were re-suspended in HEPES buffer without detergent and analysed by EM (Figure 3-10).

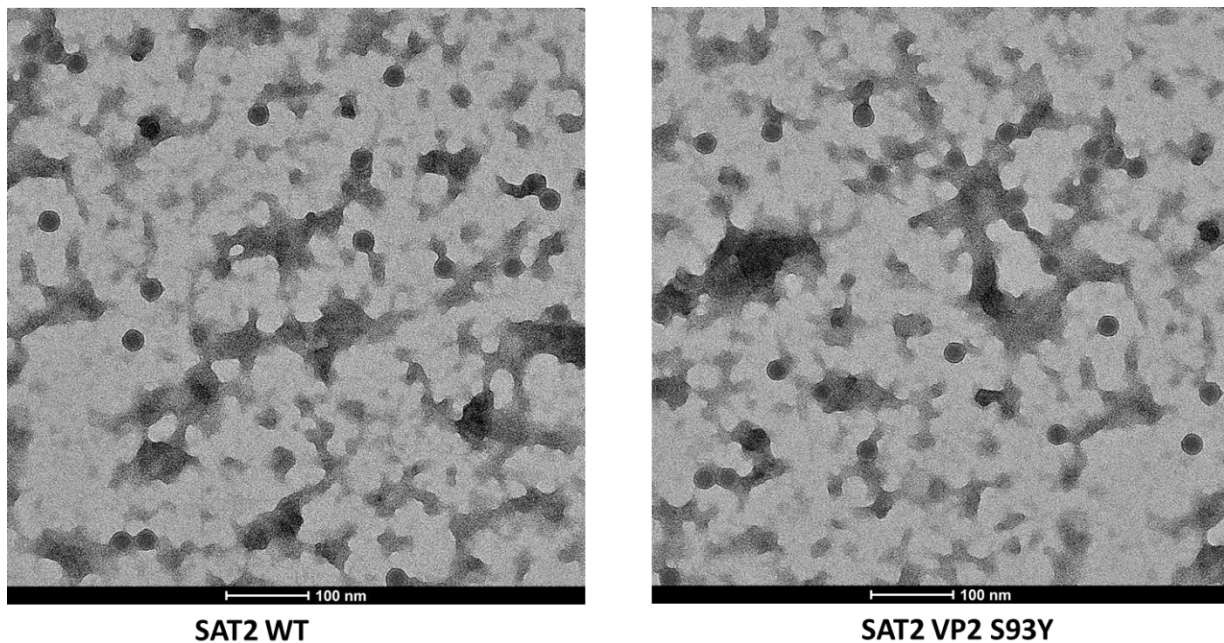


Figure 3-10: EM analysis of inactivated SAT2 pelleted through a 30% sucrose cushion. The sucrose pelleted sample was re-suspended and analysed by EM. Intact capsids were detected for both wildtype and mutant inactivated FMDV SAT2.

EM analysis evidently showed the presence of intact capsids for both wildtype FMDV SAT2 and the FMDV VP2 S93Y mutant. The capsids were then purified using 10-50% sucrose gradients prepared in 50mM HEPES pH 8.0, 200mM NaCl; fraction samples were analysed by SDS PAGE and Coomassie blue staining (Figure 3-11).

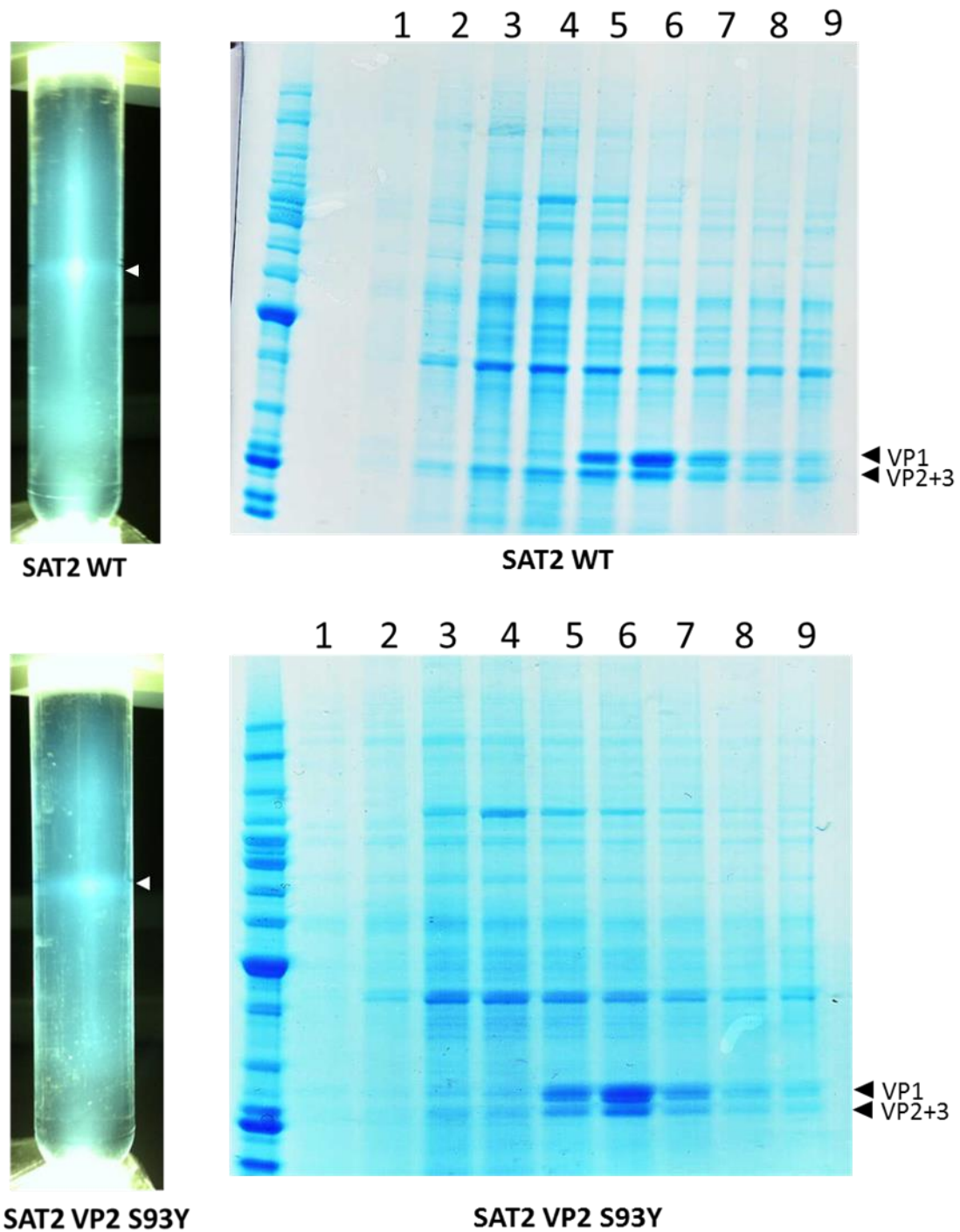


Figure 3-11: Purification and analysis of inactivated FMDV SAT2 capsid proteins. Viruses were purified using 10-50% sucrose gradients. A band was detected in both samples at the middle of the gradient. The gradient was fractionated and SDS analysis of the fractions confirmed the presence of FMDV capsid proteins with a peak in fraction 6 for both wildtype and stabilised mutant containing the VP2 S93Y mutation.

A clear band was observed at the middle of the gradient and the SDS PAGE analyses confirmed the presence of FMDV capsid proteins in fractions 3 to 9 with a peak in fraction 6. Similar results were obtained for the mutant SAT2 virus. A small amount of sample from fraction 6 of the WT and mutant sucrose gradients was analysed by EM.

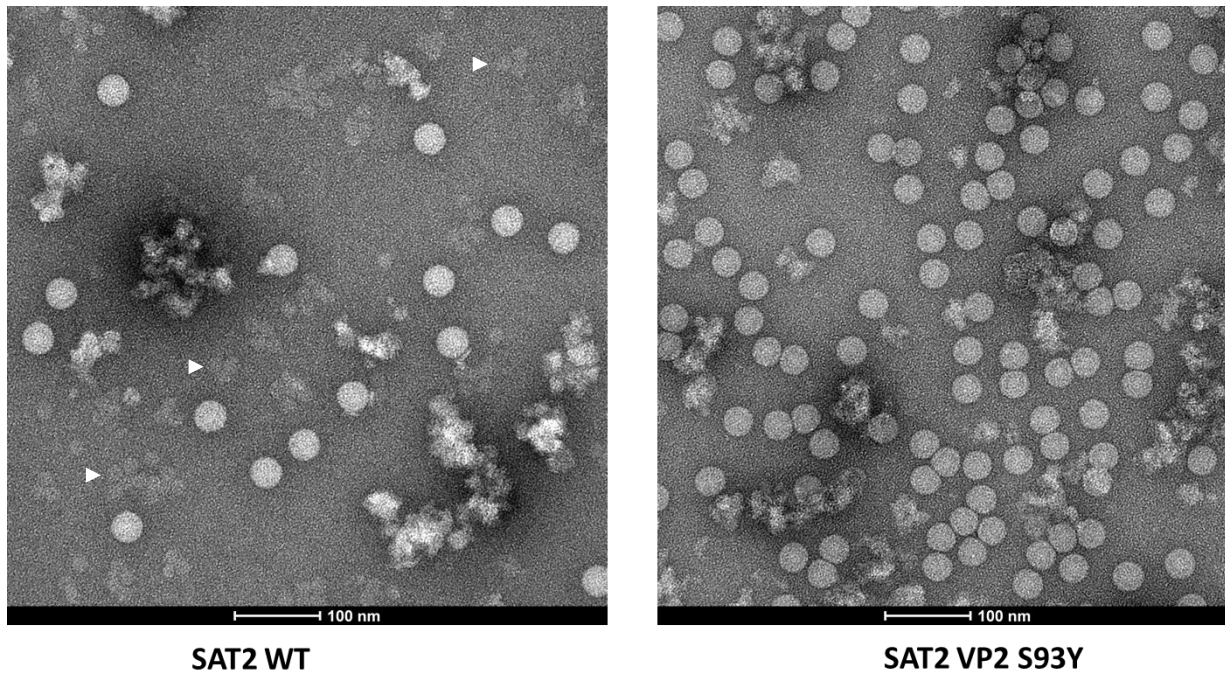


Figure 3-12: EM analysis of inactivated FMDV SAT2. EM confirmed that the band detected on a sucrose gradient contained intact SAT2 capsids. Some pentamers were also detected (white arrows) with wildtype but not with mutant.

As with O1M, the sucrose gradient fractions required further purification to remove contaminating proteins and aggregates. The peak fractions 5-7 were pooled, sucrose removed and samples concentrated. Further purification was achieved by running the samples on a second 10-50% sucrose gradient (Figure 3-13). The yield for the wildtype SAT2 was low at 0.03mg/ml whereas for VP2 S93Y it was 0.08mg/ml similar to that obtained for O1M inactivated particles.

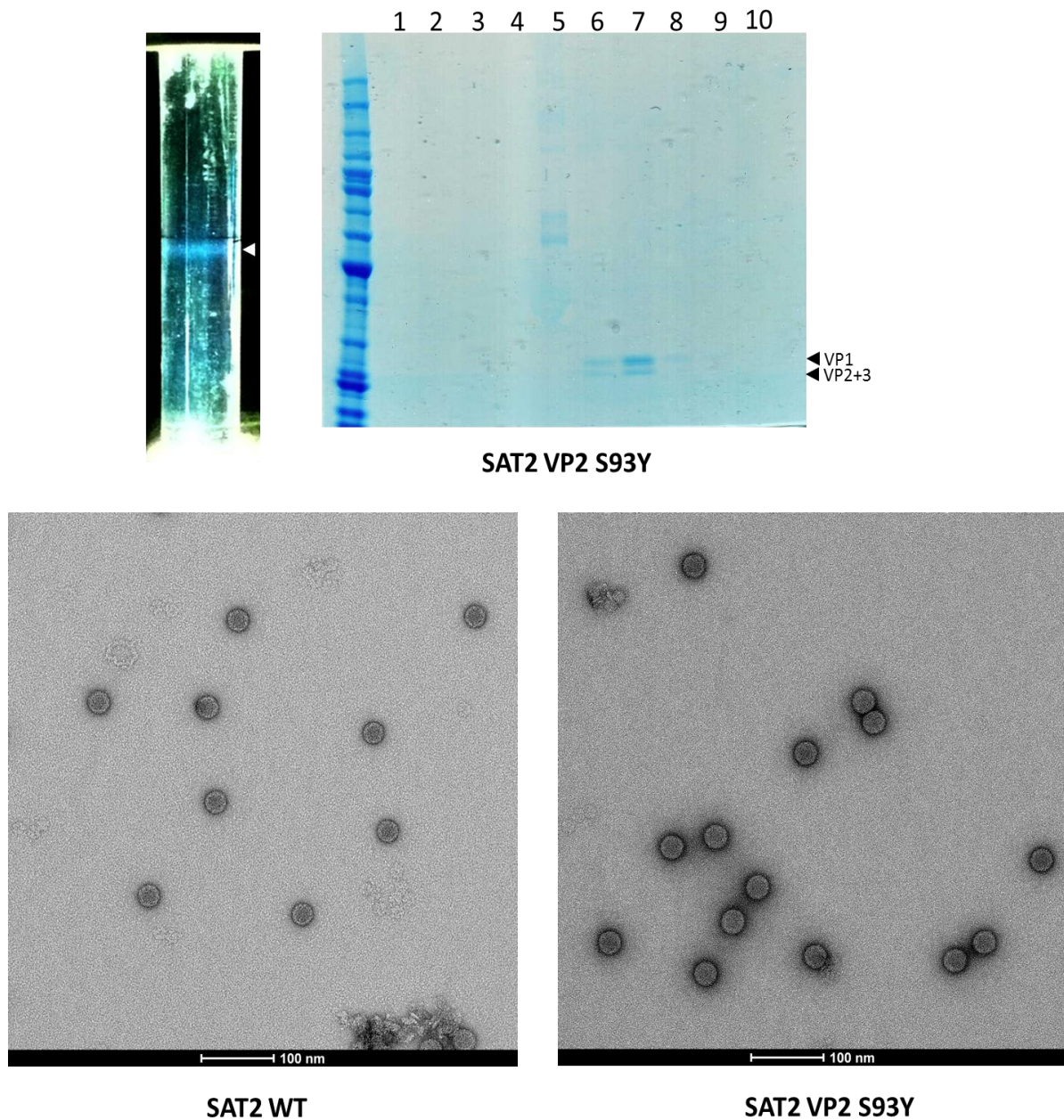
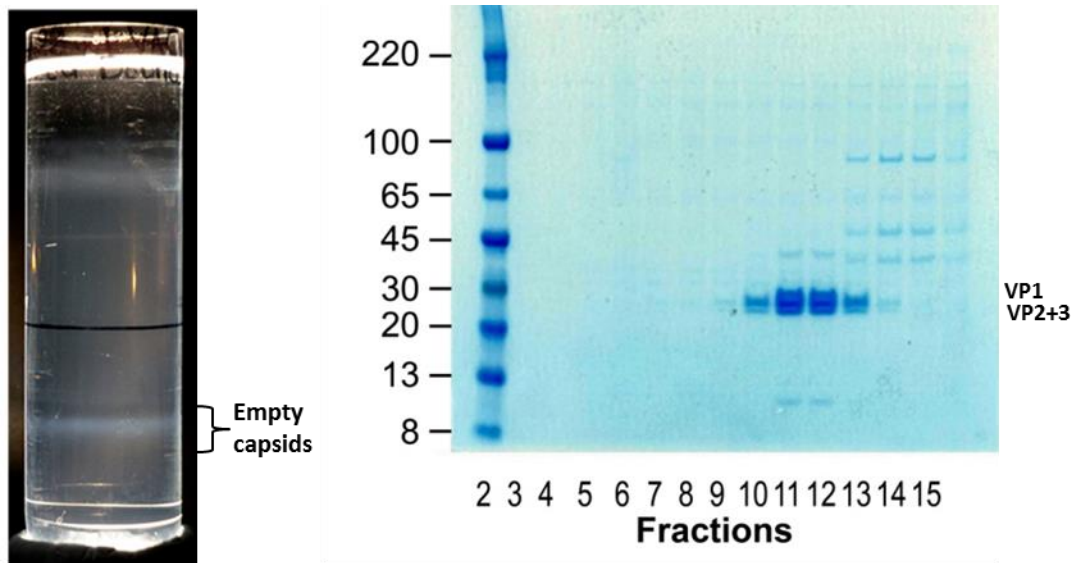


Figure 3-13: Further purification and analysis of inactivated FMDV SAT2. The contaminating proteins and protein aggregates were removed by purifying the capsids through a second sucrose gradient. A single clear band was obtained at the middle of the gradient and SDS PAGE and EM analysis confirmed the purity and intactness of the capsids.

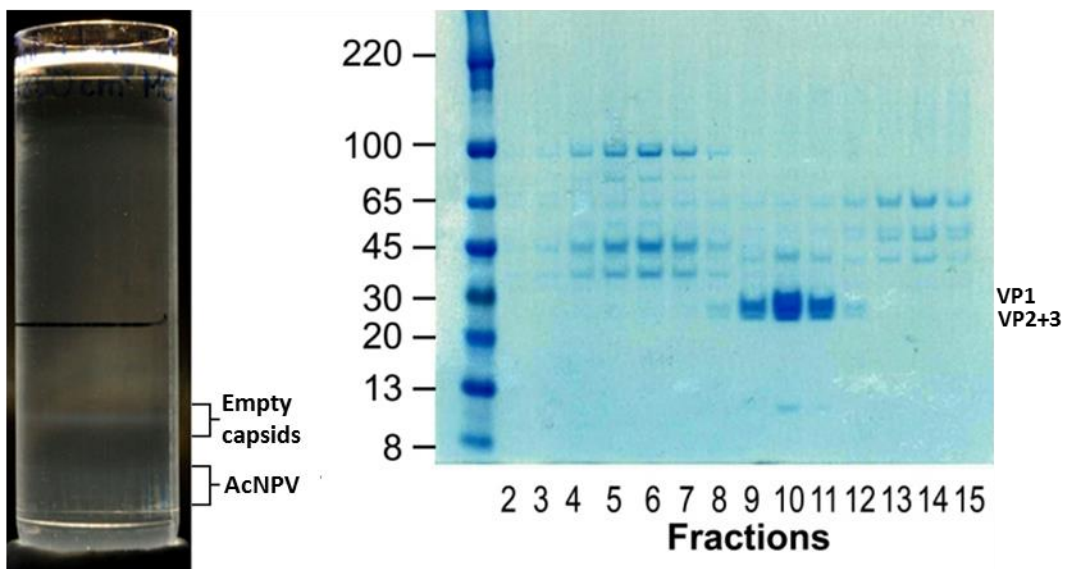
A single band was observed at the middle of the gradient and SDS analysis showed only FMDV capsid proteins. EM images also showed pure and intact SAT2 capsids for both, wildtype and mutant.

3.6.4 Purification and analysis of recombinant empty capsids

The recombinant empty capsids proteins of A22, O1M and SAT2 wildtype and mutants were successfully expressed in both vaccinia virus (VV) and baculovirus insect cell expression systems and the proteins self-assembled into the viral capsids. However, the expression and assembly of O1M and SAT2 wildtype, using an insect cell expression system was not very efficient and yielded only very small amounts of capsids (proteins could only be detected by Western bolt analysis). Capsid yields for the O1M and SAT2 serotypes using the VV system were also lower than that obtained for the A22 capsids. These results may be due to the culture medium used for growing the insect cells. The media is rather acidic and is at a pH of about 6.5, correlating to the pH at which the FMDV capsid dissociates. In addition, it takes up to 5 days before the cells can be harvested, making these conditions unfavorable for the unstable wildtype capsids. The cells expressing recombinant A22 particles were lysed by incubation with detergent (1%, NP40) for 20mins on ice and the cell debris removed by centrifugation at 48000xg. The capsids were pelleted over a 3ml, 30% sucrose cushion (105000xg, 5hrs at 12°C). The pellets re-suspended in phosphate buffer and the capsids purified over two consecutive 15-45% sucrose gradients as above. The capsids sedimented at the same position on the sucrose gradient as those purified from the inactivated capsids. The results were similar with both, VV and insect cell expressed empty capsids (Figure 3-14). A second lower band corresponding to the proteins from baculovirus nucleocapsids was also observed on the gradients loaded with the lysates from insect cells. The yields ranged from 0.8µg/ml, for A22 VP S93C, to 1.2 µg/ml, for A22 wildtype capsids.



VV expression system



Insect expression system

Figure 3-14: Purification of recombinant FMDV empty capsids of A-serotype virus, A22 Iraq. The recombinant empty capsids were expressed using VV or insect expression systems. Both systems successfully produced the capsids for the A22 serotype. Capsid expression was greatly reduced for the O1M and SAT2 serotypes using the insect expression system, however, their respective yields improved with the stabilized capsids (the expression and purification of A22 wildtype and VP2 S93C was performed by Claudine Porta).

The recombinant capsids were further analyzed by EM. The capsid proteins were found to self-assemble into spherical capsids with a diameter of 28-30nm which is identical to the inactivated FMDV capsids. The stain penetration in the capsids is consistent with the empty capsid assembly (Figure 3-15).

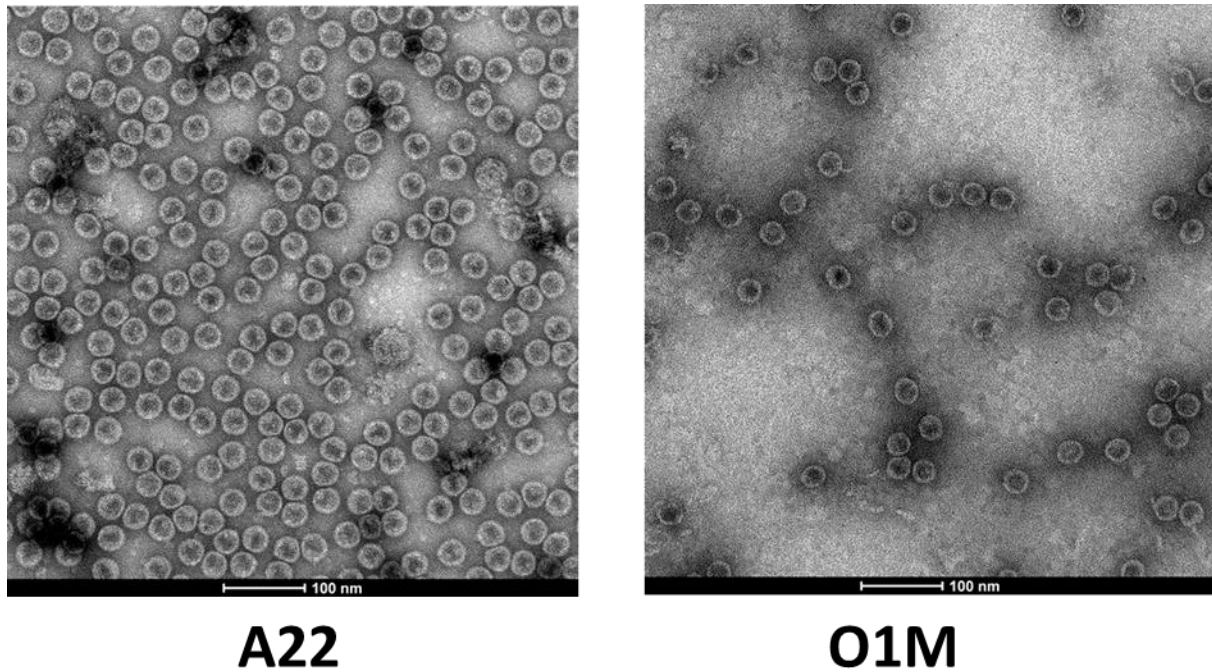


Figure 3-15: EM analysis of recombinant FMDV empty capsids. Sucrose gradient fractions containing FMDV recombinant capsids were analysed by EM. The proteins self-assembled into viral capsids for both A and O serotypes. The penetration of stain inside capsids is indicative of empty capsid assembly.

The covalent mutant strategy was not overly successful, but did work with the recombinant A22 Serotype that contained a single disulfide bond at the 2-fold axis, position 93 of VP2 (VP2 S/H93C). In the case of the O serotype, capsid yield was found to be extremely low. The double and triple disulfide mutants either did not form any capsids or only formed protein aggregates.

3.7 Conclusion

The infectious and recombinant empty capsids of FMDV were successfully produced in mammalian and insect cell lines. There was no noticeable difference in production of infectious wild type and stabilized mutant virus. The capsids were successfully purified using sucrose gradient sedimentations. However, the chemical inactivation had a severe impact on capsid stability for the particles we have examined. The destabilization effect of chemical inactivation on the viral capsid stability has been already shown by Doel and Baccarini (1981). In line with their experiment, the results from our EM experiment showed that inactivated wild type capsids were mostly dissociated. However, the inactivation had no effect on stabilized capsids and the capsids remained intact.

Recombinant capsids proteins were also successfully produced in both vaccinia virus mammalian expression system and baculovirus insect expression system. The proteins self-assembled into empty virus capsids that were identical to inactivated capsids. The expression of wild type empty capsids of unstable serotypes such as O and SAT2 was greatly reduced in the insect cell expression system. However, the expression improved with stabilized mutant capsids. The covalent disulfide bond at the interface was not successful for empty FMDV capsids of a serotype other than A22 serotype. Moreover, such mutants would only work for empty capsids as the resulting capsids are permanently fixed and infectious capsids would probably no longer be able to uncoat upon infection, essentially producing a dead virus.

A detailed description of inactivated and recombinant empty capsids thermostability and the correlation of capsid stability to the predicted binding free energy from the MD simulations are presented in chapter 6 and the use of stabilized capsids as a vaccine with improved properties is described in chapter 7.

**Chapter 4 : FMDV structure determination -
Crystallography, data collection and processing of
FMDV crystals**

4.1 Introduction

X-ray crystallography is the gold standard method for the determination of high resolution three-dimensional structures of macromolecules. The three dimensional (3D) structures of the virion particles and empty capsids determine features such as the folding of the capsid proteins and their antigenic properties. Following the rational design and production and introduction of stabilizing mutations into the capsids it is important to ensure that the external antigenic structures resemble the wildtype virion or empty capsid and that the mutations does not cause structural perturbations that may alter the antigenicity. It is also important to verify that the stabilizing mutations are present and have the expected structural effect. It is therefore necessary to obtain X-ray crystallographic structures for both wildtype and mutants for the serotypes under investigation.

This chapter describes the methods used for the production of diffracting FMDV crystals, crystal quality optimization, X-ray diffraction data collection using an *in-situ* data collection method, and data processing from multiple crystals. The chapter begins with a general introduction to protein crystallography (a generic term taken to include virus crystallography) and X-ray diffraction methods and is followed by the results obtained from the data collection and processing.

4.2 Basic principles of protein crystallography

The determination of the 3D structure of proteins by X-ray diffraction comprises several steps and starts with the purification of proteins to homogeneity and the formation of ordered crystals. Protein crystals, unlike crystals of small molecules and salts such as NaCl, are very fragile and sensitive to dehydration and are hence kept in their crystallisation solution, also called mother liquor. This is required even during the diffraction data collection.

The crystallisation of a protein from solution does not usually occur spontaneously. It is achieved by gradually altering the protein solubility in order to reach a state of super saturation. The usual approach is to use precipitants to slowly drive the proteins out of solution (Rayment 1985). Crystallisation is a thermodynamic process, driven by the minimization of the free energy of the system. The protein molecules in solution are at equilibrium with the solvent. When more molecules (precipitant) are added, the system undergoes internal changes and these precipitants compete with solvent for hydration. When the system reaches supersaturated state, it is no longer at equilibrium. The individual molecules will come together and form many new non-covalent interactions resulting in a transition of the molecules between the liquid and solid phases. In highly favourable conditions, the solid state can form stable crystal nuclei, minimizing the free energy of the system. The formation of a stable nucleus is a first order phase transition where molecules form an ordered state on which further growth occurs, leading to the formation of an ordered crystal (Chayen and Saridakis 2008; J. Drenth and Haas 1992). The entire process of crystallisation can be illustrated by a protein crystallisation phase diagram, as shown in Figure 4-1.

The process of producing highly ordered diffracting crystals is one of the rate limiting factors in crystallography. However, a number of commercial crystallisation screens are available that can be used to reduce the protein solubility and stimulate crystal formation.

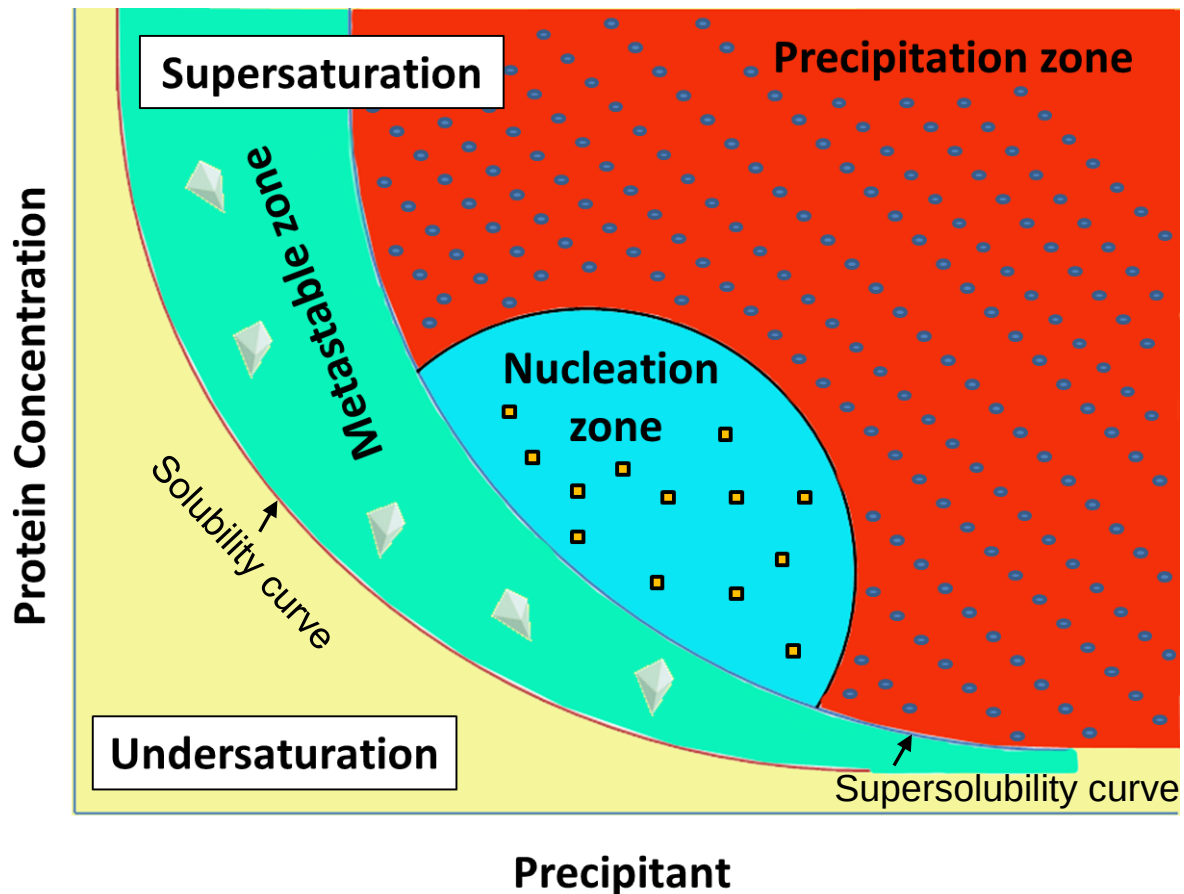


Figure 4-1: Illustration of crystallisation of phase diagram. The crystallisation process is diagrammatically represented in the form of a phase diagram. When the system reaches a supersaturation state, in some favourable conditions stable nucleation is achieved. The stable nuclei grow further forming ordered crystals. The ‘precipitant’ could include additives, changes in protein concentration, pH or temperature. Figure adapted from Chayen (2004).

There are several methods for producing protein crystals, including the vapour diffusion batch method, crystallisation by dialysis, free interface diffusion and lipidic mesophases. Each of these methods explores the phase diagram via different equilibrium kinetics. Vapour diffusion is the most widely used method for crystallogenesis. The method can be implemented using sitting drop or hanging drop techniques. In both techniques, a droplet of purified protein mixed with crystallisation solution (precipitant) is equilibrated with a reservoir containing similar precipitant in higher concentration. As the water vaporizes and diffuses into the reservoir, the precipitant concentration in the protein droplet increases

gradually driving it to supersaturation and thus resulting in the formation of crystals. These techniques are micro-techniques where by many crystallisation trials can be carried out with only a few microliters of protein (McPherson 2004; Walter et al. 2005).

4.2.1 Crystallisation screening and optimization

A common practice to produce protein crystals is the use of commercially available crystal screens, also referred to as sparse matrices which contain vast combinations of chemical solutions and help explore parameters such as precipitant concentration, pH, ionic strength, and specific additives (Jancarik and Kim 1991). Most of these screens contain a wide range of popular protein precipitants which have been used for successful crystallisation of other proteins and also include salts (eg. ammonium sulfate, ammonium acetate and lithium sulfate), polymers (eg. polyethylene glycol), organic solvents and the combinations of these at a range of different pHs (Berry et al. 2006; Stevens 2000).

When a promising condition is discovered, a “fine screening” is used to optimize the crystal quality and growth. This is achieved by making small changes in variables such as precipitant concentration, pH, temperature and additions of small molecule additives. Such optimization of the initial lead can help to get good quality diffracting crystals (Walter et al. 2005).

4.2.2 Crystal symmetry

As discussed before, a protein crystal is a pre-requisite for the determination of the 3D structure of a protein. A crystal can be described as an ordered array or lattice, of constituent protein and solvent molecules arranged in a periodic pattern of identical repeating units extending in all three spatial dimensions. The smallest repeating unit is called the unit cell that fills the space of the lattice. The lengths of the sides of a unit cell and the angles between

them are called the lattice parameters and are defined by a , b and c and the corresponding angles α , β and γ respectively (Figure 4-2). This can be also described as the vectors: **a**, **b** and **c** which through simple unit translations can generate the entire crystal.

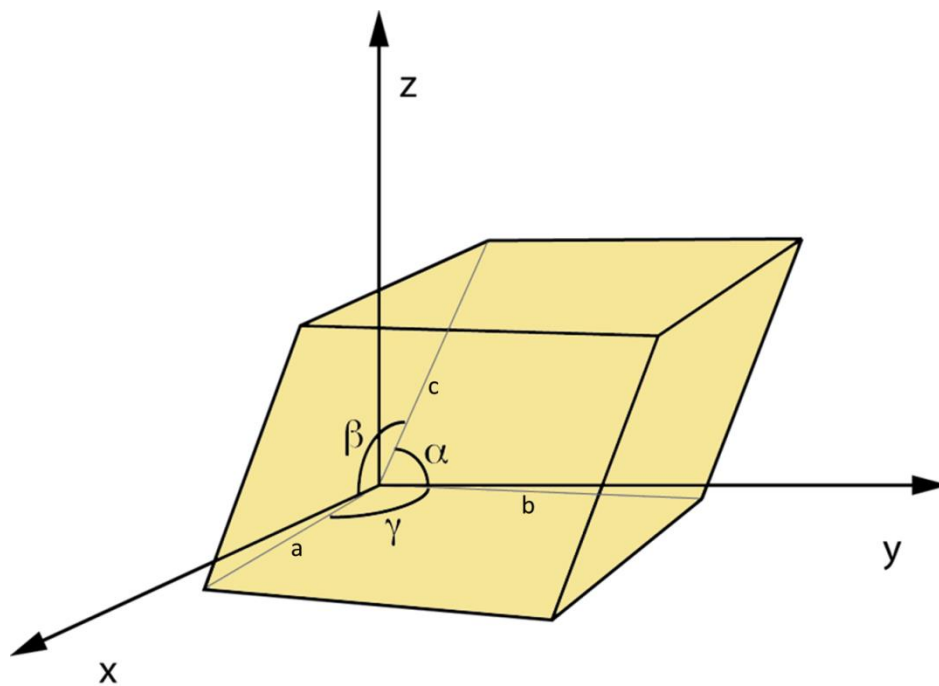


Figure 4-2 Schematic representation of a unit cell. A unit cell shown as parallelepiped with length of the edges as a , b and c and the angles α , β and γ .

The unit cell can be further divided into a unique portion called the asymmetric unit, which is the smallest unit of volume which by the application of crystallographic symmetry operations can produce the unit cell. The asymmetric unit may often contain more than one copy of the same protein molecules. The symmetry operations superimposing these multiple copies are called non-crystallographic symmetry (NCS) operators.

4.2.3 Crystal systems

There are essentially seven distinct crystal systems and every crystal belongs to one of these crystal systems. The crystal system imposes restrictions to the relative lengths of the unit cell vectors **a**, **b**, **c** and the corresponding angles between them. The seven crystal systems and their cell parameters are listed in Table 4-1. The restrictions on the lattice parameters arise from the underlying symmetry of crystal, for instance an orthorhombic crystal must contain three mutually perpendicular 2-fold rotation or screw axes.

Table 4-1: The crystal systems and conditions for cell parameters.

Crystal System	Lattice Parameters
Triclinic	$a \neq b \neq c, \alpha \neq \beta \neq \gamma$
Monoclinic	$a \neq b \neq c, \alpha = \beta = 90^\circ \neq \gamma$
Orthorhombic	$a \neq b \neq c, \alpha = \beta = \gamma = 90^\circ$
Tetragonal	$a = b \neq c, \alpha = \beta = \gamma = 90^\circ$
Cubic	$a = b = c, \alpha = \beta = \gamma = 90^\circ$
Hexagonal	$a = b \neq c, \alpha = \beta = 90^\circ;$
Trigonal (Rhombohedral)	$a = b \neq c, \alpha = \beta = 90^\circ; (a = b = c, \alpha = \beta = \gamma = 90^\circ)$

In addition, the symmetry elements of the crystal systems when coupled with the lattice centering, gives rise to 14 distinct types of lattice called Bravais lattices. These lattices may be primitive (P), body-centered (I), face-centered (F) or base-centered (C). Additional symmetry elements can be applied on the asymmetric unit to generate the contents of the unit cell, namely mirror planes, screw axes, rotation axes, glide planes and inversion centers. The combinations of all these symmetry operators that can be used to define the relationship of an asymmetric unit to any another in the unit cell gives rise to 230 space groups. Since protein molecules are chiral and only occur in L form, symmetry elements that change the hand of the object are excluded, so that only 65 space groups are available for these molecules. All these space groups and the relation of asymmetric units within the unit cells of different

crystal systems can be found in the International Tables for Crystallography (Hahn and Paufler 1984; McPherson 2003)

4.2.4 X-ray diffraction

X-rays are electromagnetic radiation with a wavelength (λ) in the range of about 0.01 to 10nm (0.1 to 100Å). Traditionally, X-rays are generated when a stream of high energy electrons collides with a metal such as copper. The collision leads to the excitation of metal electrons to the higher energy levels and X-ray photons are emitted. At synchrotron light sources, the X-rays are produced from a storage ring which contains circulating electrons that are maintained at high energy (typically 2-8 GeV for the efficient production of hard X-rays). When this high energy electron beam passes through strong magnetic fields, generated by so-called bending magnets and insertion devices, X-rays are emitted, tangential to the electron beam. The nature of the magnetic device (field strength, number of magnetic poles) together with the energy of the electrons determines the spectrum of X-rays produced. The benefits of using synchrotron radiation are manifold compared to a conventional X-ray generator. Synchrotron X-rays have many orders of magnitude higher brightness and intensity and the beams are highly polarized and collimated. An X-ray generator containing a $\text{CuK}\alpha$ anode generates X-rays with a wavelength of 1.54 Å, whereas synchrotron sources have a wide range of tunability in energy (and thus, wavelengths) making them more advantageous in comparison to lab-based X-ray generators.

The reason for using X-rays to study the 3D structure of proteins is because of their wavelength which is in the same order of magnitude as that of the bond lengths of atoms, typically about 1.5Å - thus X-rays can be utilized to visualize the geometry and the atomic structure of proteins.

Individual atoms in protein molecules can scatter X-rays; however most of the energy passes through them and the scattering is too weak to be detected. The problem can be solved by using crystals as a starting material. As discussed previously, crystals are an ordered array of identical repeating units, therefore the scattering of X-rays from the crystal is amplified, given the scattered waves interfere constructively and a diffraction maximum can be observed. The diffraction pattern is essentially the Fourier transform of the convolution of the protein molecule with the periodic crystal lattice.

W.L. Bragg described the diffraction of X-rays from a crystal as the reflection of waves from lattice planes (Figure 4-3). According to Bragg's law, only at certain positions, these reflections can be measured, where constructive interference occurs. The constructive interference between scattered waves from successive lattice planes will only occur if the path difference (d) between the waves is an integral multiple of the wavelength (λ) ($d=AB+BC$ in Figure 4-3). Thus, Bragg's law provides a relationship between the inter-planar spacing and reflected X-rays that generate constructive interference:

$$n\lambda = 2d_{hkl} \sin\theta \quad (18)$$

Where λ is the wavelength, d_{hkl} is the inter-planar spacing, n is the order of reflection and θ is the incident angle (Blow 2002; Rhode 2000). hkl are the indices of the plane and describe its fractional intercept along **a**, **b**, and **c** respectively, the so-called reciprocal lattice.

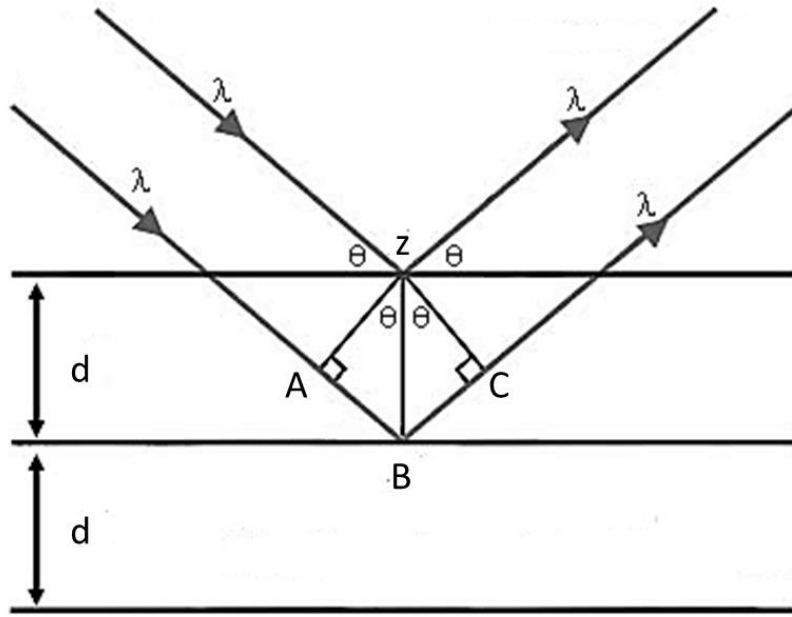


Figure 4-3: Geometric representation of Bragg's law.

The reciprocal lattice is directly related to the real lattice (crystal from which it arises) and has the same symmetry. Therefore, if the wavelength of the incident X-ray and the diffraction angle at which the constructive interference occurs are known, and the indices hkl are known, then the inter-planar spacing (d_{hkl}) can be calculated by solving Bragg's equation. Application to several reflection gives the unit-cell parameters of the crystal and thereby an indication of the likely crystal system (but usually not the space group). To achieve this, the assignment of the indices to each reflection is needed. This process is called indexing (discussed below) (Steller et al. 1997).

4.2.5 Ewald Sphere

The Ewald sphere is an imaginary geometric construction that allows the visualization of the relationship of reciprocal lattice (a lattice whose points are defined by the indices hkl) with Bragg's Law. The construction of the Ewald sphere can be represented in 2D as shown in

Figure 4-4 in which a crystal is placed at the center of the sphere that has a radius (R) corresponding to the reciprocal of the wavelength of the radiation, thus $R=1/\lambda$.

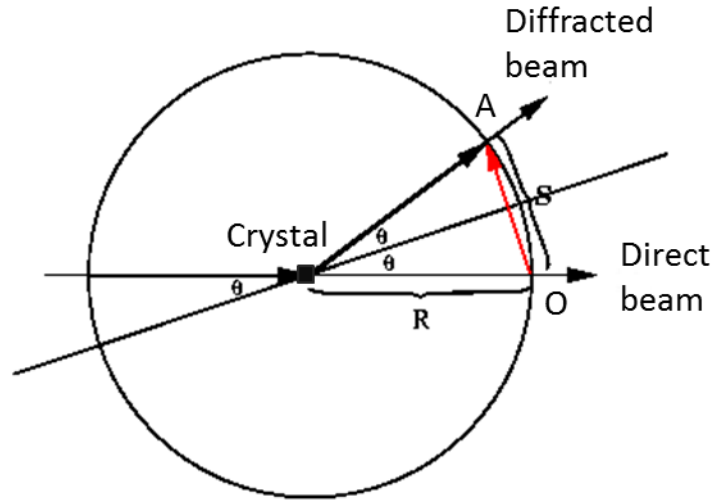


Figure 4-4: Two-dimensional representation of the Ewald sphere

Consequently from the figure above, the following equation can be derived:

$$\frac{\frac{1}{2}OA}{1/\lambda} = \sin\theta \quad \text{or} \quad \lambda = \frac{2\sin\theta}{OA} \quad (19)$$

By substituting $OA = 1/d$, we get

$$\lambda = 2d\sin\theta \quad (20)$$

Which is the same as Bragg's law with $n = 1$. The representation above shows that for any reciprocal lattice vector 'S', Bragg's law will be satisfied for only those lattice points that fall on the sphere and for these a diffraction spot will be observed. Therefore, the Ewald sphere helps to determine lattice planes that will result in a diffraction spot for a given wavelength.

Generally, a relatively small number of reciprocal lattice points will satisfy the Bragg's law, as a result the crystal must be rotated with respect to the incident beam in order to sample sufficient reciprocal space (Blow 2002).

4.2.6 Structure factors and calculation of electron density

The determination of a crystal structure is a multistep process. The major limiting factor is often obtaining diffraction quality crystals. Once the diffraction images are obtained, the second step is the determination of unit cell parameters, followed by the assignment of space groups and the estimation of the relative phases of the reflections. Once the phases are obtained, an electron density map can be calculated directly. Finally, the electron density map can be used for model building and structure refinement. The phase estimation, model building and structure refinement are discussed in Chapter 5.

Each diffraction spot, identified by the three indices hkl is termed as structure factor and each structure factor can be described as a wave with specific relative phase and amplitude. In a diffraction experiment, the intensities of the X-ray beam (Bragg reflection) are measured from the diffraction spots. These experimentally measured intensities, after appropriate correction, relates directly to the square of its amplitude. Thus:

$$I_{(hkl)} \propto |F_{hkl}|^2 \quad (21)$$

Where $I_{(hkl)}$ is the intensity of the reflected X-rays from crystal planes and $|F_{hkl}|$ is the structure factor amplitude. The observed diffraction spots are a sum of individually scattered

waves from crystal lattice planes that are characterized by their indices h,k,l . Therefore, the structure factor equation takes the following form:

$$F_{(hkl)} = \sum_{i=1}^n f_i \cdot e^{2\pi i(hx_i+ky_i+lz_i)} \quad (22)$$

Where f_i is the atomic scattering factor of the i^{th} atom of n in the crystallographic unit cell, with the fractional co-ordinates: x_i, y_i and z_i .

As an alternative, the structure can be represented by the electron density (ρ) at every point xyz . Using this formulation the structure factor equation can be integrated over all electrons within the unit cell. This gives the atomic scattering factor as electron density and can be expressed as:

$$F_{(hkl)} = V \int_{x=0}^1 \int_{y=0}^1 \int_{z=0}^1 \rho(xyz) \cdot e^{2\pi i(hx_i+ky_i+lz_i)} dx dy dz \quad (23)$$

Where V represents the volume of the unit cell, ρ is the density expressed as electrons per unit volume, F is the structure factor expressed as electrons per unit cell and $2\pi i(hx_i + ky_i + lz_i)$ is a phase factor in 3D. Three nested integrations are taken over 0 to 1 to cover the whole unit cell. The inverse Fourier transform of equation (23) gives the real space electron density as:

$$\rho(x, y, z) = \frac{1}{V} \sum_{hkl} F_{hkl} \cdot e^{-2\pi i(hx+ky+lz)} \quad (24)$$

Unfortunately, during the diffraction experiment only intensities are measurable which are equal to $|F_{hkl}|^2$. The phase angles cannot normally be measured directly. This is known as the phase problem in crystallography. The methods for estimating the phases will be discussed in the next chapter (Blow 2002).

4.2.7 Diffraction data processing

The data processing is divided into four consecutive stages, namely (i) indexing the reflections which determines the unit cell parameters and the orientation of the crystal, (ii) integration which consist of predicting the position of diffraction spots on each image and obtaining estimates of the intensity of each observed reflection, (iii) scaling, the application of a scale factor to bring the reflection intensities onto the same scale and (iv) merging of equivalent reflections. The latter two stages are often associated with a refinement of the parameters initially determined at the indexing stage. The objective of whole process is to determine from the diffraction images the best intensities for as complete a set of indices (hkl) as possible, and an estimate of the unit cell parameters.

4.2.7.1 Indexing of reflections

The diffraction image provides two separate pieces of information: (a) the intensity of the reflections - that gives partial information about the content of the lattice as discussed previously in section 4.2.6 and (b) the geometrical arrangement of the reflections. The latter gives the information about the orientation, unit cell dimensions and hints to the symmetry of the crystal. This information is obtained by the process called indexing whereby a consistent set of reciprocal lattice vectors $\mathbf{a}^*$, $\mathbf{b}^*$ and $\mathbf{c}^*$ (reciprocal lattice vectors are denoted by $*$) are associated with the diffraction pattern. The corresponding real space vectors $\mathbf{a}$, $\mathbf{b}$ and $\mathbf{c}$ define

the real space unit cell. These real space vectors are associated with the reciprocal space by the following equations:

$$\mathbf{a}^* = \frac{\mathbf{b} \times \mathbf{c}}{V} \quad \mathbf{b}^* = \frac{\mathbf{c} \times \mathbf{a}}{V} \quad \mathbf{c}^* = \frac{\mathbf{a} \times \mathbf{b}}{V}$$

where, (25)

$$V = \mathbf{a} \cdot (\mathbf{b} \times \mathbf{c})$$

Where **a**, **b** and **c** are real space unit cell vectors and V is the volume of the unit cell.

For indexing the diffraction image, a set of strong diffraction spots (typically ~50-100) are searched and subjected to auto-indexing algorithms such as (Kabsch 1988). While early indexing algorithms were based on the analysis of the difference vectors in a series of rotation images, the modern auto-indexing methods are based on Fourier methods (Michael G. Rossmann and van Beek 1999). In this method, after locating the representative spots, the 2D co-ordinates of the spots are converted to scattering vectors that correspond to their coordinates in the 3D reciprocal lattice (using the Ewald sphere construction). If the scattering vectors are projected along a real axis direction, all spots in the same plane will give rise to the projected vector of the same length, as will all spots in the next plane. This will produce a series of regularly spaced peaks in the Fourier transform. The distance between the adjacent peaks corresponds to the real cell spacing along the principle axis direction. However, if the scattering vectors are projected along a random direction, the spots in the same plane will give vectors of different lengths and the corresponding Fourier transform will only show noise. Thus, the peaks in the Fourier transform can be used to identify directions that correspond to real spaces axes and the spacing between the peaks gives the unit cell dimensions. Further refinement of parameters such as direct beam position, mosaic spread, cell edges and crystal to detector distance minimizes the error and produces

better fit of the observed spots to their calculated positions and thus improves the estimate of the unit cell parameters (Leslie 2006; Powell 1999).

4.2.7.2 Reflection integration

After the unit cell parameters and orientations have been obtained, the next step in data processing is the integration of the diffracted intensities. The integration process involves predicting the position of each Bragg reflection on all diffraction images collected and then estimation of the intensities (I) of each diffracted spots and their standard deviations (σI). There are essentially two distinct methods for integration: The simplest way to obtain an estimate of the integrated intensity is to simply sum the pixel values for all pixels under the area of the diffraction spot and then subtract the estimated background value for the same spot. This process is called summation integration. More accurate estimates of integrated intensities are obtained by a procedure known as profile fitting. In this process, reference profiles are created from the observed profile of a collection of reflections. The observed profile for each reflection is then fitted by the least squares method to the reference profile to estimate its intensity. This method generates more accurate estimates of the integrated intensity especially for weaker reflections (Leslie 2006; Pflugrath 1999). In both methods the level of background scatter is usually inferred from the region surrounding the reflection and subtracted prior to integration

4.2.7.3 Scaling and merging

The measured intensities are proportional to the square of the structure factor amplitudes, subject to geometric correction for the so-called Lorentz and polarization effects (Evans 2011). These effects are normally correct at the integration stage; however, this

proportionality is also affected by various systemic errors such as fluctuation in the X-ray beam intensity, change in illumination volume, differences in adsorption with different crystal orientation and radiation damage. Therefore, in order to obtain $|F(hkl)|^2$ from $I(hkl)$, the measured intensities must be internally consistent and on the same relative scale. This is achieved by applying a scale factor to the intensities. The scaling process therefore involves determination of a scale factor which is achieved by comparing recorded reflections with their symmetry related counterparts. After the intensity correction, the space group can be determined by taking into account the Bravais lattice. Finally, the absolute structure factor amplitudes are calculated, usually taking into account their expected statistical distribution, to allow amplitudes to be derived in the face of reflection intensities whose measured intensities are negative (French and Wilson 1978).

4.2.8 Crystallisation of FMDV inactivated virion and recombinant empty capsids

Crystallisation screening was performed using a high throughput automated procedure developed in the Oxford Protein Crystallisation Facility (Daniel et al. 2011; Mayo et al. 2005). Purified FMDV particles (inactivated virions and recombinant empty capsids) at a concentration of 2.0 to 2.5mg/ml were used for setting up crystallisation trials. Crystals were grown using the sitting-drop vapour diffusion method in 96-well Greiner Crystalquick™X plates (Greiner Bio-One). 30µl aliquots of commercial SaltRx sparse matrix crystallisation screen (96-well format, Hampton Research) were dispensed into reservoir chamber using a Hydra-96 micro-dispenser (Matrix Technologies) robot.

The crystal drops were set up by adding 100nl virus plus 100nl precipitant to the crystallisation wells using a Cartesian liquid dispensing robot (Cartesian Technologies) as previously described (Walter et al. 2005). The crystallisation plates were stored at 294K in a

Rock Imager 1000 (Formulatrix) vault and the crystal drops were imaged regularly with an in-built imager and viewed with XtalPIMS (Daniel et al. 2011).

The hit conditions giving micro-crystals were optimized using two different optimization strategies. In one screening method crystallisation solution, containing either 1.5M ammonium sulphate, 0.1M bis-Tris propane pH 7.0 or 4M ammonium acetate, 0.1M bis-Tris-propane pH 7.0, was serially diluted over 12 rows of a 96-well plate with sterile distilled water in 1% - 3% steps using a Robogo liquid handling robot. This resulted in a decrease in concentration of the precipitant from 100% to 89% or 67%. In a second screening method a 3-row optimization was used, enabling serial dilution, changes in pH between 7.0 to 8.5 and changes in buffer to protein ratios of 1:1, 1:2 and 2:1 (capsids : reservoir) (Walter et al. 2005).

4.2.9 FMDV data collection and processing

X-ray diffraction experiments on FMDV crystals were performed at the Diamond Light Source, Oxfordshire, UK. Although the samples were non-infectious, since they were FMDV related/derived, a tracking sheet was kept with us and signed at each destination. Diffraction images were collected using the *in-situ* data collection method (Axford et al. 2012) at 294K on a Pilatus 6M detector. A micro-focus beamline (Diamond, I24) was used for the data collection with $\lambda=0.9778\text{\AA}$. Depending on the size of the crystals, a 20x20 or 50x50 μm^2 beam, focused downstream from the crystals (towards the detector) was used. The crystals, being very sensitive to radiation, yielded only 4 to 6 useful images, with 0.1° oscillation per image, from one position of a crystal. In general, the data were collected from two different positions however, with some larger crystals data were collected from up to 8 different

positions. As a result, the data were collected from 40 to 80 different crystals to sample the maximum reciprocal space.

The data was processed using the HKL2000 software package (Otwinowski 1997). Each dataset was first individually indexed to assign Miller indices to each reflection and to determine the unit cell dimensions and space group. The point group was chosen based on the lowest penalty scoring, unit cell parameters and highest symmetry lattice. This was followed by integration of the images. Finally, datasets with the same space group and similar unit cell parameters were merged together and the intensities scaled using Scalepack software (Otwinowski 1997). The quality of the merged data was assessed by the merging R-factor (R_{merge}), which is a measure of internal consistency of the scaled data and is defined by equation (26). The resolution limit was evaluated by the ratio between the mean intensity I and the standard deviation of the measurements σI , the so called signal to noise ratio ($I/\sigma I$).

$$R_{merge} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)} \quad (26)$$

Where $I_i(hkl)$ is all observed intensities and $\langle I(hkl) \rangle$ is the average intensity of all observed reflections. Finally the structure factors for each dataset were calculated from the reflection intensities using the TRUNCATE module of the CCP4 package (Winn et al. 2011).

4.3 Results and discussion

X-ray crystallography was used to determine the structures of rationally engineered mutants that were designed and evaluated by *in-silico* modeling, MD simulations and binding free energy calculations with a view to analyzing the structure of the modified inactivated virion and recombinant empty capsids.

4.3.1 Crystallisation of FMDV O1M and SAT2 serotype

Purified inactivated O1M wildtype and VP2 S93Y inactivated virus particles were concentrated and excess sucrose removed using a desalting spin column as described in Chapter 3 prior to crystallisation trials. 2.0mg/ml wildtype and 2.2mg/ml mutant were used for setting up crystallisation trials using a sitting drop vapour diffusion method. Micro-crystals were obtained within 3 days at 294K for both, wildtype and mutant viruses, in two different crystallisation conditions (i) 1.5M Ammonium Sulfate, 0.1M bis-Tris propane, pH 7.0 and (ii) 1M lithium sulfate, 0.1M bis-Tris propane, pH 7.0. Optimisation using serial dilution of the ammonium sulfate condition produced crystals with dimensions of $50 \times 50 \times 5 \mu\text{m}^3$ (Figure 4-5). The crystals of recombinant O1M empty capsids (wildtype) were grown by dilution of the Lithium sulfate condition.

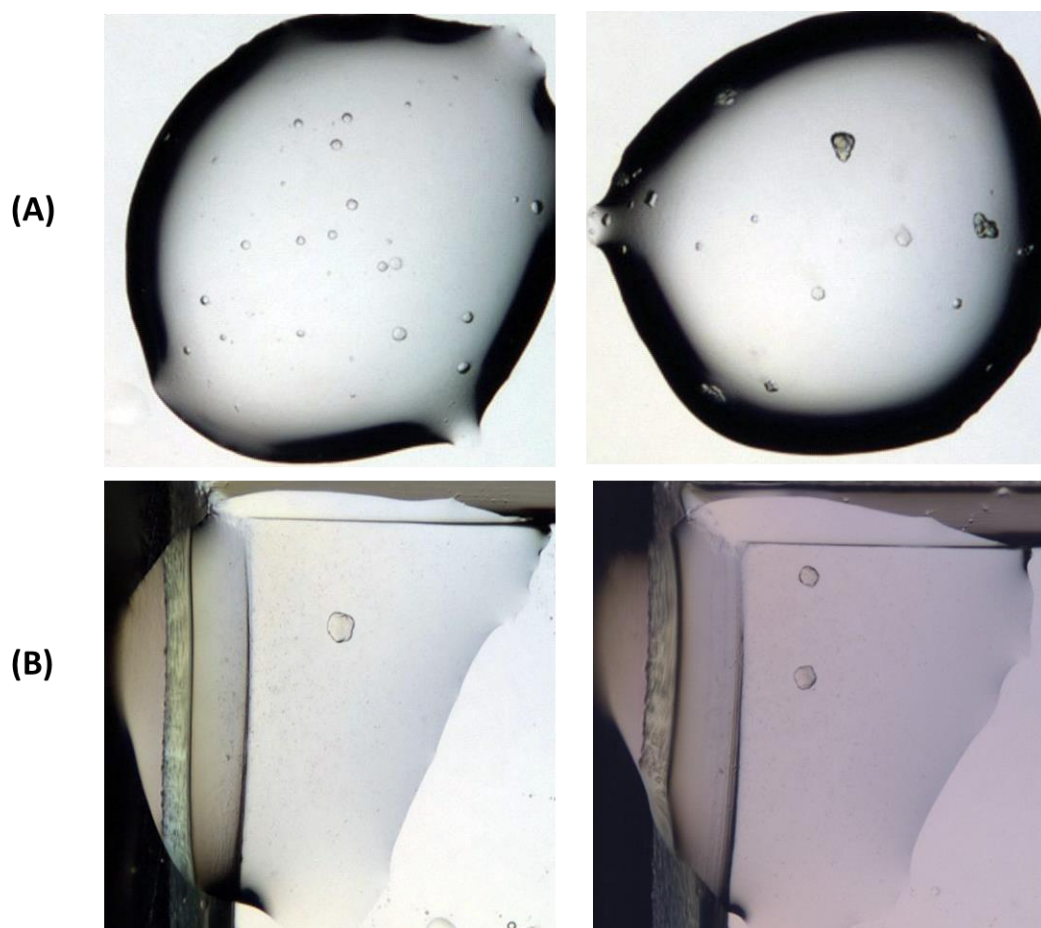


Figure 4-5: Crystallisation of inactivated FMDV O1M serotype virion, Wildtype and VP2 S93Y mutant. Inactivated O1M wildtype and VP2 S93Y crystals grew within 3 days at 294K in two different crystallisation conditions (A) containing either ammonium sulfate (left panel) or lithium sulfate (right panel). (B) The optimization of ammonium sulfate concentration yielded bigger crystals with a final size of about $50 \times 50 \times 5 \mu\text{m}^3$

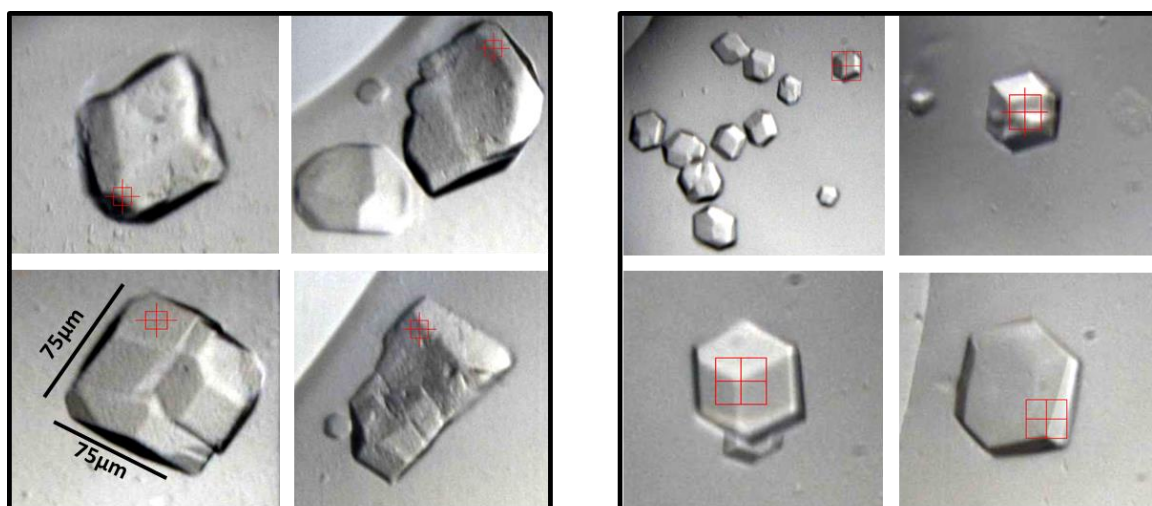
With inactivated SAT2, the yields were lower compared to inactivated O1M virus. Purified SAT2 containing the VP2 S93Y mutation was concentrated to $30 \mu\text{l}$ giving a final concentration of $1.5 \mu\text{g}/\mu\text{l}$. With the limited amount only a coarse SaltRx crystallization screen could be tested. Micro-crystals measuring $20 \times 20 \times 5 \mu\text{m}^3$ appeared within 3 days to 1 week with 5 different crystallisation conditions as listed in (Table 4-2). These conditions will be optimized to produce bigger crystals when more material becomes available. The concentration of the wildtype was not sufficient to set up crystallisation trials.

Table 4-2: Crystallisation conditions for inactivated SAT2 VP2 S93Y virions.

Salt	Buffer
3.5 M Sodium Formate	0.1 M bis-Tris Propane pH 7.0
3.5 M Sodium Formate	0.1 M Tris pH 8.5
1.5 M Ammonium Sulphate	0.1 M bis-Tris Propane pH 7.0
1.0 M Lithium Sulphate	0.1 M bis-Tris Propane pH 7.0
1.5 M Lithium Sulphate	0.1 M Tris pH 8.5

4.3.2 Crystallisation of recombinant FMDV A22 empty capsids

Recombinant empty particles of FMDV A22 serotype, wildtype and mutants, VP2 H93C (produced in vaccinia virus mammalian expression system) and VP2 H93F (produced in baculovirus insect expression system) were similarly purified from sucrose gradient fractions by desalting and concentrated by ultrafiltration prior to crystallisation as described previously. 3mg/ml wildtype and 2.3 mg/ml mutants were used for setting up crystallisation trials. Micro-crystals for all three types of capsids with average dimensions of $30 \times 30 \times 5 \mu\text{m}^3$ grew within 1 week at 294K in two similar conditions containing 4M ammonium acetate. The difference in the condition was the buffer and pH. One condition contained 100mM bis-Tris propane, pH 7.0 and another, 100mM Tris-HCl pH 8.5. Bigger crystals were produced by mixing the two conditions 1:1 and optimizing by serial dilution of the new mixture by 1% in each step over rows F-H as described in section 4.2.7 (Figure 4-6).



Recombinant A22 empty wildtype

Recombinant A22 empty VP2 H93C

Figure 4-6: Crystallisation of recombinant FMDV A22 serotype. Crystals of recombinant A22 serotype grew within 1 week at 294K by optimization of the initial condition containing 4M ammonium acetate. Crystals for VP2 S93F were also obtained using the same crystallisation condition (red square shows 20x20µm beam size).

4.3.3 *In-situ* diffraction data collection

The diffraction data were collected at 294K with a microfocus beam (Diamond, I24 beamline) and using a recently developed *in-situ* data collection method (Axford et al. 2012). The I24 beamline delivered some 10^{12} photons/sec onto the sample, with the beam focusing being adjusted to provide between $20 \times 20 \mu\text{m}^2$ or $50 \times 50 \mu\text{m}^2$ beam at the crystal (depending on the size of the crystal) and with actual focus somewhat downstream of the crystal to minimize divergence of the beam on the detector.

There are multiple advantages with the *in-situ* method: The traditional method of virus crystallography required mounting crystals in sealed capillaries, which is not only time consuming but also impractical for very fragile micro-crystals (Fry et al. 1993). In contrast, the *in-situ* data collection method is a high-throughput method that allows the diffraction data to be collected directly from crystals within the plate in which they were grown. Software allows the user to easily maneuver around the plate to collect data from many crystals in a

short space of time (Figure 4-7). Such *in-situ* analysis from sealed plates also provides a safe alternative for pathogenic material like virus crystals.

Although this method is very useful for data collection from virus crystals, there are some limitations with this method. Firstly, the data can only be collected at 294K as a result only 3-4 frames with 0.1° oscillation could usually be collected per crystal position and many crystals were required to achieve the completeness of the dataset. This is not as problematic for virus crystallography as the data from virus crystals are often required to be collected at 294K and from numerous crystals, because cryo-cooling of virus crystals tends to damage crystals and the mosaic spread of the crystals increases. The large mosaic spread leads to the overlapping of diffraction spots from the adjacent reciprocal lattice planes (Gilbert et al. 2003). In addition, the data were collected on a Pilatus 6M detector which has a large active area (423.6 x 434.6 mm²) short read-out time (< 3.0 msec) compared to the typical CCD detectors (~1sec), very low point spread function and very high signal-to-noise ratio (the detector works as a photon counting device, rather than being an integrating detector). This results in faster data collection and with a very short exposure time, sharp spots and reduced background (Taguchi et al. 2008) .

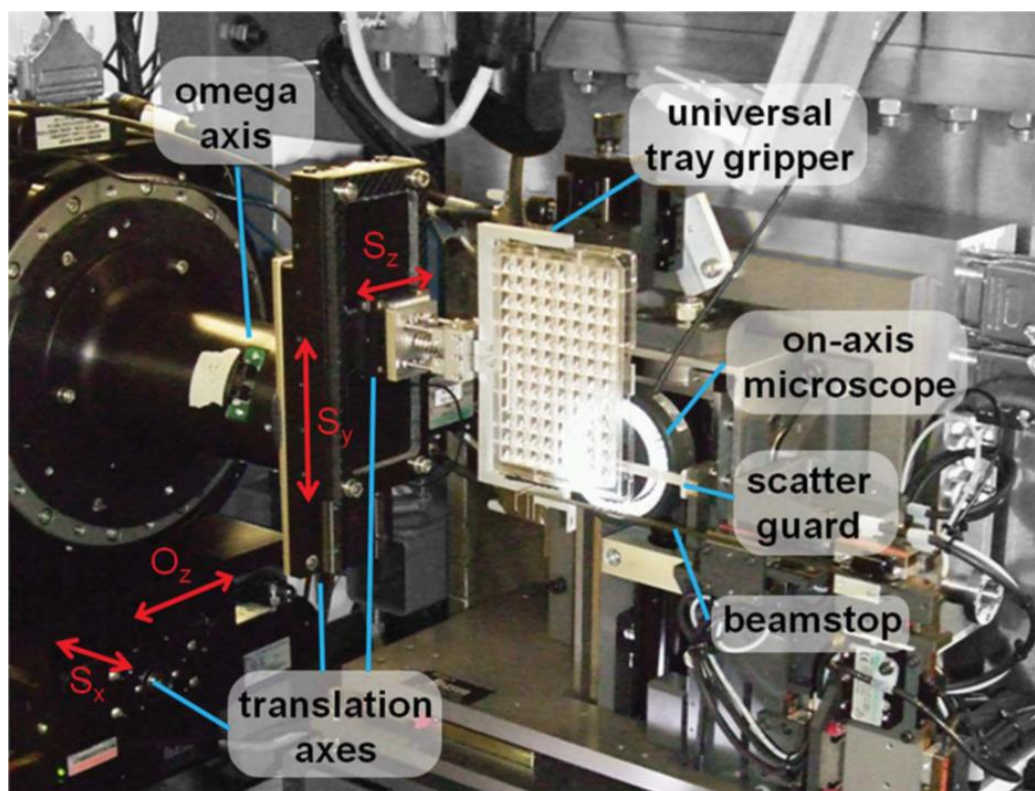


Figure 4-7: The schematics of *in-situ* data collection method. A photograph showing a schematic diagram of in-situ data collection setup at Diamond I24 micro-focus beamline. In this method the diffraction data can be collected directly from the crystallisation plate, making it a very suitable and high-throughput method for virus crystallography (taken from Axford et al. (2012)).

Secondly, standard crystallisation plates result in high background scatter from the significant path length (1.03 mm) of plastic that the X-ray beam passes through (see Appendix V for more details). The problem was solved by using low scattering, thin walled, diffraction capable CrystalQuick-X plates from Greiner Bio-one (these were designed in collaboration with STRUBI, and EMBL-Grenoble). The CrystalQuick-X plates have a reduced thickness of the base (0.26 mm) of each well resulting in minimization of X-ray scattering and reduction in the refraction effects at higher angles when the plates are rotated. The scattering is further minimized by using optimized sealing tape, ThermalSeal RT (50 μ m thickness). In addition, there is a rotational restriction on the goniometer for handling the crystallisation plate, as a result of spatial limitations the crystal plates can be rotated only by ± 18 to $\pm 25^\circ$ depending on

the type of crystallisation plates used. However, due to high symmetry of virus crystals, the variety in the orientation of the crystals in different crystallisation wells and the non-crystallographic redundancy, this rotation range allows more than enough reciprocal space to be sampled for structure determination and refinement.

The inactivated O1M VP2 S93Y crystals diffracted to about 3.5Å. Because of the radiation damage, between 4 and 6 diffraction images with 0.1° oscillation could be collected per crystal. The inactivated wildtype capsids being very unstable (see Chapters 3 and 6 for more details) only four crystals were obtained after optimization which diffracted to about 8Å. All diffraction data were processed and diffraction images from isomorphous crystals were merged using HKL2000 (Otwinowski 1997). Both, inactivated wildtype and VP2 S93Y crystals belong to the body centered cubic space group I23 with unit cell dimensions of $a=b=c=344\text{\AA}$ and $\alpha=\beta=\gamma=90^\circ$ which is similar to that of the published structure of the native FMDV serotype O subtype O1BFS (Acharya et al. 1989). The crystals of recombinant O1M wildtype empty capsids diffracted to 3Å with space group and cell dimensions very similar to that of inactivated viruses.

The crystals of recombinant A22 empty wildtype and VP2 S93F diffracted to 2.1 and 2.4Å respectively (Figure 4-8). Both crystals belonged to the body centered orthorhombic space group I222 with unit cell dimensions of $a=327.6$, $b=341.3$ and $c=363.7$ and $\alpha=\beta=\gamma=90^\circ$. The crystals of the covalent mutant VP2 S93C diffracted to 2.9Å and were also in I222 space group with similar unit cell dimensions. Details of the data collection statistics are shown in Table 4-3 and refinement statistics are presented in Chapter 5.

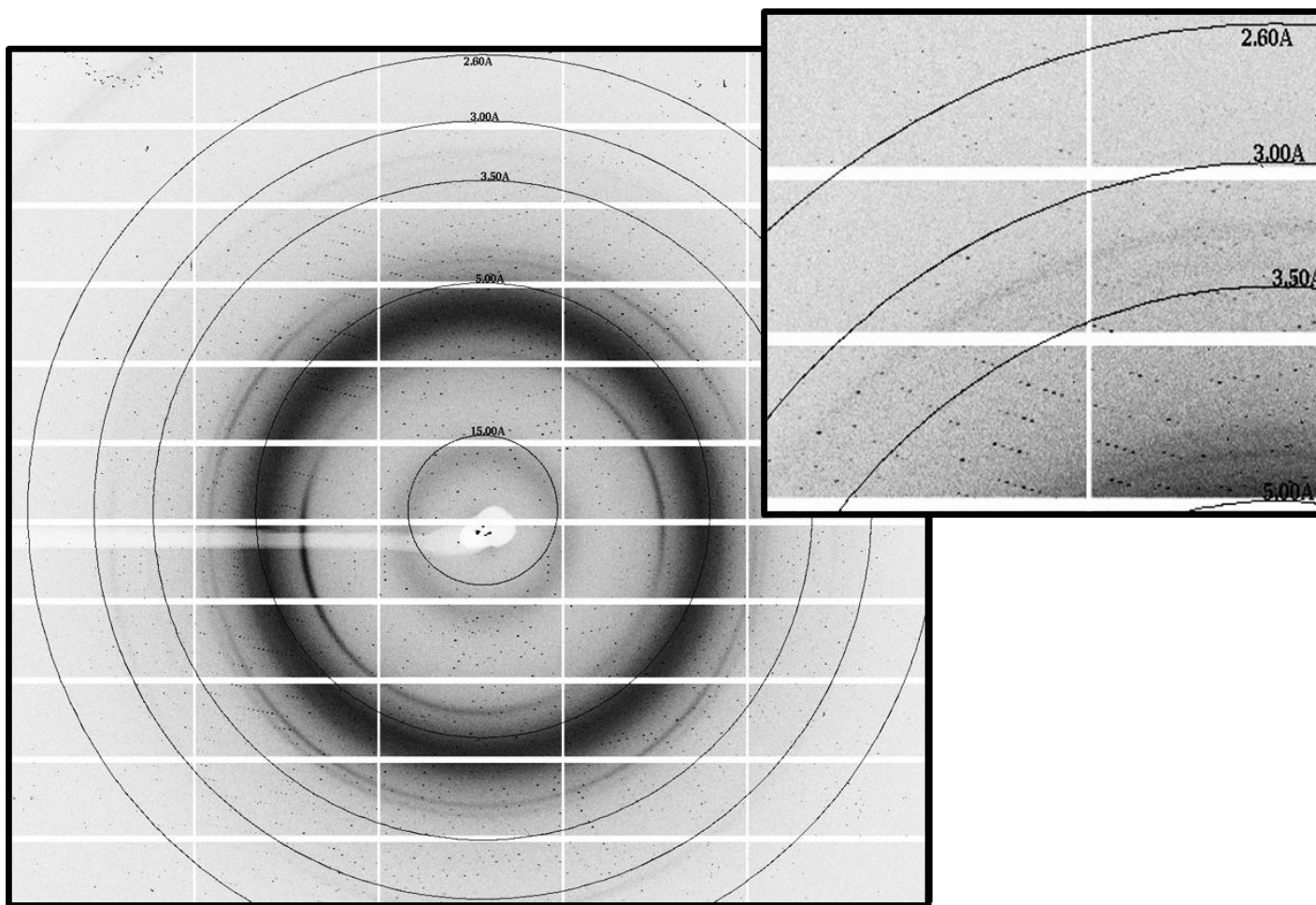


Figure 4-8: Diffraction image of recombinant FMDV A22 serotype. The diffraction data from FMDV crystals were collected using the *in-situ* method directly in crystallisation plates. The diffraction image from the crystals of recombinant FMDV A22 wildtype diffracting to 2.1 Å at 294K is shown above. VP2 S93F crystals diffracted to 2.4 Å and VP2 S93C to 2.9 Å.

Table 4-3: Data Collection Statistics.

X-ray source		Diamond, beamline I24		
Data collection	A22 wildtype	A22 VP2 S93F	A22 VP2 H93C	A22 VP2 H93C reduced
Wavelength (Å)	0.9778	0.9778	0.9778	0.9778
Number of crystals/positions	47 (125)	4 (2)	77 (191)	12 (19)
Number of images	991	64	764	208
Rotation per image (°)	0.1	0.1	0.1	0.1
Space group	<i>I</i> 222	<i>I</i> 222	<i>I</i> 222	<i>I</i> 222
Unit cell (Å)	$a = 327.6, b = 341.4, c = 363.7$	$a = 327.6, b = 341.3, c = 363.6$	$a = 328.0, b = 341.5, c = 363.4$	$a = 328.0, b = 341.4, c = 363.3$
Resolution range (outer shell) (Å)	50 – 2.1 (2.2-2.1)	50 – 2.4 (2.5-2.4)	50 – 2.9 (3.0-2.9)	50 – 2.9 (3.0-2.9)
Unique reflections	869280 (29881)	107002 (10017)	353968(23484)	206765(16895)
Completeness (%)	74.6 (25.8)	16.8 (15.2)	79.7 (53.2)	46.7 (38.4)
Multiplicity	2.7 (1.2)	1.2 (1.2)	2.2 (1.6)	1.4 (1.3)
Average I/σI	3.5 (0.5)	1.8 (0.4)	2.2 (0.7)	1.8 (0.7)
R _{merge}	0.244	0.259	0.407	0.360

4.4 Conclusion

X-ray crystallography is a powerful tool that enables the evaluation of protein structures at atomic level. The determination of 3D structures requires the formation of highly ordered diffraction quality crystals, which is often a major bottleneck in the structure determination process. Micro-crystals of three different serotypes of FMDV were readily obtained using a gradual salting-out and sitting drop vapour diffusion crystallisation method. The crystals were obtained in three different types of salt conditions consisting of 1.5M ammonium sulphate, 4M ammonium acetate or 1M lithium sulphate. Optimization of concentration and pH around the initial hit conditions yielded crystals with average dimensions of $50 \times 50 \times 5 \mu\text{m}^3$. The diffraction data was collected using an *in-situ* data collection method. This method was recently developed at the Diamond I24 micro-focus beamline and facilitates data collection directly from crystallisation plates, making it a high-throughput method for virus structure determination. The *In-situ* data collection method also made it possible to collect diffraction images from several crystals within short periods of time which would not have been possible with traditional methods involving mounting crystals in capillaries. This method is also a safe alternative for collecting the data from pathogenic samples.

All FMDV crystals diffracted to a resolution sufficient to solve the atomic structures. The data were collected from multiple crystals and processed using the HKL2000 software. Almost all the crystals of the A serotype were isomorphous with recombinant A22 wildtype crystals diffracting to $2.1 \text{ \AA}$ and mutants VP2 S93F and VP2 S93C diffracting to 2.4 and $2.9 \text{ \AA}$ resolution. The inactivated O1M VP2 S93Y crystals were isomorphous with O1BFS crystals obtained previously and diffracted to $3.5 \text{ \AA}$. Inactivated O1M wildtype crystals were very unstable and only diffracted to about $8 \text{ \AA}$ resolution. The inactivated SAT2 wildtype and VP2 S93Y material was very limited due to the necessity for its production under high levels of

containment which rendered scale-up difficult. The concentration of inactivated wildtype was very low and hence was not used in crystallisation trials. The mutant produced micro-crystals with average dimensions of $20 \times 20 \times 5 \mu\text{m}^3$ with 5 different crystallisation conditions. Crystal optimization will be performed when more material becomes available. The phasing of the diffraction data, generation of electron density, model building and refinement and model validation will be discussed in the next chapter.

Chapter 5 : FMDV structure determination - Phase estimation, structure refinement and model validation

5.1 Introduction

The determination of the three-dimensional structures of proteins by X-ray crystallography is a multistep process. As discussed in Chapter 4, the initial steps involve purification and crystallisation of the protein of interest (FMDV capsids in this case), followed by collection and processing of diffraction data. Each reflected X-ray from the crystal planes can be described by amplitude and phase. In diffraction experiments only the diffraction intensities and the directions of the diffracted beam can be measured. The directions allow indices to be assigned to the reflections and there is a well-defined relationship between the diffracted intensities and the structure factor amplitudes, allowing the latter to be derived directly. The next step in the process is to obtain the phase angles for each of the measured reflections, since this is lost in a classical diffraction experiment; we are faced by what is known as the ‘phase problem’. Since it is impossible to calculate a meaningful electron density map in the absence of phases, estimates must be obtained.

In this chapter, I will briefly discuss the methods used to derive the initial phases and phase refinements in real and reciprocal space, to allow the calculation of electron density maps that are used for the generation of atomic models of viral capsids.

5.2 Phasing methods

There are several methods available to recover the phase information in diffraction experiments. These methods include Single Isomorphous Replacement (SIR), Multiple Isomorphous Replacement (MIR), Single wavelength or multiple wavelength Anomalous Dispersion (SAD/MAD) and Molecular Replacement (MR). In isomorphous replacement methods phase information is retrieved by making isomorphous structure modifications. Such modifications often involve the incorporation of heavy atoms by soaking crystals of native

protein with metals (SIR/MIR) or by incorporating metal atoms into the proteins of interest typically via selenomethionine substitution for methionine (usually followed by SAD/MAD analysis). The heavy atoms perturb the scattering and from the differences (either between substituted and unsubstituted crystals or between reflections showing opposite anomalous scattering effects within the same data set) it is often possible to determine the position of the heavy atoms/ anomalous scatters. Once these positions have been determined, phase information can be derived for the non-heavy atom scattering components (Blow 2002). However, neither of these methods was used in this thesis and hence they will not be discussed further. The phase information of all FMDV structures presented in this thesis was derived using the Molecular Replacement method.

5.3 Phasing Using the Molecular Replacement Method

Molecular replacement is a powerful method for deriving phase information (M. G. Rossmann 1990). It can be used for obtaining approximate phase information for an unknown structure when a related or partially similar protein structure is available. Usually, a model with at least 40% sequence identity with the unknown structure is required to ensure a successful MR solution. However, it has been shown that MR solutions can be obtained in some cases with phasing models that exhibit much lower sequence identity (Acharya et al. 1989; Jones 2001).

The goal of the MR method involves the positioning of a homologous search model (probe) in the unit cell of the target crystal such that the calculated diffraction pattern that would result from the probe coincides with the orientation and position of the unknown experimental target within the crystal. The placement of the probe and correlation with the target can be achieved by six dimensional searches, composed of three rotational and three

translational searches, however, these searches would be computationally very demanding. In practice the searches are usually reduced to two, each of three-dimensions, a three-dimensional rotation search followed by a three-dimensional translational search, once the correct rotation is found.

This is achieved by using the information from a Patterson function. The Patterson function is essentially a Fourier transform of the square of the structure factor amplitudes and can be calculated directly from the diffraction intensities without the phase information:

$$P(u, v, w) = \frac{1}{V} \sum_{hkl} |F(hkl)|^2 \cos[2\pi(hu + kv + lw)] \quad (27)$$

Where V is the volume of the unit cell and $|F(hkl)|$ are the observed amplitudes. The Patterson function is essentially a map of all interatomic distance vectors in the crystal. The vectors in the Patterson map can be divided into two categories: (i) Intramolecular vectors that depend only on the orientation of the molecule and thus can be used for rotational searches and (ii) Intermolecular vectors that depend on both the orientation and the position of the molecule in the unit cell and hence, once the orientation is known, such interatomic vectors can be used for translational searches. Therefore, in practice two Patterson maps are calculated, one from the unknown structure and one from the search model (homologous protein) and correlation scores are used to find the correct solution. When the two maps are in similar orientation and position, higher correlations are observed. The scoring function is improved using maximum likelihood statistics as a target.

Once the model is correctly oriented and translated, it can be used to calculate phases which are combined with the experimental amplitudes to get an approximate electron density map of the unknown structure (Blow 2002; Jan Drenth 2007). In cases where the crystal of the unknown structure is isomorphous to that of the known structure, the phases can be directly obtained from the model and the structural differences can be seen by difference Fourier analysis, via calculation of a map with amplitude coefficients $(|F_{obs}(hkl)| - |F_{calc}(hkl)|)$, where $|F_{obs}(hkl)|$ is the measured amplitude and $|F_{calc}(hkl)|$ is that derived from the model).

5.4 Self-Rotation Function

In a cross-rotation function, a Patterson functions calculated from the search model is compared with the Patterson function calculated from the measured data. However, in order to look for the internal symmetry of the unknown crystal structure, the observed Patterson can be compared with itself in all possible orientations; this is called a self-rotation function. If there is non-crystallographic symmetry then for certain sets of rotation angles the rotated map will have a large number of vectors matching the un-rotated map, and this will lead to a peak in the rotation function (which is simply the sum over the whole map, within certain limits, of the product of the two Patterson maps at each point) at those angles. This will reveal internal symmetry within the crystallographic asymmetric unit. In practice two identical Patterson maps are calculated from the observed data and one copy is held stationary while the other is rotated about its origin. The rotation angles are conventionally specified as (ϕ, ψ, κ) in spherical polar co-ordinates (Figure 5-1), where the orientation of the rotation symmetry axis is specified by ϕ and ψ and κ specifying the angle of rotation required to bring the symmetry related atomic vectors to overlap.

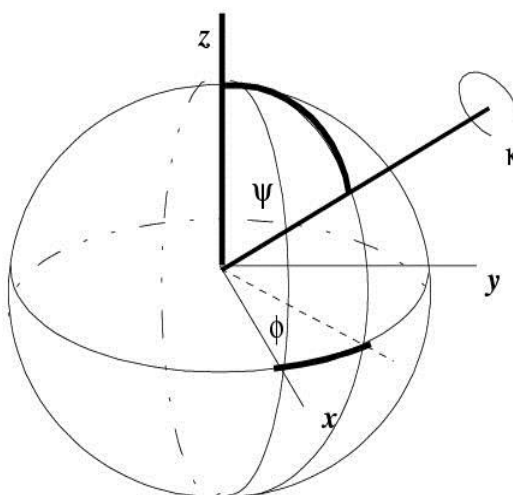


Figure 5-1: Schematics of rotational space shown in spherical polar co-ordinates. In the spherical polar coordinate system, ϕ is the rotation angle that coincides with the x-axis, ψ is the rotation angle that coincides with the z-axis and κ is the rotation about the actual rotation axis.

The self-rotation function is usually plotted as stereographic projections of a unique hemisphere in ϕ and ψ for a series of κ sections. Within this, both crystallographic and non-crystallographic symmetry peaks may be seen and provide information about the orientation of the protein/viral capsid within the unit cell. For example the peaks on a $\kappa=180^\circ$ section indicate the presence of 2-fold rotational symmetry. For an icosahedral virus the most informative section is usually the $\kappa=72^\circ$, which shows the 5-fold axes, this is because there are fewer 5-fold axes and the signal is particularly strong. For virus crystallography this information can sometimes be enough to initiate phasing, since the position of the particle may be defined by the coincidence of some crystallographic and icosahedral symmetry axes (Doubl   2007; Fry et al. 1993; Luo et al. 1989).

5.5 Twinning in crystallography

Twinning is a common problem in crystallography at least with certain higher symmetry space groups. A twinned crystal contains two or more single crystals with identical packing, but the domains within the crystals are in different orientations even though their lattices overlap in three-dimensions. In protein crystallography, two types of twinning can be observed: In non-merohedral or partially twinned crystals, the lattices of the two domains do not overlap in all three dimensions and the diffraction pattern is interpretable. The diffraction data can be processed from the major lattice while ignoring the minor lattice – or both lattices can be processed (Abrescia et al. 2011).

In the case of merohedral twinning, lattices of the two domains overlap in all three dimensions and the diffraction pattern from the domains superimpose as if observed from a single crystal. Therefore, each observed intensity is a sum of the intensities of two reflections that are related by the twin operator:

$$\begin{aligned}
 I_{obs}(d1) &= (1 - \alpha)I(d1) + \alpha I(d2) \\
 \text{and,} \quad I_{obs}(d2) &= (1 - \alpha)I(d2) + \alpha I(d1)
 \end{aligned}
 \tag{28}$$

Where $d1$ and $d2$ are twinned domains and α is the twin fraction.

Merohedral twinning can be further divided into two categories (a) Partial merohedral twinning, where the contribution from the twin domain is less than 50% to the diffraction intensities and (b) Perfect merohedral twinning, where the contribution from the twin domain is equal to 50% of the diffraction intensities. The equal proportions of twin domains and symmetry imposed by the twin operator leads to a higher apparent symmetry than the true

symmetry. The partial twinning does not affect the true crystallographic symmetry and using de-twinning programs the data can be corrected to get the true intensities with little loss of information. However, it becomes very difficult to de-twin the perfectly twinned diffraction intensities (Efremov et al. 2004; Yeates and Fam 1999), although it has been shown that this can be achieved for viruses where there is non-crystallographic symmetry (Lea and Stuart 1995)

5.6 Temperature Factor (B-factor)

The temperature factor or B-factor is often thought to be a measure of the thermal movement of atoms around the positions specified in the model. This is not strictly true; since the rate at which a diffraction event occurs is so much greater than the rate of movement of the atoms due to thermal vibration. Therefore, it is not possible to distinguish static from dynamic disorder from the effect on the X-ray diffraction pattern. However, whatever the cause side-chain atoms exhibit more freedom for movement/disorder than the main-chain atoms. The motion/disorder of atoms around its mean position in a crystal spreads the electron density which means that the density becomes ‘blurred’, attenuating the diffraction at higher resolution. The temperature factor is related to the magnitude of vibration of the atoms as follows:

$$B = 8\pi^2\{u^2\} \quad (29)$$

Where $\{u^2\}$ is the root mean square displacement of the atom from its initial resting position.

As a result it is essential to assign a temperature factor to each atom and to include the B-factor among the parameters optimised during the structure refinement process. The B-factor can also indicate errors in the model. If the atoms are incorrectly positioned, their B-factor will be higher than the surrounding atoms that are built correctly.

5.7 Density Modification and Structure Refinement

A diffraction experiment provides a map showing distribution of electrons within the crystallographic asymmetric unit. The positions of atoms are then inferred from this, taking into account prior knowledge, typically the sequence of amino acids in the protein chains. However, using the MR phasing method the initial electron density will be imperfect, since the phases are inappropriate so that the electron density may show bias towards the model. It is therefore usual to attempt to improve phases before interpretation in terms of an atomic model. Several methods have been developed to improve the phases and they fall into two categories. The first approach is to work directly with the atomic model and to refine it against the observed structure factors, using the usual stereo-chemical restraints to avoid over-fitting (Brünger et al. 1987), this method of structure refinement is described in a later section.

The second method does not directly use the atomic phasing model, but rather uses information about the presence of non-crystallographic symmetry and/or solvent regions to apply constraints on the electron density – hence this process is termed density modification. A cyclic process is then used to iterate between real and reciprocal space, to reach the point at which the electron density constraints and the match to the observed data may be, as far as possible, satisfied simultaneously. In the process it is assumed that the phase angles are refined towards their true values so that the electron density map becomes, overall, more

reliable. For a discussion of density modification methods see, for instance (Kleywegt and Read 1997). In brief the key elements are:

Solvent flattening: A protein crystal typically contains 50% solvent molecules and 50% of protein molecules (for a virus capsid the effective solvent content is typically far higher since the interior of the particle usually contains disordered viral genome). Since the vast majority of the solvent is disordered the electron density should, in these regions be flat. In practice the electron density will not be flat, since the errors in the phases lead to errors that propagate throughout the map. Therefore, if the solvent region can be located, the electron density map can be improved by setting the density of the solvent region to the expected mean solvent density. This improvement in the electron density should, upon Fourier transformation of the modified map, lead to a concomitant improvement in the phases. When these modified phases are combined with the experimental data to calculate a new electron density map this map should in term be improved. Repeating this process leads to an iterative procedure whereby the phases and electron density may converge to better approximations to their true values. In the protein regions of the map this corresponds to an improved signal-to-noise ratio.

Histogram Matching: A complimentary method to solvent flattening is histogram matching which can be applied to the protein region. Protein molecules are made up of broadly similar atoms and the bond length and angles are largely conserved across a wide range of structures, as a result the density values in the maps are similar for different proteins. If there are errors in the phases, the electron density map of a protein region will not have the expected distribution of low and high densities. The phases might then be improved by modifying the observed electron density distribution to match to that expected theoretically.

Averaging: Protein crystals often contain more than one protein subunit in the asymmetric unit. If these multiple copies have approximately the same conformation, then they are related to each other by non-crystallographic symmetry (NCS). Such non-crystallographic symmetry is a local symmetry within the asymmetric unit and does not map the whole crystal lattice back onto itself. For example 5-fold symmetry (generally observed with icosahedral viruses) can only be expressed as non-crystallographic symmetry, as it is not possible to have 5-fold crystallographic symmetry.

In practice NCS can be of two types, proper and improper. For the purposes of this thesis only proper symmetry is considered, whereby the subunits form a closed group so that if a mask is constructed to contain the entire set of NCS related subunits then the application of the NCS averaging operators within this mask will correctly average the structure. The procedure resembles solvent flattening except that the modification of the electron density is, in this case, replace each of the electron densities by the average of that point with the four other densities which are expected (in case of 5-fold NCS), by virtue of the NCS, to have identical values. Such averaging of the electron density map across the NCS related subunits should reduce the errors by a factor which is the square root of the number of fold averaging applied. Naturally Fourier transformation should, as for solvent flattening, lead to improved estimates of the phase angles.

In practice all three elements of density modification can be applied simultaneously as part of a cyclic process, starting from a first map calculated with the initial phases (Cowtan and Zhang 1999). For viruses the process is extremely powerful and can yield highly accurate phases from extremely poor starting values (Fry et al. 1993; M. G. Rossmann et al. 1985).

5.8 Structure refinement

Density modification is typically followed by rebuilding of the atomic model and structure refinement. The goal of refinement is to maximise the agreement of the model with the experimental data whilst applying as many stereo-chemical (and other) restraints as possible so as to avoid overfitting. The process of density modification, structure rebuilding and refinement is iterative and involves sequential real and reciprocal space refinements to optimise the agreement between the diffraction data calculated from the model and those measured experimentally.

Real space rebuilding/refinement includes manual fitting of individual amino acid side chains into the electron density map and adjusting the model geometry with restraints on known parameters such as bond length, angles and dihedrals (Brünger et al. 1987; Hendrickson 1985). This is followed by restrained reciprocal refinement whereby four model parameters such as atomic positions (x,y,z) and an atomic displacement parameter, B-factor, are refined against the observed data. Additional restraints or constraints may be imposed in the presence of NCS, as is invariably found for icosahedral virus structures.

The success of the refinement depends on the observation to parameter ratio. However, the number of parameters at the moderate resolution at which virus/capsid crystals typically diffract may exceed the number of observations. This leads to poor observation : parameter ratio, means that it is very easy to overfit the data, leading to unrealistically good agreement between the model and the observed intensities. The way to get around this issue is to add more observations from already available information in the form of geometrical restraints which keep the parameters such as bond lengths, bond angles and the planarity of peptide-bonds and aromatic ring systems close to reference values expected from very high resolution

studies. In addition the number of parameters can be reduced by adding constraints. With certain programs (such as CNS, Brunger et al. (1998)) NCS can be treated in this way, where only a single coordinate is supplied, plus NCS operators. Overall the principle is to minimise the residual between the observed structure factors (F_{obs}) and those of the calculated structure factors (F_{calc}) from the model, under as rich a set of restraints and constraints as possible. This is achieved by using target functions such as the maximum likelihood target function implemented in modern refinement programs.

During the process of refinement, the overall agreement between the model and the observed data is measured by monitoring several parameters such as the R-factor (R_{work} and R_{free}) and the root mean square deviations of the bond lengths and the bond angles from their ideal values. The R-factor represents the deviation of F_{obs} from the F_{calc} and it is represented as:

$$R = \frac{\sum |F_{obs}(hkl) - F_{calc}(hkl)|}{\sum F_{obs}(hkl)} \quad (29)$$

Where $F_{obs}(hkl)$ are the observed structure factor amplitudes and $F_{calc}(hkl)$ are the calculated structure factor amplitudes from the model. Generally, a small fraction (typically 5% in order to give a statistically useful sample, but less than 5% in the case of virus structures since there are more reflections overall) of reflections randomly chosen and excluded from the refinement to be used as a means of cross-validation. R_{free} is then calculated in a similar manner as above only using the excluded reflections. Thus it is a useful tool to avoid overfitting. During the process of refinement and model rebuilding, when both R-factors decrease continuously it suggests a good agreement between the model and the observed

(Brunger 1992). However, *R_{free}* is of limited significance in virus crystallography. Because of the high NCS, as is inevitably in the case for virus crystals, the *R_{free}* reflection set is not properly independent since the NCS introduces correlations in reciprocal space that make it effectively impossible to define a truly independent set of reflections (Fabiola et al. 2006). Nevertheless, in part, due to the expectations of many referees it is conventional to use *R_{free}* even in this situation.

5.9 Structure determination of inactivated and recombinant FMDV capsids

The orientation of inactivated and recombinant FMDV capsids were determined by using the self-rotation function implemented by the MOLREP program of the CCP4 package (Winn et al. 2011). The structures of recombinant A22 capsids (wildtype and mutants: VP2 S93F, VP2 S93C) belonging to space group *I*222, and recombinant O1M wildtype and inactivated VP2 S93Y capsids belonging to space group *I*23, were solved by using molecular replacement. As the orientation of the particles and the unit cell dimensions of the crystals were isomorphous to the crystal structure of native A22 (PDB id: 4GH4) and O1BFS (PDB id: 1BBT) viruses, the co-ordinates and NCS operators from the native virus were used for the molecular replacement and refinement.

All structural determination steps used the Crystallography and NMR system (CNS) – macromolecular structure determination software package (Brunger et al. 1998). First, all the water molecules were removed from the starting PDB model and then the model was converted to CNS readable topology and coordinate files using the CNS_SOLVE program and using appropriate CNS input files. All input files for CNS were downloaded from <http://cns.csb.yale.edu/v1.2/> and modified according to the experimental data. The observed structure factor amplitudes (MTZ file, CCP4 format) obtained from the TRUNCATE

programme (see chapter 4 for details) were also converted to a CNS readable ASCII reflection file using the MTZ2VARIOUS module of the CCP4 package.

Initial estimates of phases were obtained by rigid body refinement using the native virus as a search model. The model was refined and phases improved by iterative cycles of minimisation and anisotropic B-factor refinement using strict NCS constraints. The phases were further improved by cyclic NCS averaging.

5.10 NCS averaging and solvent flattening

The cyclic averaging and solvent flattening were performed using the General Averaging Program (GAP, D. Stuart and J. Grimes, unpublished). The procedure used for averaging is summarised in Figure 5-2:

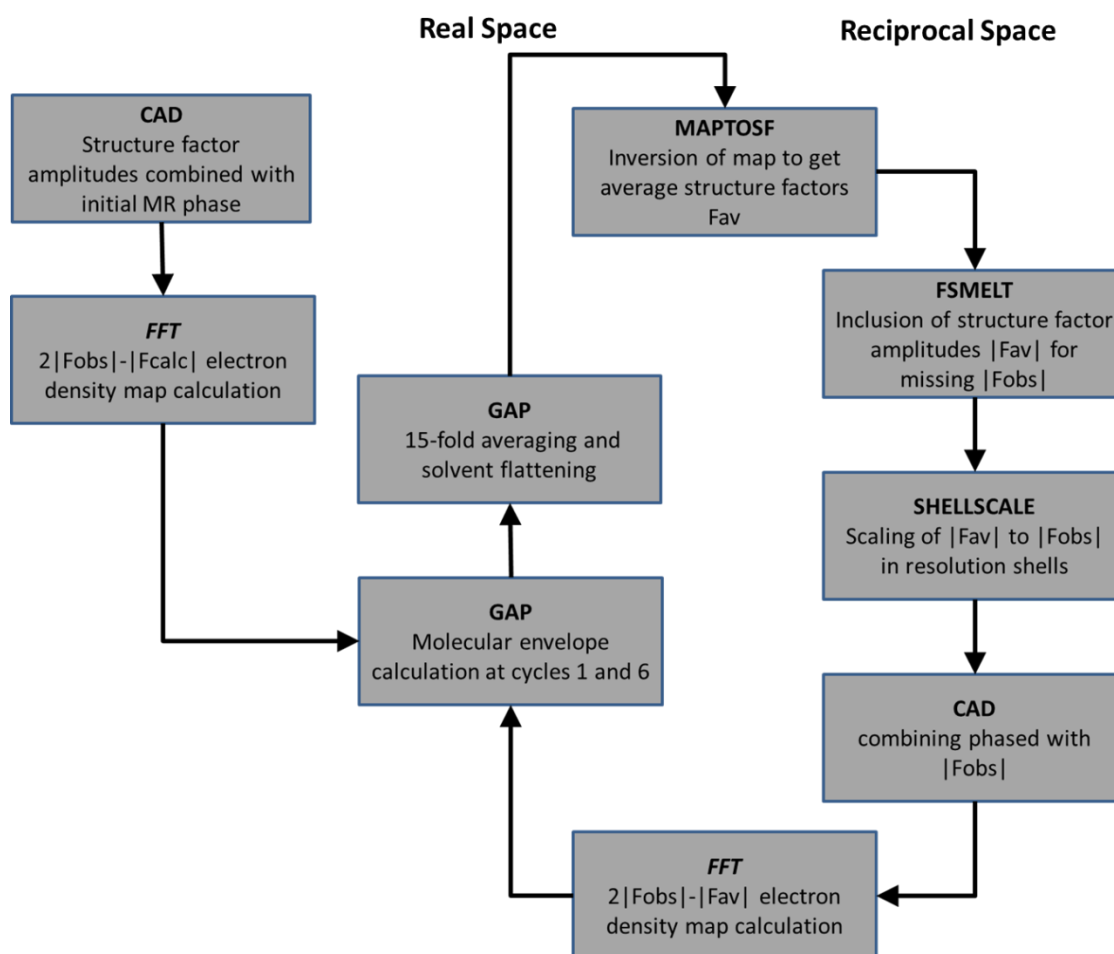


Figure 5-2: Schematic diagram showing NCS averaging protocol: The phases were improved by cyclic NCS averaging and solvent flattening using the General Averaging Program (GAP).

The weighted phases from the $2|F_{obs}| - |F_{calc}|$ map obtained from MR (CNS) were combined with the observed structure factor amplitudes using the CAD module of the CCP4 package and a new map was calculated using FFT (CCP4). The molecular envelope was created to cover the asymmetric unit using GAP on the first and sixth averaging cycle. This was followed by averaging the non-crystallographic portion of the virus (either 15-fold or 5-fold depending on the NCS). The spherical mask covering the inner and outer surfaces of the virion protein capsid was defined. Electron density inside and outside of these spheres was considered solvent density and flattened to the average value to improve the signal to noise

ratio. Finally the averaged map was back transformed to generate the calculated amplitudes and phases. The calculated structure factors were scaled and recombined with the observed amplitudes and a new $2|F_{obs}| - |F_{av}|$ map calculated with improved phases.

The progress of averaging was monitored by using real space R-factor (R) and correlation coefficients (CC). The resulting maps were very clean and clearly showed the density for the mutated amino acid residues. Water molecules were added and atomic models manually adjusted to match the observed density using COOT (Emsley and Cowtan 2004) and further refined using the CNS package. The progress of refinement and the agreement of the model with the experimental data were monitored using crystallographic R-factor calculated using equation (29). The atomic models were further validated by side-chain rotamer analysis using COOT and assessing their stereo-chemical properties using a Ramachandran plot implement within COOT. The structures were further validated using MOLPROBITY (V. B. Chen et al. 2010).

5.11 Results and Discussion

The crystal structures of the FMDV recombinant empty capsids and inactivated virion capsids were determined at high resolution by means of *in-situ* room temperature X-ray crystallography using molecular replacement and NCS averaging.

5.11.1 Structural analysis of recombinant FMDV A22 serotype

The crystals of recombinant A22 wildtype empty capsids diffracted to 2.1Å and the crystals of recombinant A22 mutants VP2 S93F and VP2 S93C diffracted to 2.4Å and 2.9Å respectively. All A22 crystals were scaled and merged in space group $I222$. Analysis of the unit cell dimension and self-rotation function suggested the crystals were isomorphous to that

of the native A22 virus (Curry et al. 1996), with two whole particles in the unit cell. The crystallographic asymmetric unit contained three pentamers generating 15-fold non-crystallographic symmetry. As the crystals were isomorphous and the particle orientation similar to the native virus structure, the phases were obtained by MR using the atomic model of the native virus (4GH4) as a template and rigid body refinement with strict NCS constraints in the CNS package.

The phases were further improved by 15-fold NCS averaging and solvent flattening. The averaging essentially involved superimposing the electron density map onto the NCS related protomers, followed by summation of the density values of the superimposed maps. These were then divided by the number of NCS operators thereby reducing random errors. There was a good agreement between the observed data and the data calculated from the averaged electron density map; with *R-factor* = 10.4% and *CC* = 97% for wildtype, *R*=15.5% and *CC* = 92% for VP2 S93F hydrophobic stacking mutant and *R*=12.3% and *CC* = 95% for the VP2 S93C covalent mutant. Averaging and solvent flattening significantly improved the signal-to-noise ratio and produced reliable atomic resolution maps for all three empty capsid structures (Figure 5-3).

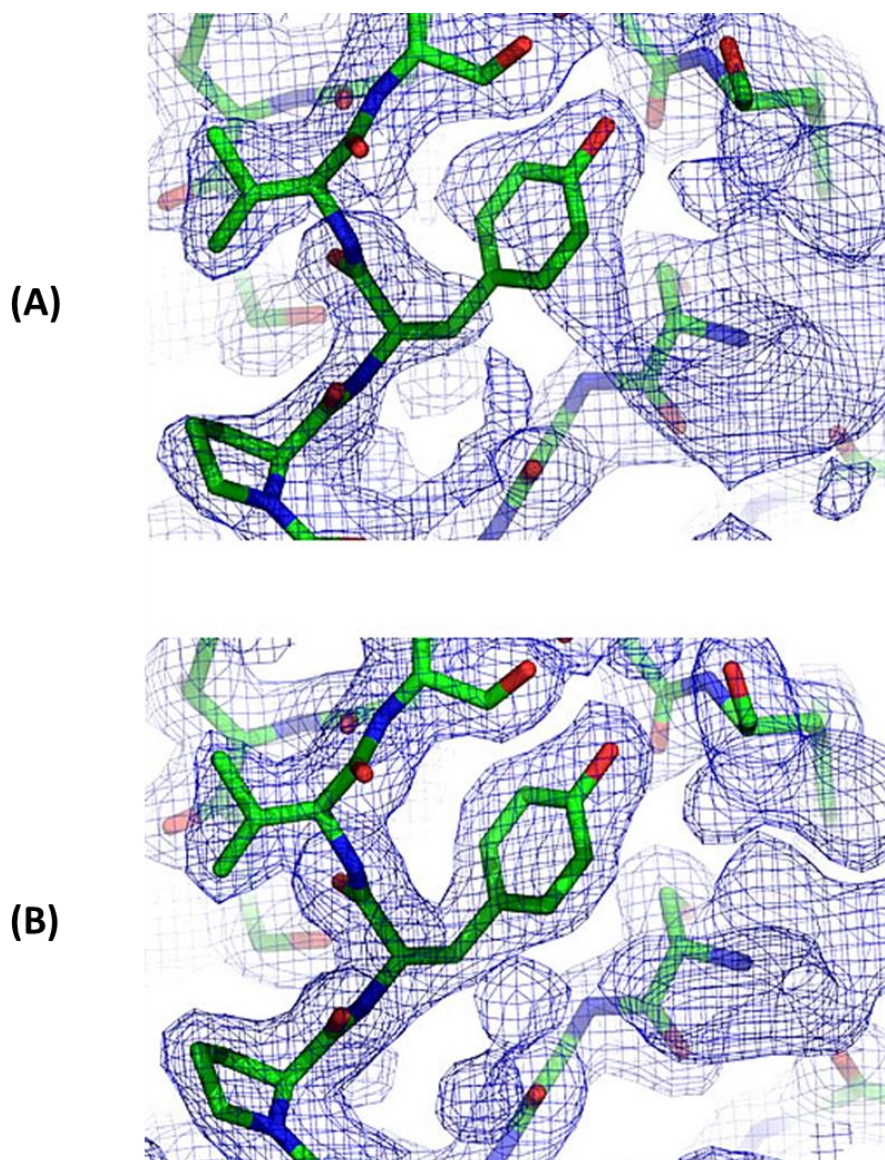


Figure 5-3: Phase improvement by NCS averaging and solvent flattening. The phases of the FMDV structure were improved by cyclic averaging and solvent flattening using GAP. An example of improvement obtained after averaging is shown with the electron density map of A22 wildtype. (A) Electron density map before averaging (B) Electron density map after 10 cycles of averaging and solvent flattening

Local refinement of the side-chains to fit electron density, model rebuilding and addition of water molecules using COOT, and further reciprocal refinement using CNS, showed good agreement between the model and the observed data with R_{work} = 19% for wildtype, 24.1% for VP2 S93F and 23.5% for VP2 S93C. The details of data collection and refinement statistics are shown in Table 5-1.

Table 5-1: X-ray data collection and refinement statistics for recombinant A22 empty capsids structures.

X-ray source	Diamond, beamline I24			
Data collection	A22 wildtype	A22 VP2 S93F	A22 VP2 H93C	A22 VP2 H93C reduced
Wavelength (Å)	0.9778	0.9778	0.9778	0.9778
Number of crystals/positions	47 (125)	4 (2)	77 (191)	12 (19)
Number of images	991	64	764	208
Rotation per image (°)	0.1	0.1	0.1	0.1
Space group	<i>I</i> 222	<i>I</i> 222	<i>I</i> 222	<i>I</i> 222
Unit cell (Å)	<i>a</i> = 327.6, <i>b</i> = 341.4, <i>c</i> = 363.7	<i>a</i> = 327.6, <i>b</i> = 341.3, <i>c</i> = 363.6	<i>a</i> = 328.0, <i>b</i> = 341.5, <i>c</i> = 363.4	<i>a</i> = 328.0, <i>b</i> = 341.4, <i>c</i> = 363.3
Resolution range (outer shell) (Å)	50 – 2.1 (2.2-2.1)	50 – 2.4 (2.5-2.4)	50 – 2.9 (3.0-2.9)	50 – 2.9 (3.0-2.9)
Unique reflections	869280 (29881)	107002 (10017)	353968(23484)	206765(16895)
Completeness (%)	74.6 (25.8)	16.8 (15.2)	79.7 (53.2)	46.7 (38.4)
Multiplicity	2.7 (1.2)	1.2 (1.2)	2.2 (1.6)	1.4 (1.3)
Average I/σI	3.5 (0.5)	1.8 (0.4)	2.2 (0.7)	1.8 (0.7)
R _{merge}	0.244	0.259	0.407	0.360
Refinement statistics:				
Resolution range (Å)	50 – 2.1	50 – 2.4	50 – 2.9	50 – 2.9
No. of reflections(working/test)	814431/43049	118415/6336	334790/17689	195890/10435
R-factor (R _{work} /R _{free})	0.190/0.193	0.24/0.25	0.235/0.239	0.255/0.261
Protein atoms	5199	5200	4793	4793
Non protein atoms	349	N/A	58	N/A
r.m.s.d. bond length (Å)	0.014	0.011	0.006	0.006
r.m.s.d. bond angle (°)	1.8	1.67	1.30	1.36
Average B-factor (protein/water Å ²)	31/42	N/A	35/39	N/A

The $2|F_{obs}| - |F_{av}|$ electron density map for the VP2 S93C mutant capsids, calculated from the amplitudes and phases derived from the cyclic NCS averaging clearly showed that the disulphide bond is correctly formed across the 2-fold axis between two pentamers whereas the recombinant wildtype showed the expected histidine density (Figure 5-4).

With the VP2 S93F crystals, only four out of the 10 crystals diffracted in space group *I*222, other crystals diffracted in *P*2. As a result, the completeness was only 17% (15% at 2.4Å). Nevertheless, with 15-fold NCS constraints, the averaged electron density map had a high signal-to-noise ratio and clearly showed electron density for the phenylalanine side-chain, making a hydrophobic stacking interaction at the 2-fold axis as predicted from the MD simulations and binding free energy calculations (for details see chapter 2).

As there was a very small difference between the histidine and phenylalanine density at 2.4Å, the presence of phenylalanine was further verified by sequencing. The difference electron density map calculated from the amplitudes of recombinant wildtype and mutant empty capsids $|F_{obs-mut}| - |F_{obs-wt}|$ showed an extra patch of positive density around the phenylalanine side-chain further confirming the density for phenylalanine (Figure 5-5). The refinement also showed a good agreement between the *F_{obs}* and *F_{calc}* with *R_{work}* = 24%. More diffraction data have been collected and will be added to complete the structure and further improve the statistics.

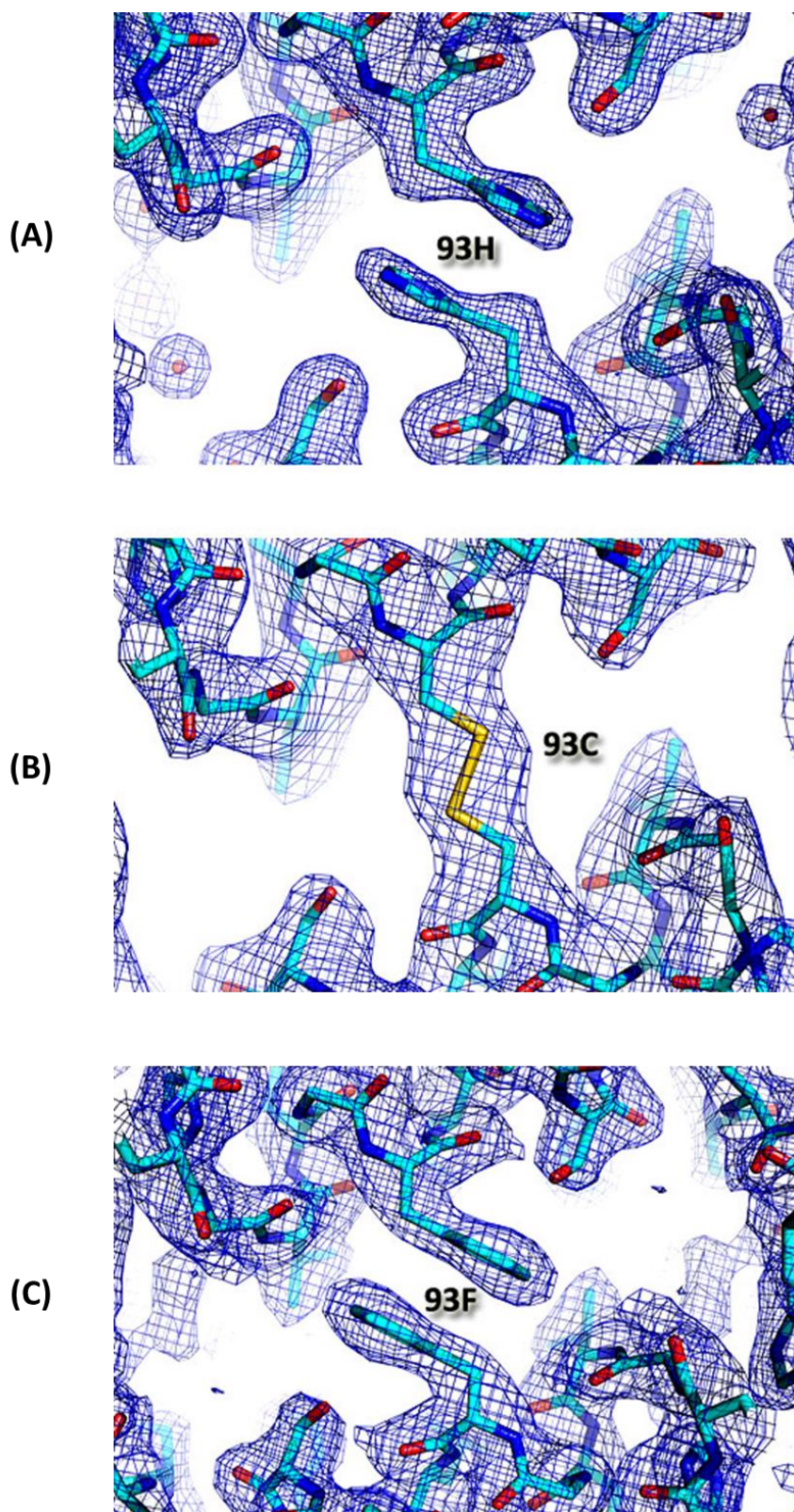


Figure 5-4: Structure analysis of recombinant FMDVA22 empty capsids. The three-dimensional structures of wildtype and mutants were solved at atomic resolution. (A) The structure of recombinant A22 wildtype showed the expected electron density for histidine side chain. (B) The structure of recombinant A22 mutant VP2 H93C showed the disulphide density at the 2-fold axis between two pentamers and (C) The structure of recombinant A22 mutant VP2 H93F showed hydrophobic stacking of phenylalanine side chains at the 2-fold axis.

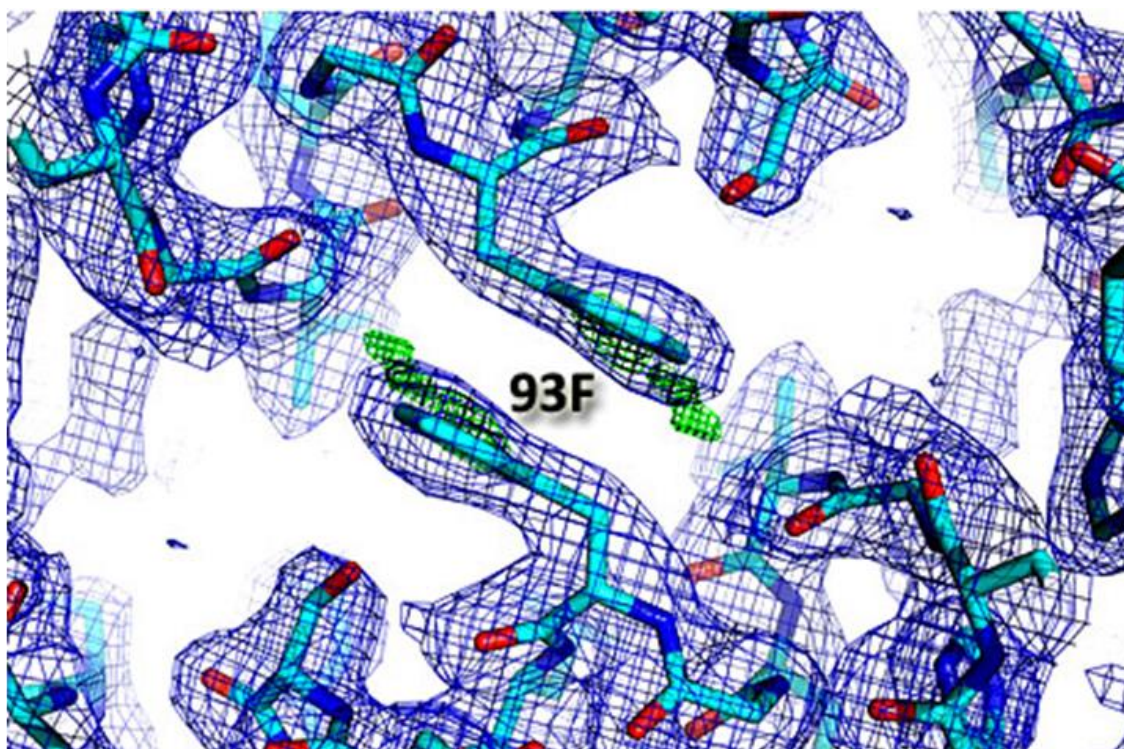


Figure 5-5: Electron density difference map showing positive density for phenylalanine. The presence of positive peak (shown in green) in electron density difference map $|F_{obs-mut}| - |F_{obs-wt}|$ further validated the phenylalanine side-chain.

The structures of native A22 virus and its natural empty capsids were previously reported by Curry et al. (1996) and (1997). When compared, the structures of recombinant FMDV A22 empty capsids were found to be very similar. The structural superimposition gave rms deviations in *C α* s of 0.4Å between the native and recombinant wildtype capsids and 0.36Å between recombinant mutant capsids. Despite the high degree of similarity, one region in VP3 (residues 171-181) on the surface of the particle, termed the VP3 GH loop (Acharya et al. 1989), showed significant structural differences. The conformation of this GH loop is essentially identical in the natural A22 structures (native and natural empty capsids) whereas in the recombinant empty capsids (wildtype and mutants) the conformation is more extended (Figure 5-6) and almost identical to that previously observed with another A serotype virus, subtype A10 (Fry et al. 2005). It has been shown previously that the conformation of the VP3

GH loop is modulated by changes in the VP1 GH loop, amino acid residues 136 to 156 (Curry et al. 1996). The sequence alignment of the VP3 GH loops of A22 and A10 serotypes showed only a single change V174T whereas there were several changes in the VP1 GH loops. Therefore, it is possible that one or more mutations occurred in the highly variable VP1 GH loop during replication of native A22 virus which accounts for the different conformation of the VP3 GH loop in native A22 virus capsids. The VP1 GH loop region is very flexible and density for it was not observed either in natural (Curry et al. 1996; Curry et al. 1997) or recombinant capsids.

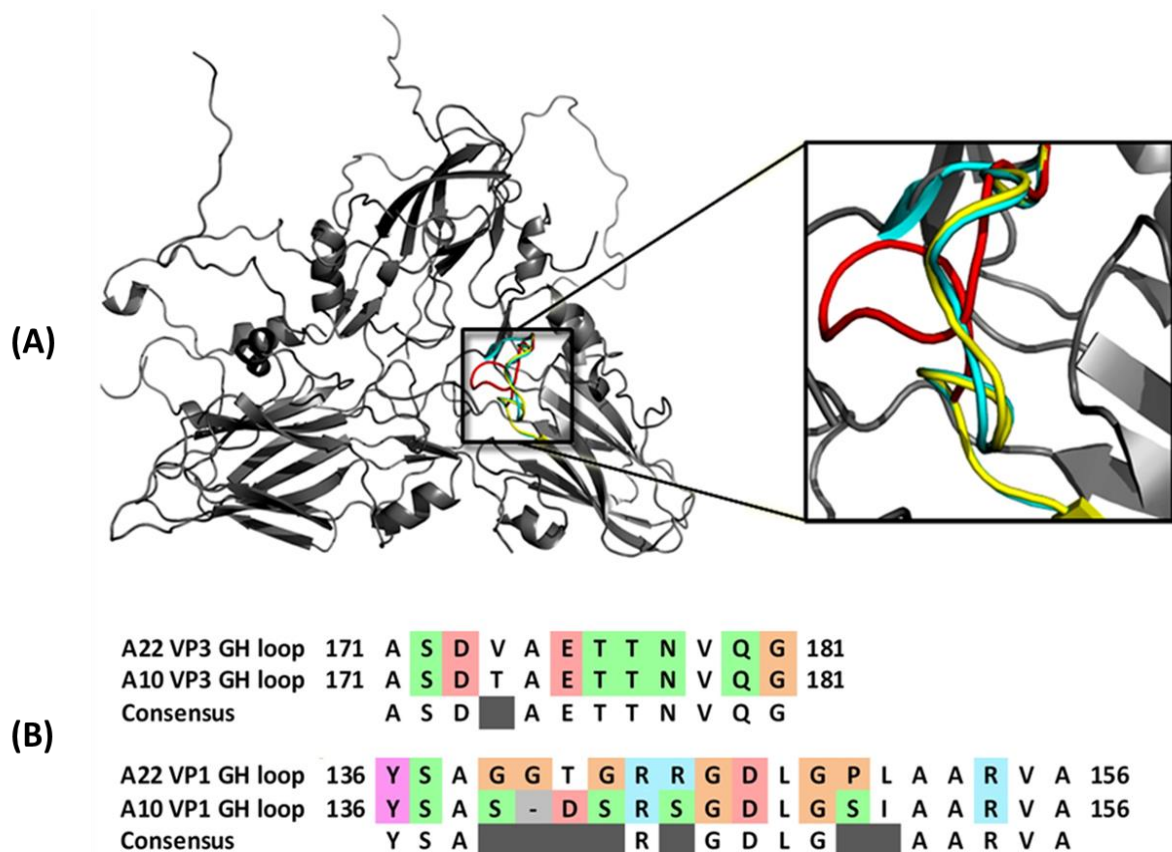


Figure 5-6: Extended conformation of VP3 GH loop and its sequence alignment to A10 subtype. A significant structural difference was observed in the VP3 GH loop between natural (red) and recombinant (yellow) A22 structures. (A) The VP3 GH loop in recombinant A22, wildtype and mutants, is more extended and almost similar to A10 virus structure. (B) Sequence alignment of the VP1 and VP3 GH loops between A22 and A10 viruses.

In accordance with observations made by Curry et al. (1997) on the A22 natural empty structure, VP0 was found to be cleaved into VP4 and VP2 in the recombinant empty capsid. A difference electron density map between the wildtype and mutant recombinant structures showed no significant structural differences on the particle surface. However, changes were apparent on the interior of the particles, VP4 was better ordered and the conformation was identical between the recombinant wildtype and VP2 S93F mutant to that of A22 virus structure, whereas with VP2 S93C, it was completely disordered and the density for only 5 residues was observed (Figure 5-7).

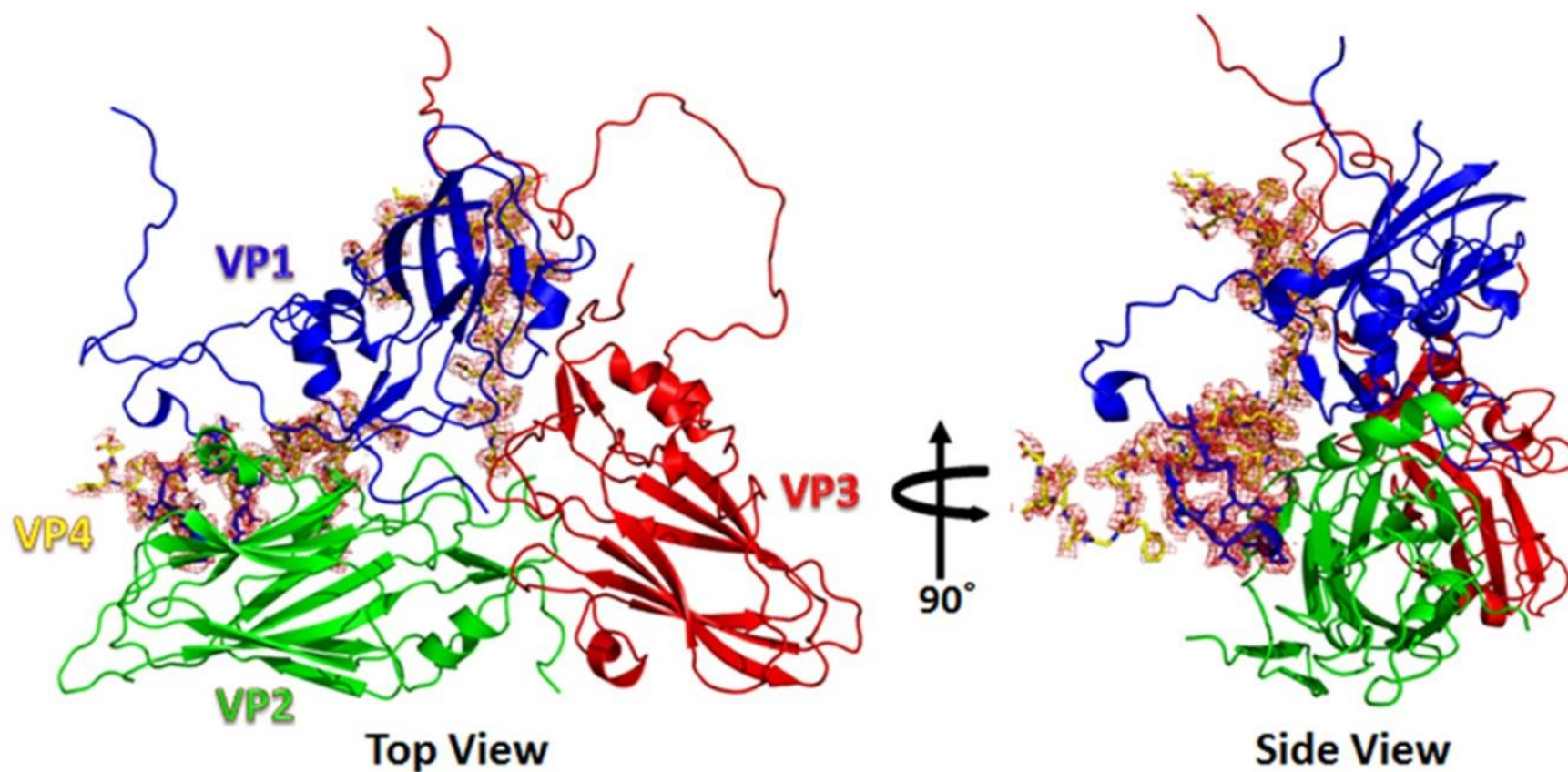


Figure 5-7: Disordered regions of VP4 in VP2 S93C mutant capsids, A22 Iraq. The structures of the recombinant capsids were similar to that of A22 virus on the exterior surface. However, on the interior, the VP4 was disordered in VP2 S93C (shown in red density).

The reason for this is unclear; however it is possible that the greater rigidity due to the disulphide bond between two pentamers inhibits the movements required for VP4 to settle into an ordered conformation following the cleavage of VP0. The N-terminus of VP1 (residues 1 to 12) and residues 34-36 in VP2 were also disordered. Nevertheless, the disordered regions are located on the interior of the capsids and do not affect the antigenic properties of the mutant capsids (See Chapter 7 for more details). To further verify, the disulphide bond was reduced by soaking the crystals in 10mM dithiothreitol (DTT), 2 hours prior to data collection (Table 5-1). The reduction of the disulphide bond was verified by an electron density difference map between the amplitudes of the reduced and non-reduced structures which showed a positive peak for the free thiol-sulphates (Figure 5-8). However, no additional density was observed for the disordered regions and the reduced structure was overall similar to that of VP2S93C non-reduced.

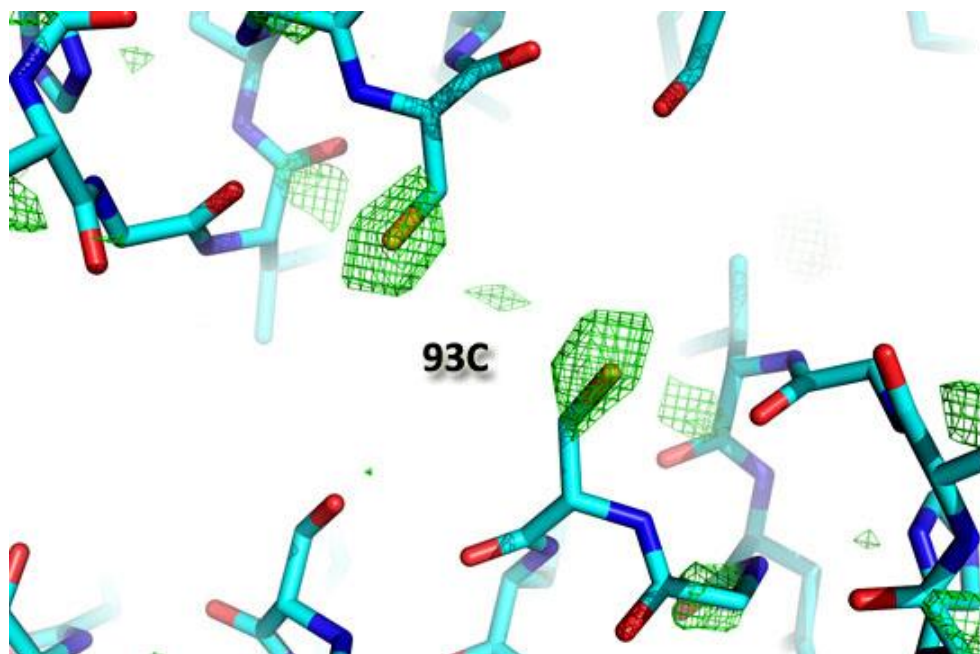


Figure 5-8: Structure of the VP2 S93C mutant with reduced disulphide bond. The disulphide was reduced by soaking the VP2 S93C crystals with 10mM DTT. Free thiol-sulphate density was verified by an electron density difference map calculated from the amplitudes of the reduced and non-reduced structures.

5.11.2 Analysis of O1M crystals.

The crystals of recombinant O1M wildtype capsids (obtained with 1.5M lithium sulphate) and inactivated VP2 S93Y virion capsids (obtained with 1.5M ammonium sulphate) diffracted to 3.0Å and 3.5Å respectively in space group *I*23 with cell dimensions ($a = b = c = 344\text{\AA}$ and $\alpha = \beta = \gamma = 90^\circ$) similar to that observed with the O1BFS structure (Acharya et al. 1989) with five protomers in the asymmetric unit.

In the *I*23 space group, the icosahedral virus can be packed, on a specific 3-fold axis, in two different ways that are related by a 90° rotation about a particle 2-fold axis (Figure 5-9). Within the same crystal, all particles are generally packed in the same orientation; however, the choice of orientation will be random between different crystals.

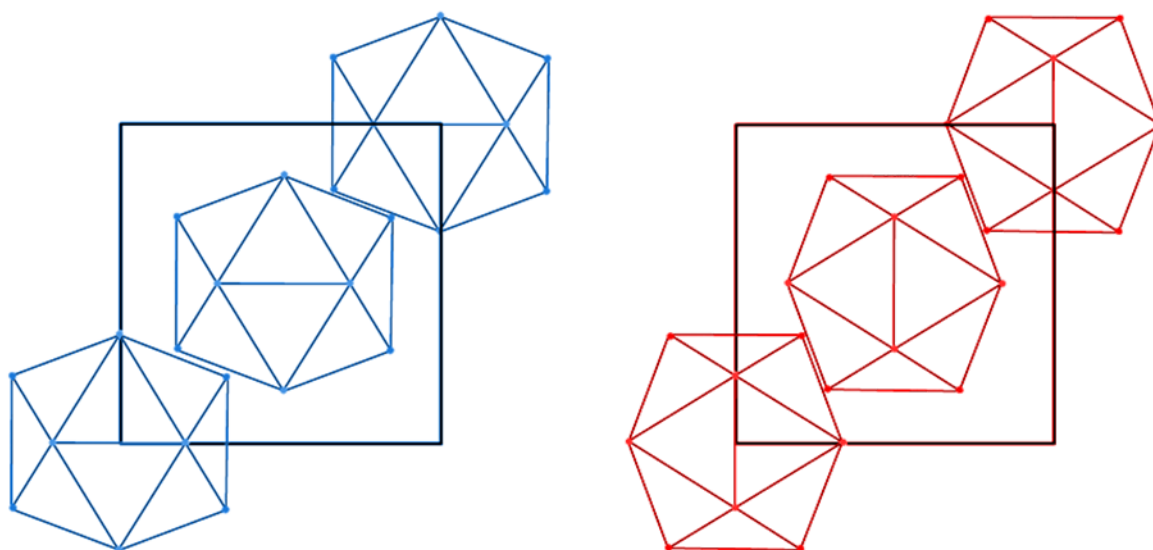


Figure 5-9: Orientations of virus capsids in a cubic space group. In space group *I*23, icosahedral viral capsids can be packed in two different ways which are related by 90° rotation symmetry to each other.

As a result, when merging the data from multiple crystals, special care should be taken to correct for the orientation. This can be achieved by two different methods. In one method, an individual crystal merged in *I23* can be used as a reference and compared with the other crystal during the merging process. If the crystal is indexed differently to the reference data, the χ^2 and *Rmerge* will be significantly higher. The orientation can be corrected to match the reference data by simply swapping a pair of reciprocal indices *hkl* to $-khl$. However, when the method was applied on O1M data, no differences were observed in either orientation. As the reference set contained only 4-5 diffraction images (because of the radiation damage only 4-5 images could be collected per crystal), it is possible that the number of reflections was not significant enough to show an observable difference between orientations. In a second method, a dataset of O1K (2.6Å resolution and 67% completeness) was used as a reference to orient the O1M crystals but this method was also unsuccessful. The inability to distinguish between the correct orientations suggested the datasets to be twinned.

The twinning of the O1M crystals was further verified by plotting a self-rotation function of the individual datasets. Figure 5.10 shows the peaks on a self-rotation function of the untwinned dataset of O1K in two different orientations and the twinned dataset from the individual O1M crystal. The presence of additional non-crystallographic symmetry peaks on the 2-fold ($\chi=180$), 3-fold ($\chi=120$) and 5-fold ($\chi=72$) sections of the self-rotation plot and the presence of peaks on the $\chi=90$ (4-fold symmetry) section suggested merohedral twinning of the O1M crystals. A 4-fold symmetry is not possible with icosahedral viruses since they have no 4-fold symmetry and the unit cell is too small to accommodate viral capsids around a 4-fold crystallographic symmetry.

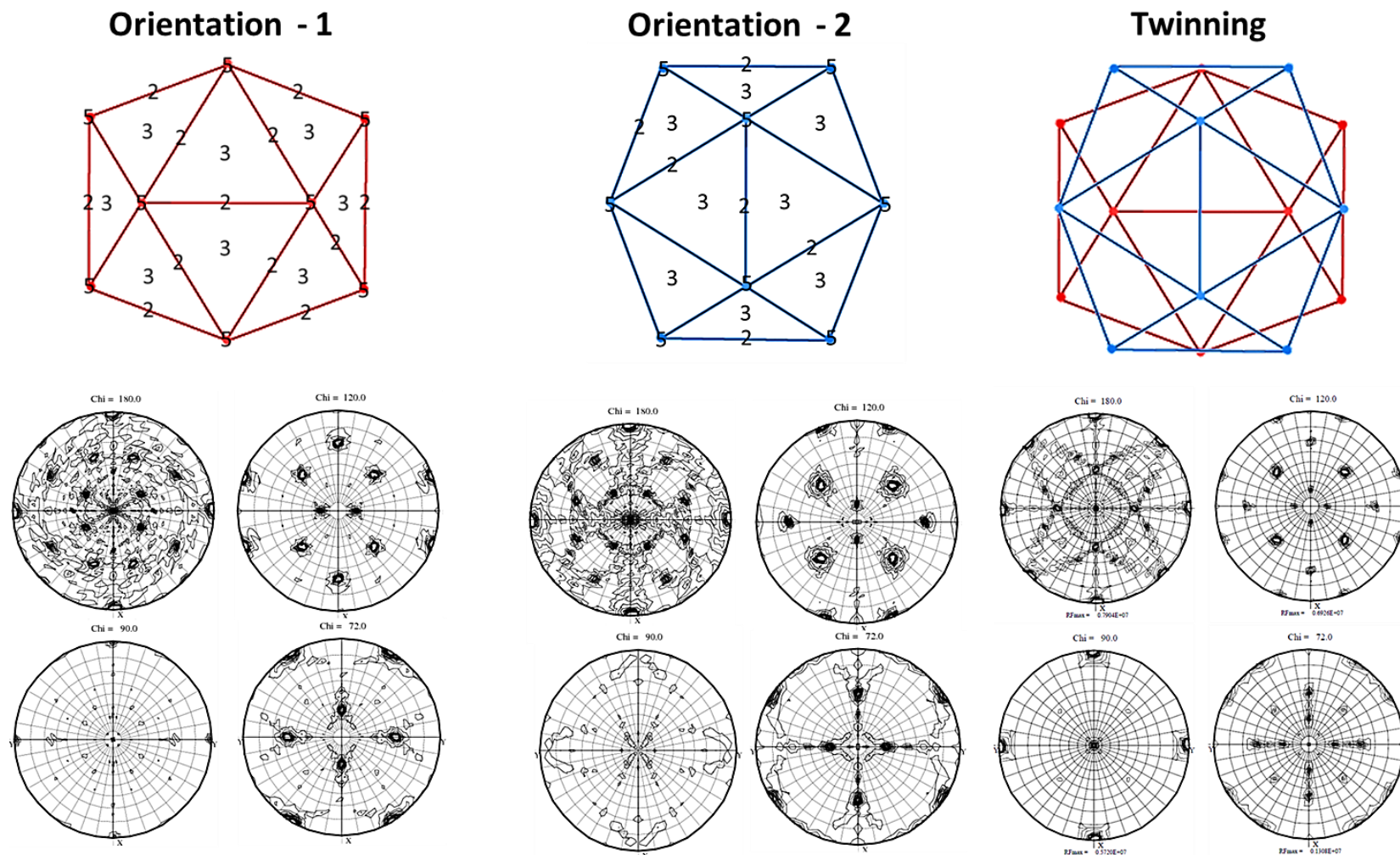


Figure 5-10: Twinning of O1M crystals. The crystals of recombinant O1M wildtype and inactivated O1M VP2 S93Y were found to be twinned. The twinning was confirmed by additional symmetry peaks, including 4-fold symmetry, by plotting a self-rotation function using MOLREP module of CCP4 package.

Twinning is not uncommon especially with virus structures because of their approximate spherical nature and such twinning cases have previously been reported with another variant of the FMDV O-serotype by Lea and Stuart (1995). Accordingly, O1M crystals could be scaled and merged in point group 432. The data processed in space group *I432* were almost identical to those obtained in space group *I23* and no information was lost by processing the O1M data in the higher symmetry point group, further confirming the twinning of the dataset. Thus the diffraction data consists of superimposition of two sets of non-equivalent lattices and the usual procedure is to screen for a more favourable crystal form that is not twinned. However, Lea and Stuart (1995) successfully used a cyclic procedure to de-convolute the overlapped lattices by reconstructing the structure-factor amplitudes for the *I23* cell from *I432* using the 5-fold NCS and solvent flattening constraint on the twinned reflections. A similar process is being developed for de-convoluting the O1M dataset in order to provide reasonable estimates of the electron density for the O1M capsids.

5.12 Conclusion

Three-dimensional structures of two of the designed mutants and the recombinant wildtype of the FMDV A22 serotype were solved to high resolution. The structures were solved by molecular replacement and the phases improved by real space NCS averaging. There was no significant change in the three-dimensional structure of the recombinant wildtype capsids suggesting no change in the antigenicity of the recombinant capsids. The structure was almost identical to that of the A22 virus and its natural empty capsids. The VP3 GH-loop was more exposed on the particle surface but this just seems to be an alternative conformation as it has been previously observed with another A-serotype, A10 possibly linked to a sequence variation in the adjacent VP1 GH loop. As predicted from the MD simulations, the mutant VP2 S93F formed a hydrophobic stacking interaction of the phenylalanine side-chain and the

mutant VP2 S93C formed the expected disulphide bond at the 2-fold axis relating two FMDV pentamers. Datasets for O1M wildtype and VP2 S93Y were also collected at high-resolution. However, the crystals showed merohedral twinning. Such twinning has been observed previously with another O-serotype virus and work is in progress to de-twin the diffraction intensities and to obtain good electron density map for the structures. Similarly, crystallisation conditions for the inactivated SAT2 VP2 S93Y were also identified and work is in progress to optimize the conditions to grow diffraction quality crystals. Experiments showing the thermo- and pH stability of these and other predicted mutants are discussed in the next chapter and the application of mutants as a next generation of stable vaccines with improved properties are discussed in Chapter 7.

Chapter 6 : Assessing the thermostability of the stabilised virus particles and recombinant capsids

6.1 Introduction

The potency of all vaccines is temperature dependent. The majority of currently used vaccines are stored at 2-8°C and have a shelf life of 1 to 2 years. Temperature plays a crucial role in maintaining this shelf life and the vaccines are highly unstable at higher temperatures. As a result, cold chain systems have been implemented to ensure that the potency of the vaccines are maintained and the vaccines are stored under refrigerated conditions until required. Building and maintaining cold chains is a major undertaking and requires huge investments. It also complicates the transportation and storage of vaccines particularly in developing countries where the diseases are endemic (D. Chen and Kristensen 2009).

Inactivated FMDV capsids are very sensitive to temperature and rapidly dissociate into pentameric subunits, reducing the potency of the vaccines. Even with the use of cold-chains, the shelf life is only about 6 months. The situation is worse with inactivated O and SAT serotypes, the capsids of which are notoriously unstable and have a very limited shelf-life of only a month; the potency of the vaccines is severely decreased even when administered multiple times. A and C serotypes are to some extent more stable than O and SAT serotypes, but chemical inactivation usually reduces the thermo-stability of the respective capsids (Doel and Baccharini 1981; Doel and David 1984).

In this chapter, I will describe the methods used to characterize the thermo-stability of FMDV particles containing capsids with targeted mutations (designed using the MD simulations). Several different methods were used in parallel to verify the stability of the inactivated and recombinant empty capsids. A brief introduction to each of these methods is given, followed by the respective results obtained. Finally, a correlation of predicated stability derived from the MD data to the capsid stability is shown.

Once again the work presented in this chapter was done as part of collaboration with The Pirbright Institute, ARC-OVI and STRUBI. Thermo-stability assays on recombinant O1M wildtype and mutant empty capsids were performed by Alison Burman (The Pirbright Institute). Thermo-stability assays on recombinant A22 wildtype and VP2 H93C (covalent mutant) empty capsids were performed by Alison Burman and Claudine Porta (The Pirbright Institute). Thermofluor assays on infectious O1M wild type and mutant viruses were performed by Julian Seago (The Pirbright Institute), and thermofluor assays on infectious SAT2 wild type and mutant viruses were performed by Francois Maree and Katherine Scott (ARC-OVI). Thermofluor assays on inactivated viruses and temperature stability of inactivated and recombinant empty capsids by electron microscopy were performed by myself.

6.2 Stability assay using sucrose density gradient

Intact FMDV capsids are routinely purified using ultracentrifugation over a sucrose density gradient. The capsids are sufficiently dense that they migrate within the gradient and form a characteristic banding pattern (see Chapter 3 for more details). As FMDV capsids readily dissociate into pentameric subunits at elevated temperatures, the sucrose density gradient method was utilized to test the heat stability of the wild type and mutant empty capsids. After heat treatment stabilised empty capsids should remain intact and migrate through the sucrose gradient. Dissociated subunits will remain at the top of the gradient.

Sucrose gradient purified samples of FMDV empty capsids were diluted with phosphate buffer and incubated in a water bath at either 45°C for 1 hour or 56°C for 2 hours. They were then loaded onto 15-45% sucrose density gradients and sedimented as discussed in Chapter 3. The gradients were fractionated into 12 fractions and an equal volume of saturated

ammonium sulfate was added to each in order to precipitate any empty capsids present. The pelleted precipitates were then analysed by SDS PAGE and Western blot analysis for the VP1 capsid protein. In similar experiments, samples containing empty capsids were diluted with 50mM sodium acetate buffer pH 4.6 to give a final pH of 5.2 and incubated for 15 minutes at room temperature. After neutralization the empty capsids were loaded onto 15-45% sucrose density gradients and centrifuged as before. SDS PAGE and Western blot analyses for VP1 capsid protein were performed on the gradient fractions.

6.3 Thermofluor Assay

A thermofluor assay is a biophysical technique used to study protein stability. It is routinely used in biochemistry labs to test protein solubility and stability in different buffer conditions. The protein of interest is screened against a matrix of buffers, ions, additives and ligands using thermal denaturation. The mixture is gradually heated to about 95°C and protein unfolding with the rise in temperature is probed using a fluorophore such as SYPRO Orange. The unfolding of protein exposes the hydrophobic residues to which a fluorophore can bind resulting in an increase in fluorescence. The process is also frequently used for screening crystallization conditions (Ericsson et al. 2006; Lavinder et al. 2009).

In Oxford the general approach has been further developed to produce a rapid and sensitive assay for virus particle stability, specifically by the simultaneous use of two, non-interfering dyes which monitor the presentation of hydrophobic protein surfaces and the release of viral RNA. This has been termed PASTRY (Particle Stability Thermal Release Assay) by Walter et al. (2012).

The thermofluor (PASTRY) assay was performed in 96-well PCR plates using an Agilent MX3005 PCR machine. All assays were performed in a total of 150µl reaction volume and comprised of the minimum volume of virus (300ng of virus, between 3 - 15µl), 15µl of SYBR green-II dye (Molecular Probes, Invitrogen; diluted 1:100 in the same buffer used for virus purification), with the volume made up with buffer. The temperature was ramped from 25°C to 95°C in 0.5°C increments with intervals of 10 seconds. SYBR green-II fluorescence was read with excitation and emission wavelengths of 490nm and 516nm respectively. The release of RNA and hence the dissociation of capsids was detected by an increase in fluorescence signal and the melting temperature was taken as the minimum of the negative first derivative of the fluorescence curve.

6.4 Results and discussion

Improvements in capsid stability can be assessed by using a variety of methods. In this thesis, three different methods were used to verify the thermo- and pH stability of the modified capsids. The thermo-stability of the recombinant empty capsids was verified by incubating the capsids at different temperatures and the pH stability was verified by incubating the capsids at pH 5.2. This was followed by sedimentation through 15-45% sucrose density gradient. Thermo-stability of the capsids of infectious viruses was investigated by detecting the release of RNA using a thermofluor assay. The intactness of both inactivated viruses and empty capsids after heat treatment was verified by electron microscopy.

6.4.1 Thermo- and pH- stability of the capsids measured using sucrose gradient analysis

The purified capsids of serotype O, subtype O1M wildtype and mutants were diluted with phosphate buffer and heated to 45°C for 1 hour. The samples were cooled and loaded onto 15-45% sucrose density gradient and sedimented as before. The mutant empty capsids remained intact and migrated through the gradient forming a band at the bottom half of the

gradient, corresponding to the position previously determined for intact capsids (see Chapter 3 for details). No such band was detected for the wildtype empty capsids, suggesting their dissociation during heat treatment (Figure 6-1).

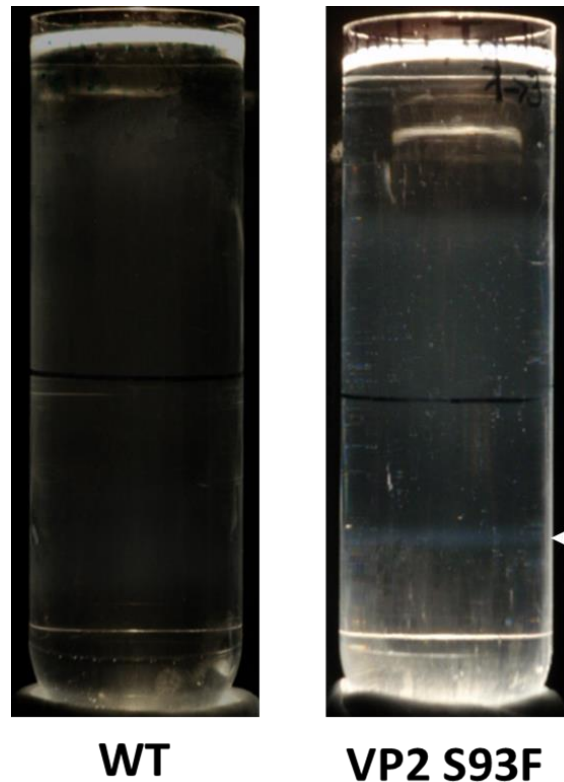


Figure 6-1: The modified recombinant empty capsids are thermo-stable. Wild type and stabilised mutant capsids of O1M serotype were heated to 45°C and the intactness of the capsids verified by sucrose density gradient. The mutant VP2 S93F capsids were intact and sedimented at the bottom half of the gradient whereas wild type capsids dissociated and did not form a band.

The gradient was fractionated (11 fractions were collected from the bottom to top of the gradient) and analysed by SDS PAGE and Western blot using anti-FMDV polyclonal antibodies. As expected, the VP2 S93F capsids remained intact, with the VP1 capsid protein detected in fraction 5 near the bottom of the gradient. In comparison, the wild type empty capsids remained near the top of the gradient in fractions 10 and 11 confirming their dissociation. Similar results were obtained with two other stabilised mutants, VP2 S93H and VP2 S93Y (Figure 6-2).

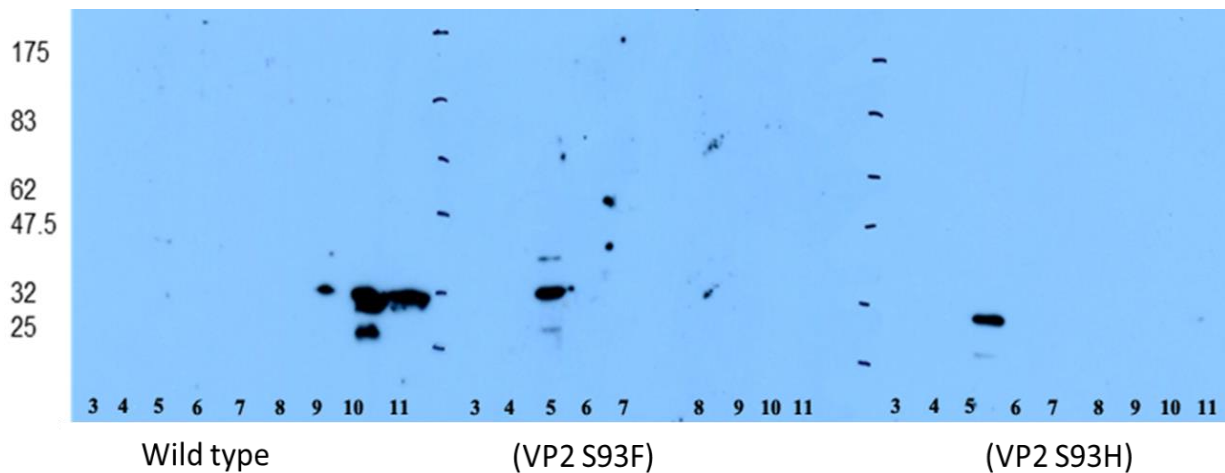


Figure 6-2: Heat treatment at 45°C for O1M recombinant empty capsids (produced in vaccinia expression system). Wildtype and mutant capsids of FMDV O1M were purified and treated at 45°C for 1hr and then sedimented through a 15-45% sucrose gradient. The wildtype capsids dissociated and remained at the top of the gradient and the capsid protein VP1 (31kDa) was detected in fractions 10 and 11 on the Western blot, whereas mutant capsids remained intact and were detected in fraction 5. Fractions are labelled at the bottom of the gel.

To further test their respective thermo-stabilities, the mutant capsids were re-incubated at 56°C for 2 hours and the sucrose density gradient sedimentation repeated (Figure 6-3). Mutant VP2 S93H was found to be unstable (all material in fractions 10-11); S93Y was found to be partially stable (about 25 % was in fraction 10 and the rest in fraction 4); and S93F was found to be completely stable (all in fractions 4 and 5). The results confirmed that the favourable hydrophobic interactions, as predicted by MD simulations, increased the stability of the FMDV capsids.

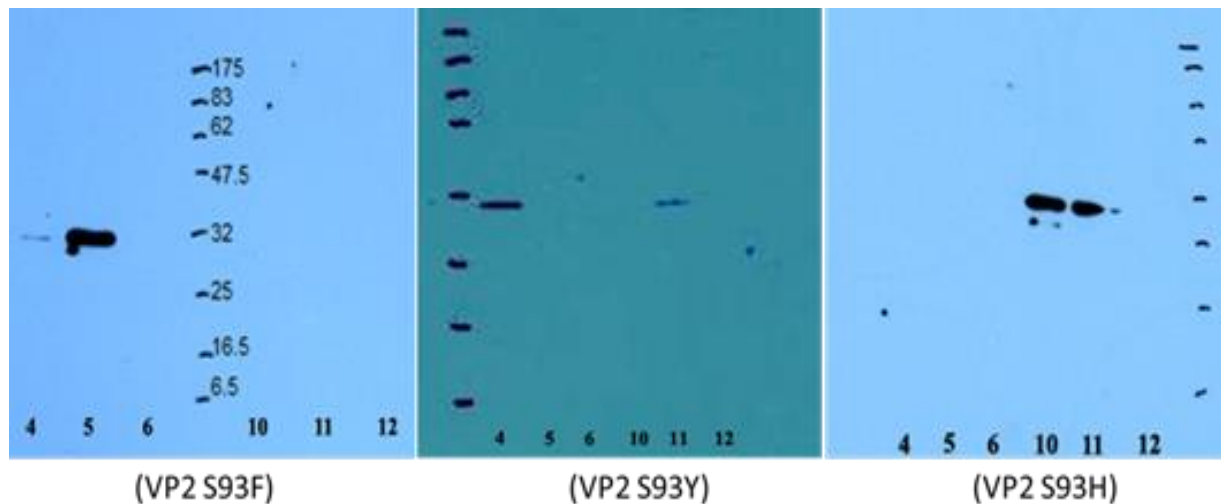


Figure 6-3: Heat treatment at 56°C for O1M recombinant empty capsids. Stable mutant capsids were further treated at 56°C for 2hrs, then sedimented through a 15-45% sucrose gradient and analysed by Western blot. VP2 S93H mutant was stable at 45 °C, however at 56°C it was found to be unstable and was detected in fractions 10 and 11; VP2 S93Y was partially stable and VP2 S93F was completely stable. (Experiments performed by Alison Burman)

The results also correspond remarkably well with the predicted $\Delta\Delta G$ values for these mutants (discussed in Chapter 2): VP2 S93H: -7.7; S93Y: -11.8; and S93F: -13.8 kcal/mol.

A sample from the VP2 S93F capsid material from the 2hrs, 56°C heat treatment was analysed by electron microscopy. The capsids were found to be completely intact with only a few pentamers detected (Figure 6-4), confirming the Western blot results and predicted $\Delta\Delta G$ value for this mutant. These results show that the thermo-stability of FMDV empty capsids of one of the least stable serotypes can be markedly improved by increasing the hydrophobic interactions at the interface, in a way fully compatible with particle assembly.

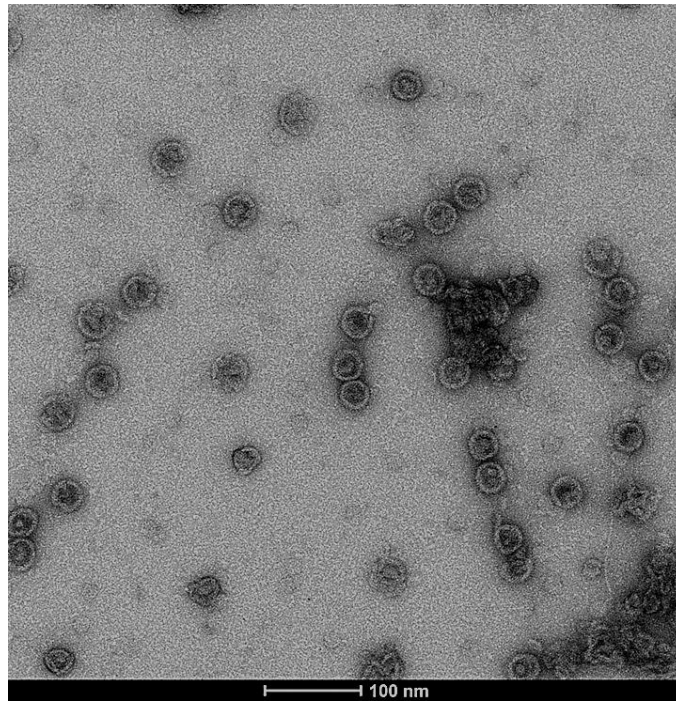


Figure 6-4: EM analysis of heat treated type O1M recombinant mutant empty capsids. VP2 S93F mutant was heated to 56°C for 2hrs and capsids sedimented over a 15-45% sucrose gradient. The EM analysis of the peak fraction showed intact capsids suggesting improved stability.

To investigate the pH stabilities of the type O empty capsid mutants, purified empty capsids were acidified to pH 5.5, incubated at room temperature for 15 mins and sedimented onto a 15-45% sucrose gradient. The fractions were analysed by SDS PAGE and Western blot as previously discussed. The wildtype capsids dissociated and remained near the top of the gradient in fraction 10, whilst the VP2 S93F and S93Y mutant capsids appeared to be stable. The empty capsids of FMDV type A (subtype A22 Iraq) containing the covalent mutation VP2 H93C, were also heat treated at 56°C for 2hrs or acidified to pH 5.2 and incubated at room temperature for 15 mins. Subsequent analysis by sucrose gradient and Western blot methods showed the mutant empty capsids were present in fraction 4, indicating they were stable and completely intact after either treatment. In comparison, the wildtype capsids dissociated, remaining at the top of the sucrose gradient in fraction 10.

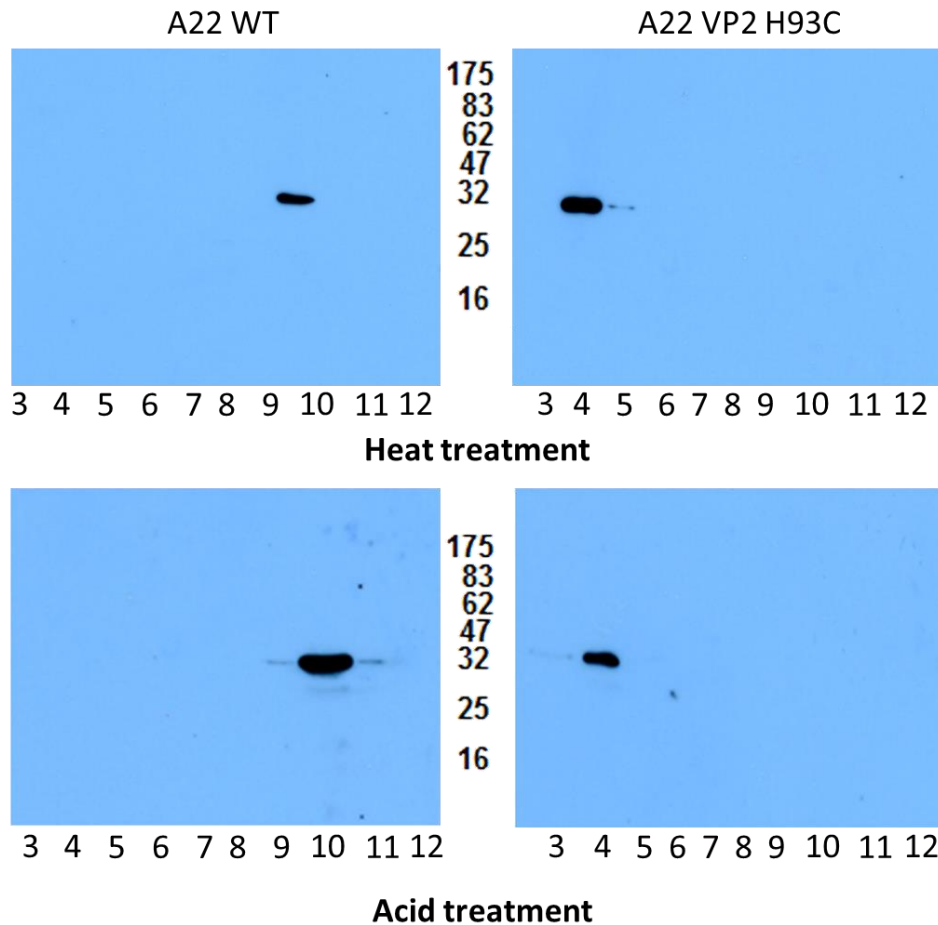


Figure 6-5: Heat and pH stability of covalent mutation of type A virus, A22 Iraq (produced in baculovirus insect expression system), recombinant empty capsids of A22 Iraq containing a covalent mutation making a single disulfide bond at the 2-fold axis VP2 H93C was found to be heat and pH stable. The fractions 3 to 12 were collected from the bottom of the gradient (labeled below the gel) and analysed by Western blot using antibodies against FMDV VP1 protein (31kDa). The mutant capsids remained intact after both heat and pH treatment and the capsid protein was detected in fraction 4, whereas capsid protein for wildtype was detected at the top in fraction 10. (Experiment performed by Alison Burman and Claudine Porta)

6.4.2 Thermofluor (PASTRY) analysis of infectious virus

To study the dissociation of the capsids of infectious viruses, the more sensitive thermofluor assay was used whereby the release of RNA (and hence the dissociation of FMDV capsids) with the rise in temperature was monitored, using an RNA specific fluorescent dye. The assays were performed on infectious FMDV O1M and infectious SAT2 Zim/7/83 wildtype and stabilised mutants. The wildtype O1M capsid dissociation as detected by release of viral

genome, occurred at 52°C whereas the mutants were found to be more stable and required higher temperatures for genome release and capsid dissociation; VP2 S92Y dissociated at 53.5°C and VP2 S93F at 54°C (Figure 6-6).

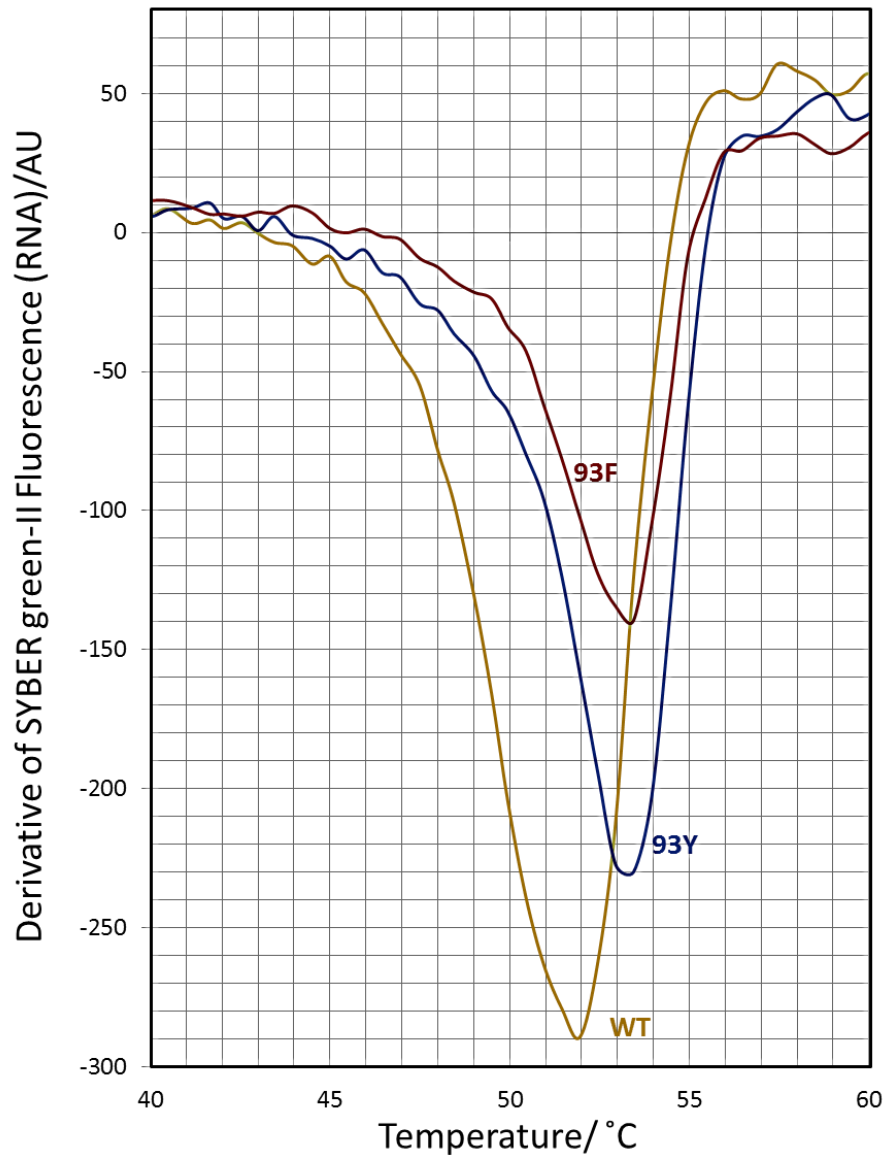


Figure 6-6: Thermo-stability of infectious FMDV O1M virus. Peaks from the thermofluor assay showed increased stability for both hydrophobic mutants. The mutant virion capsids dissociated at 53.5°C compared to wildtype which dissociated at 52°C. (Experiments performed by Julian Seago)

When the same experiments were performed on purified inactivated virion (inactivated twice as discussed in Chapter 3), both wildtype and mutant capsids became more unstable (Figure 6-7). Wildtype O1M capsids dissociated at 45°C, whereas mutant VP2 S93Y dissociated at 46.5°C.

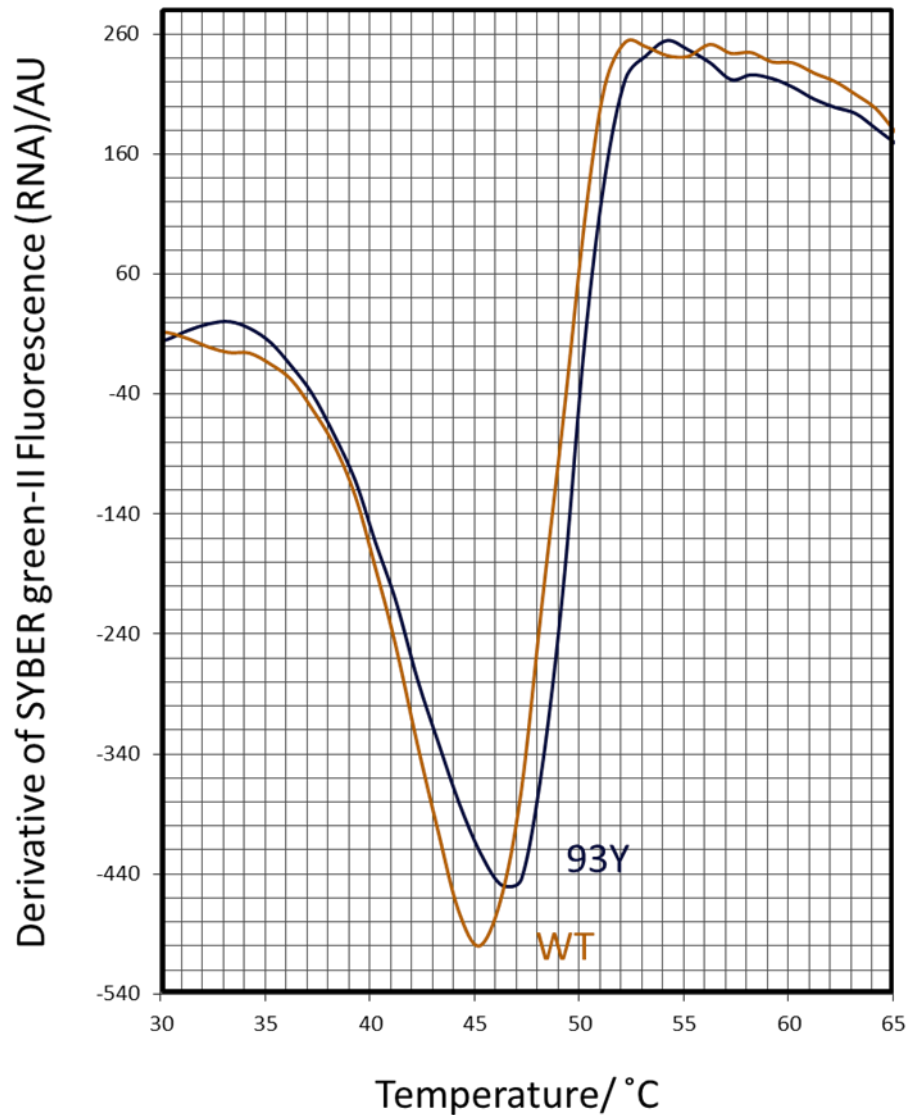


Figure 6-7: Thermo-stability of inactivated O1M capsids. After BEI inactivation, the capsids became more unstable. The wildtype capsids dissociated at 45.2°C compared to 52°C for infectious copy virus and mutant dissociated at 47.2°C compared to 53.5°C.

Although there was a decrease in the thermo-stability of BEI inactivated capsids, the mutant was 2°C more stable than wildtype. While the increase of 2°C in the thermo-stability is not very large, the EM analysis shown in 6.4.4 confirmed that even small differences can have a huge impact on the storage and shelf-life of the inactivated capsids.

In comparison to the O1M serotype, the increase in thermal stability between wildtype and mutant was greater for the SAT2 serotype. Thermofluor assays performed at The Pirbright Institute showed the wildtype infectious SAT2 virion capsids dissociated at 51°C, whereas those of VP2 S93Y dissociated at 54°C (Figure 6-8). The results are in good agreement with the predictions from MD simulations and ΔG calculations (-12.2 kcal/mol increase in binding free energy compared to wildtype capsids).

Surprisingly, thermofluor assays performed at STRUBI showed similar thermostability values for the inactivated SAT2 wildtype and mutant VP2 S93Y virion capsids. Nevertheless, these results are not directly comparable since the thermofluor assay is very sensitive to very small changes in conditions such as buffer, pH and presence of other additives like sucrose. For instance, the effect of sucrose concentration on inactivated SAT2 VP2 S93Y mutant showed a linear increase in the thermo-stability with increase in concentration of sucrose from 0% to 40% (Figure 6-9).

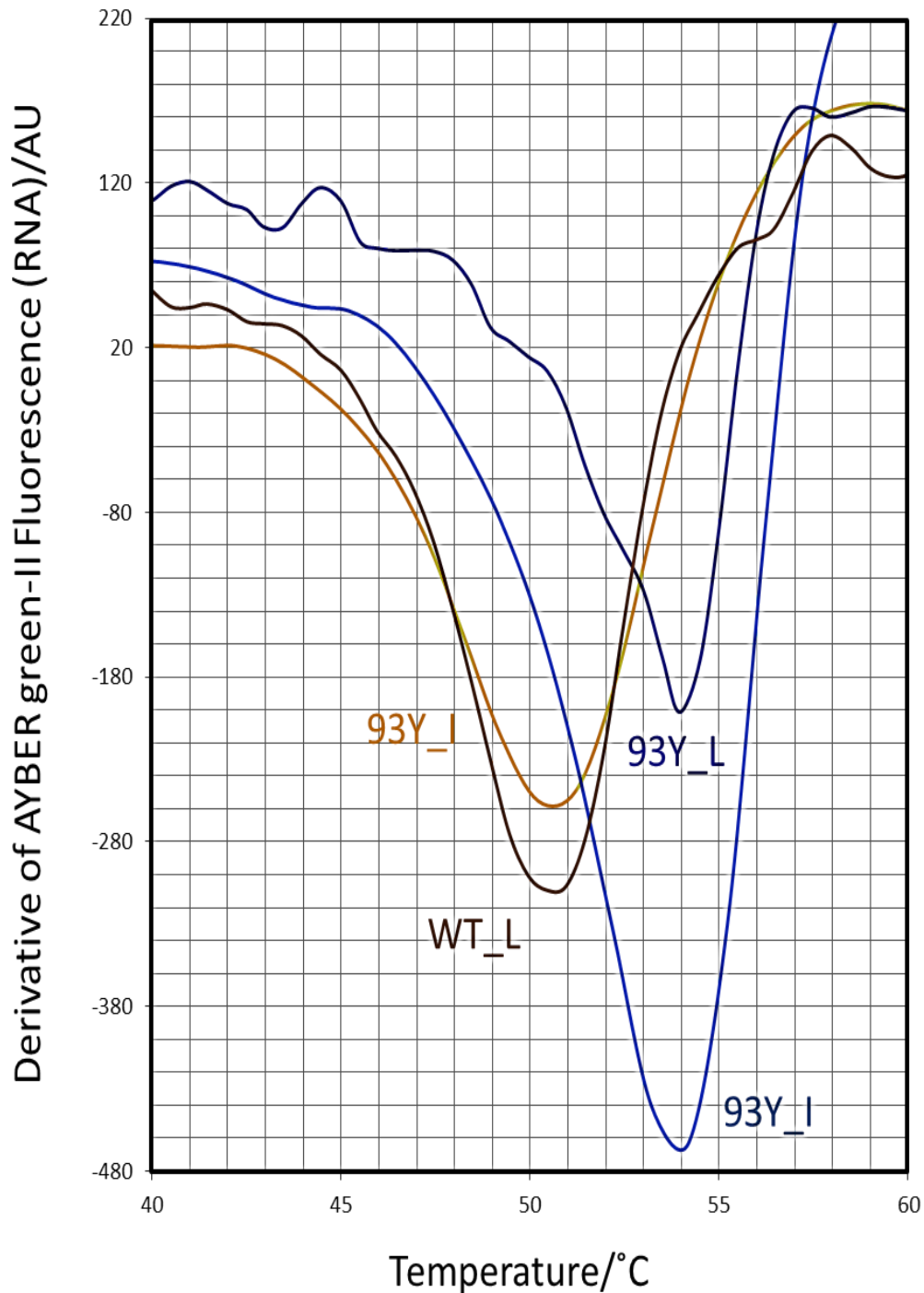


Figure 6-8: Thermo-stability of FMDV SAT2. The thermo-stability of FMDV SAT2 serotype was also improved. Infectious wildtype SAT2 (WT_L) dissociated at 51°C and mutant VP2 S93Y (93Y_L) dissociated at 54°C. Inactivated capsids showed similar results; inactivated wildtype: WT_I; inactivated VP2 S93Y: 93Y_I. (Experiments were performed by Julian Seago and myself).

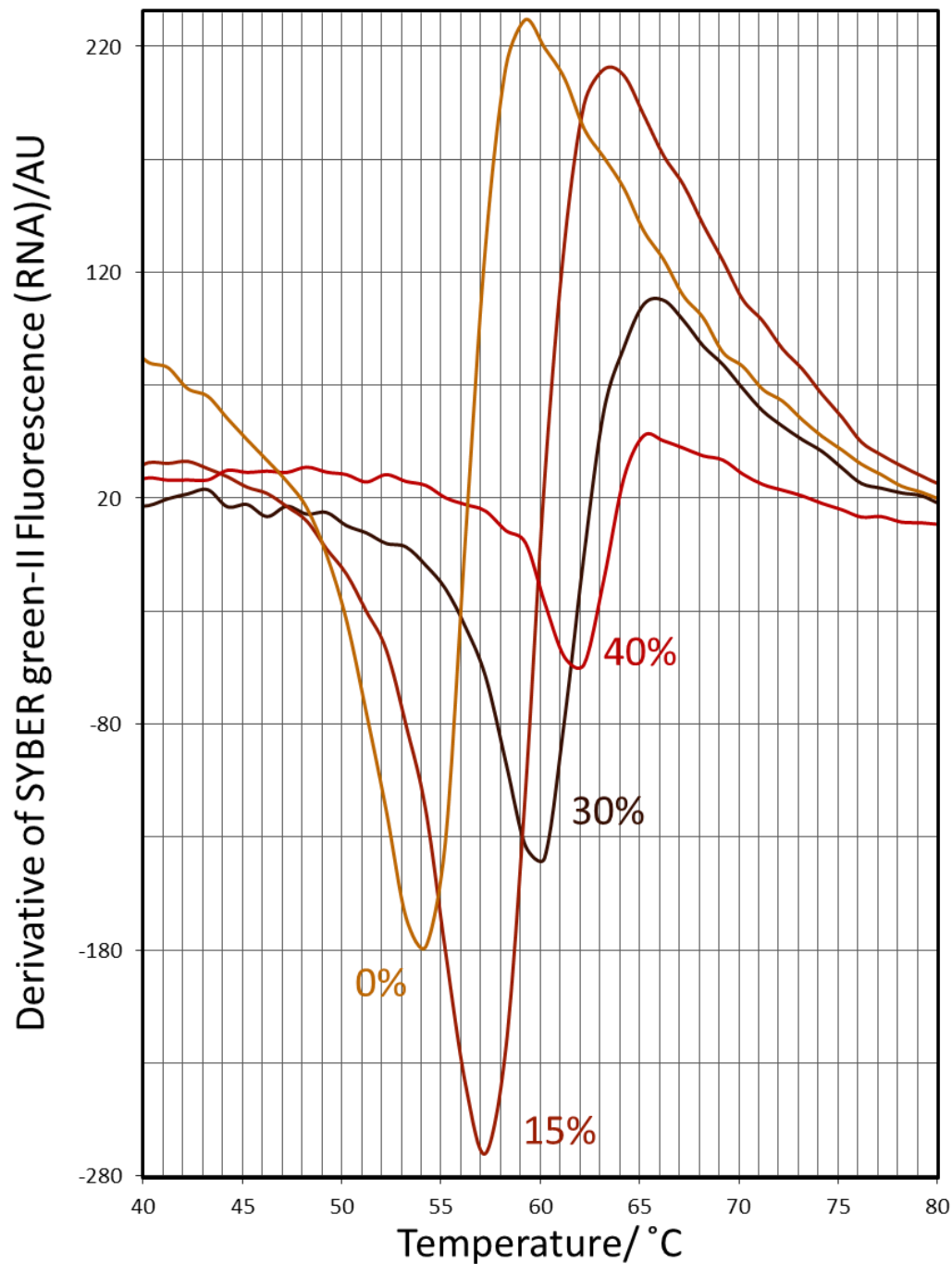


Figure 6-9: Effect of sucrose on thermo-stability of inactivated SAT2 capsids. Sucrose has a stabilising effect on FMDV capsids. Thermofluor results showed a linear increase in thermo-stability of upto 8°C with an increase in sucrose concentration from 0 to 40% with inactivated SAT2 VP2 S93Y capsids.

The experiments on infectious viruses were performed using PBS buffer at pH 7.4 whereas experiments on inactivated capsids were performed using HEPES buffer pH 8.0, 200mM NaCl.

There was also a discrepancy with the data received from the ARC-OVI. In their experiments the capsids of wildtype infectious SAT2 dissociated at 47°C, compared to 51°C observed above. However, in their experiments the capsids of mutant VP2 S93Y dissociated at 53.5°C, corresponding to the VP2 S93Y mutant in SAT2 and O1M. A mutant predicted to be less stable (VP2 S93H, predicted $\Delta\Delta G$ value of -5.3 kcal/mol) was also tested and dissociated at 51°C (Figure 6-10). The discrepancy observed for wild type SAT2 could be due to a variation in the experimental conditions, as discussed below.

A positive control consisting of a stable A serotype (A24 cruiserio) was also used which dissociated at 55°C (Figure 6-10). It is very encouraging that, as for the results for recombinant empty capsids, the results of the infectious virus are in very good agreement with the predicted $\Delta\Delta G$ values from the MD simulations: VP2 S93H = -5.3 kcal/mol and VP2 S93Y = -12.2 kcal/mol.

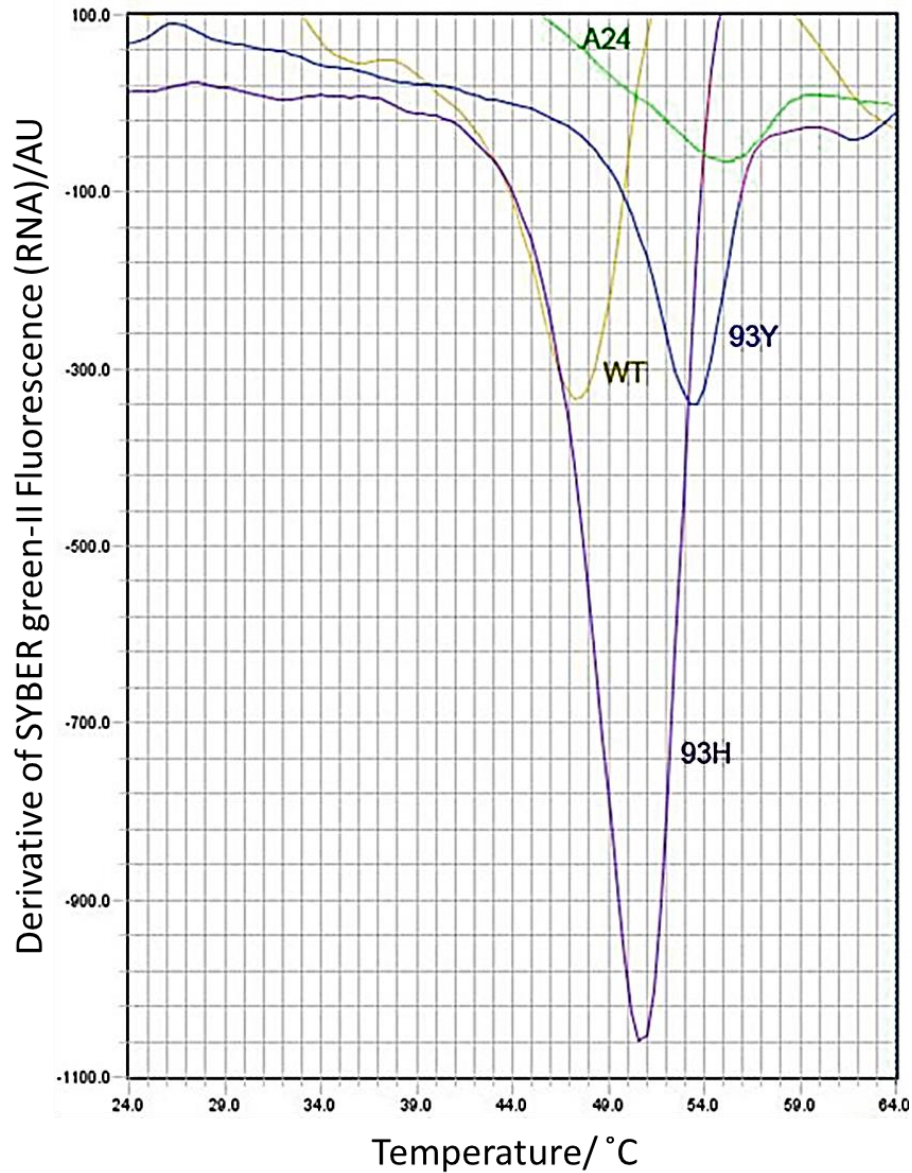


Figure 6-10: Thermo-stability of FMDV SAT2 native virus. Similar results were obtained by our collaborators with native SAT2 serotype. VP2 S93Y dissociated at 53.5°C similar to O1M VP2 S93Y, whereas wildtype capsids being more unstable dissociated at 47°C. VP2 S93H was also stable and dissociated at 51°C. (Experiments performed by Katherine Scott and Francois Maree).

6.4.3 Effect of pH and salt on the thermostability of viral capsids

To investigate the effect of pH, the purified O1M viruses were incubated for 5 min with a range of pH buffers between pH 6.0 to 9.0 at 4°C. The release of RNA and capsid stability was verified by thermofluor analysis between 25°C and 95°C as described previously. The results showed that FMDV capsids are extremely sensitive to changes in pH. The capsids were very unstable below pH 7.5 and the capsid instability increased above pH 8.0. The highest stability was observed at pH 8.0 (Figure 6-11). Furthermore, the effect of pH was also tested for the mutants. The wildtype completely dissociated below pH 6.5 however, VP2 S93Y and VP2 S93F were stable. At pH 6.5 wildtype capsids dissociated at 28°C whereas the mutant capsids were stable up to 36.5°C. The stability increased for both wildtype and mutants between pH 7.0 and 8.0 (Figure 6-12). Thus, the mutants are consistently more stable in terms of both heat and pH stability. Similarly, other mutants: VP2 S93W, VP2 S90Q and VP2 Y98F were also found to show increased pH stability.

In addition, the results from the purification of inactivated ASIA1 virion capsids (see Chapter 3 for more details) showed that 0.2 to 0.5M NaCl prevented the aggregation of the viral capsids. Consequently, the effect of salt concentration on the stability of FMDV capsids was investigated, by using MD simulations. Due to the limitations of the parameterisation available in the AMBER package, only NaCl could be straightforwardly used to model the electrostatic effect of increasing ionic strength on the binding free energy of FMDV protomers. As can be seen from the Table 6-1, the increase in NaCl concentration and hence the ionic strength led to increased stability of the FMDV capsids.

Table 6-1: Effect of ionic strength on the stability of FMDV capsids

Salt Concentration (mM)	WT ΔG (kcal/mol)	VP2 S93F ΔG (kcal/mol)
0	-32.9(11.5)	-48.3(10.7)
150	-42.6(11.4)	-51.9(11.1)
300	-43.6(11.4)	-53.0(11.5)
500	-44.8(11.3)	-53.9(11.2)
800	-48.5(11.2)	-55.0(12.5)
1000	-50.2(11.2)	-62.6(10.1)
2000	-50.3(11.3)	-61.7(10.1)

The results from the MD simulations and binding energy calculations were verified by thermofluor analysis on infectious and inactivated O1M wildtype capsids. The purified O1M wildtype capsids were diluted in buffer containing increasing concentrations of NaCl (0.15M to 2M). As predicted, the thermo-stability of the capsid increased from 52.5°C to 56.5°C with increasing concentration of NaCl. Other salts such as potassium chloride (KCl), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$) and ammonium sulfate ($(NH_4)_2SO_4$) were also investigated. KCl and $MgCl_2$ also increased the stability to some extent. The highest stability was observed with $(NH_4)_2SO_4$ with an increase in stability of up to 10°C with 1M $(NH_4)_2SO_4$ (Figure 6-14). As discussed in Chapter 2, the protomeric interfaces of FMDV capsids are hydrophilic compared to other enteroviruses and are held together by electrostatic interactions. In particular, the interface of the O-serotype is electrostatically negative. Therefore, it is possible that the presence of salt is stabilising the electrostatic properties of the protomeric interfaces and hence the increase in the capsid stability. The experiments will be repeated with the mutant capsids when more material becomes available.

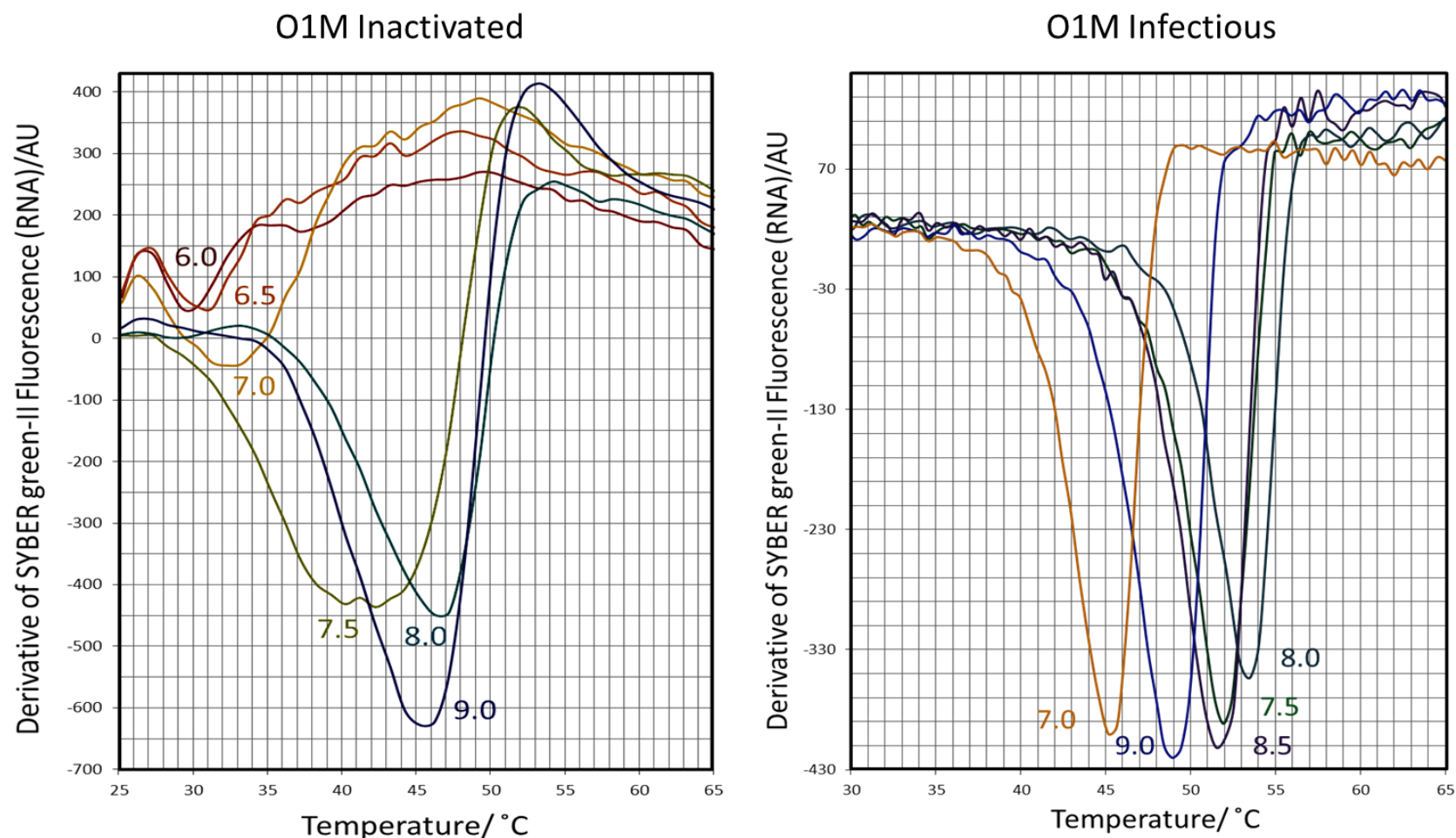


Figure 6-11: Effect of pH on thermo-stability of FMDV capsids. FMDV (O1M serotype) capsids were found to be extremely sensitive to pH. Significant changes in stability were observed even with pH change of 0.5. As expected, capsids readily dissociated at pH 6.0 and 6.5. However, capsids were also very unstable at pH 7.0 as well. The stability was better at pH 7.5 and pH 8.0 with the highest stability observed at pH 8.0. Higher pH had an adverse effect, and stability decreased at pH 8.5 and 9.0. Similar results were obtained with inactivated and infectious virus capsids. (Experiments performed by Julian Seago and myself).

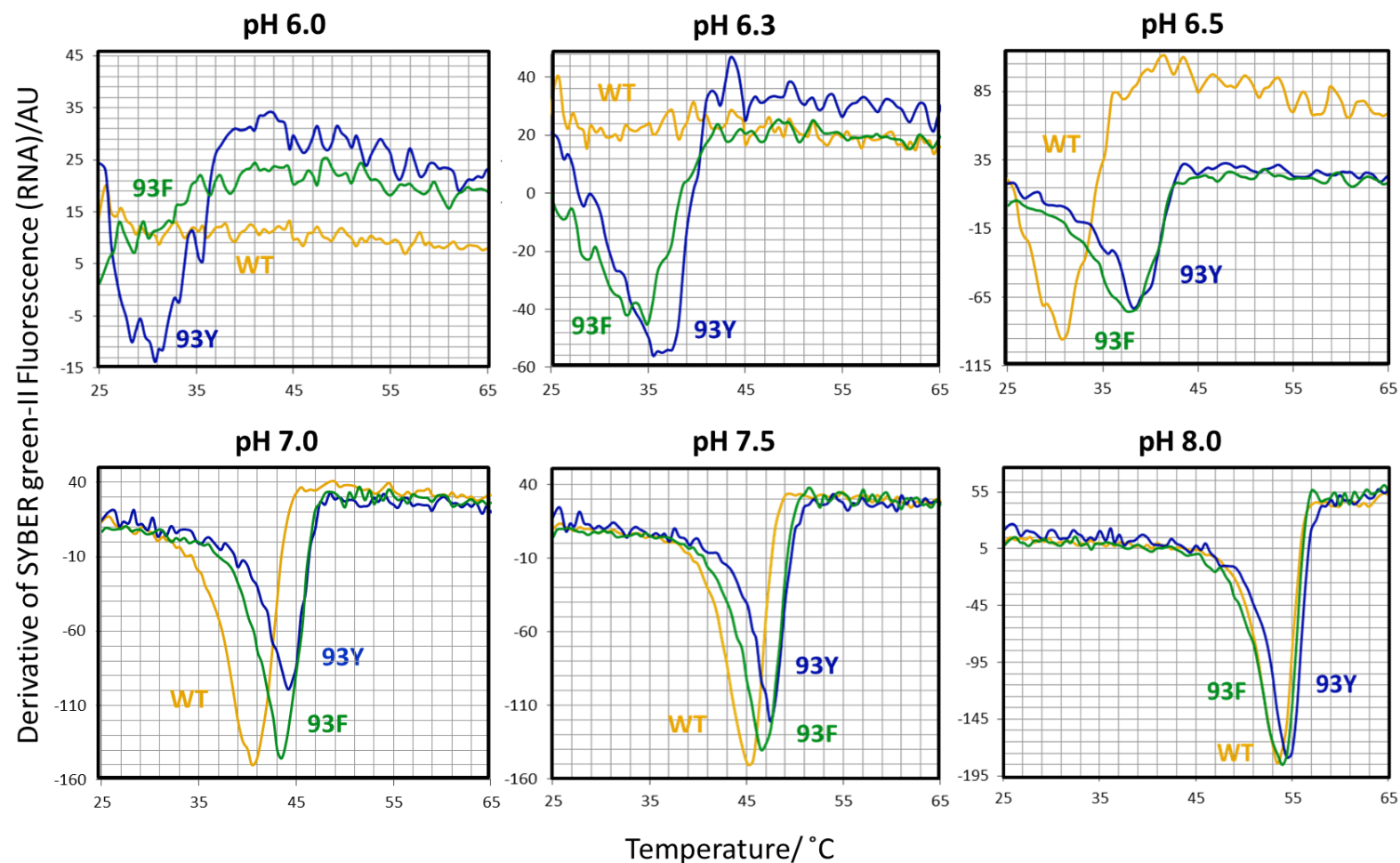


Figure 6-12 Effect of pH on stability of mutants – 1. The stability of the wildtype and mutant capsids was tested at various pHs. The wildtype capsids were very unstable below pH 7.5. The mutant capsids were also unstable, however the degree of stability at lower pH was much higher compared to the wildtype capsids. For example wildtype capsids completely dissociated below pH 6.0, whereas VP2 S93Y capsids were stable to 35.5°C and VP2 S93F to 34.5°C. The difference in stability was significant at pH 6.5, whereas wildtype capsids dissociated at 28°C whilst the mutant capsids were stable up to 36.5°C. The stability of both the wildtype and mutant increases at higher pH although the mutant capsids were relatively more stable.

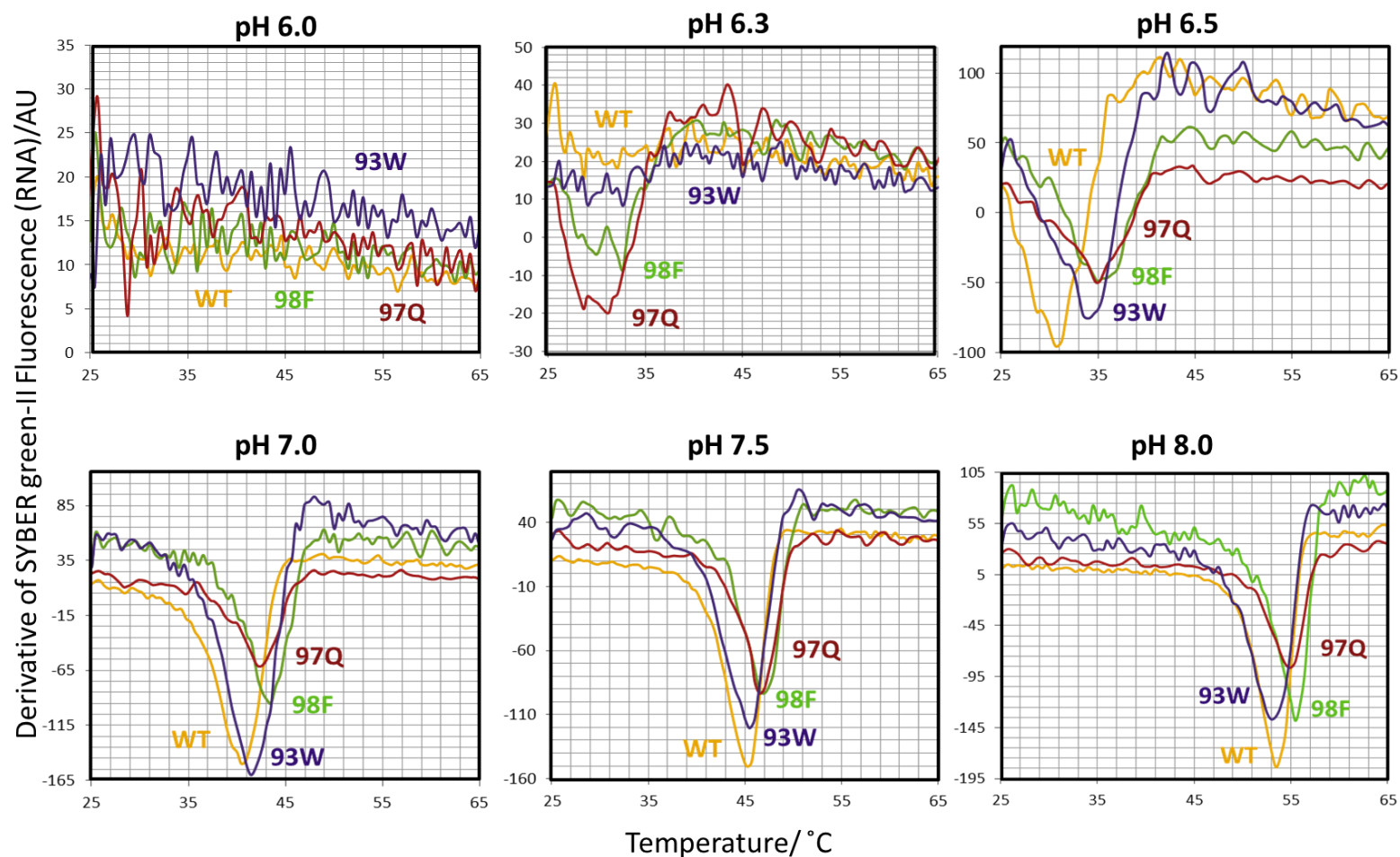


Figure 6-13: Effect of pH on stability of mutants – 2. Other mutants such as at S93W at the 2-fold axis were also found to be stable at lower pH compared to the wildtype capsids. Although, 93W was not as stable below pH 6.5 as 93Y and 93F the stability increased with pH 6.5 and the results are in agreement with the predicted stability. Mutants near the 2-fold axis such as VP2 V90Q and VP2 Y98F were also more stable.

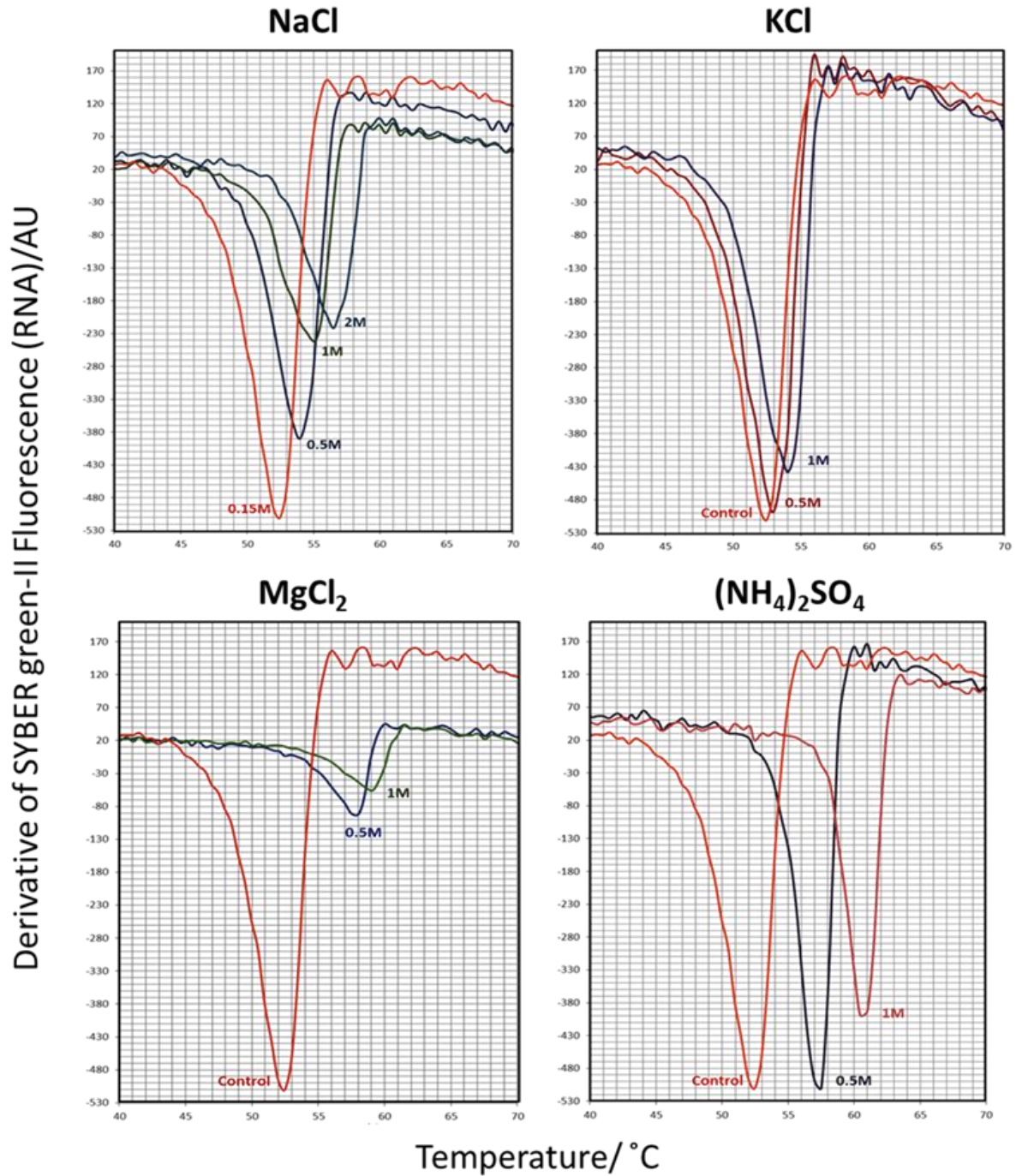


Figure 6-14: Effect of salt on capsid stability. The effect of salt concentration on the stability of FMDV capsids was also investigated by thermofluor analysis. As predicted by MD simulations, the thermo-stability of the FMDV capsids increased with increase in ionic strength. The highest increase of up to 10°C was observed with 1M (NH₄)₂SO₄. The increased ionic strength is believed to stabilize the electrostatic properties of the capsid interfaces.

6.4.4 EM analysis of the stability of BEI inactivated virus

The stability of chemically inactivated purified virions was analysed by electron microscopy. This was done with BEI inactivated O1M wildtype and the corresponding mutant containing VP2 S93Y. The intactness of the capsids was verified either directly after the purification, or after 10 days storage in PBS buffer at 4°C.

The initial ratio of intact virion capsids to dissociated pentamers was found to be 9:1 for the wildtype, whilst no dissociation was detected for the mutant virion. After cold storage the difference became much larger; for the wildtype the ratio was 1:4, with only a quarter of intact capsids, as compared to 9:1 for the mutant, *i.e.* only 10% dissociation (Figure 6-15).

To further investigate the thermo-stability, samples were incubated at 37°C for 20 minutes and the intactness analysed by EM. The results showed an even more remarkable difference; only 5% wildtype capsids remained intact compared to the 60% of the mutant virion capsids (Figure 6-16).

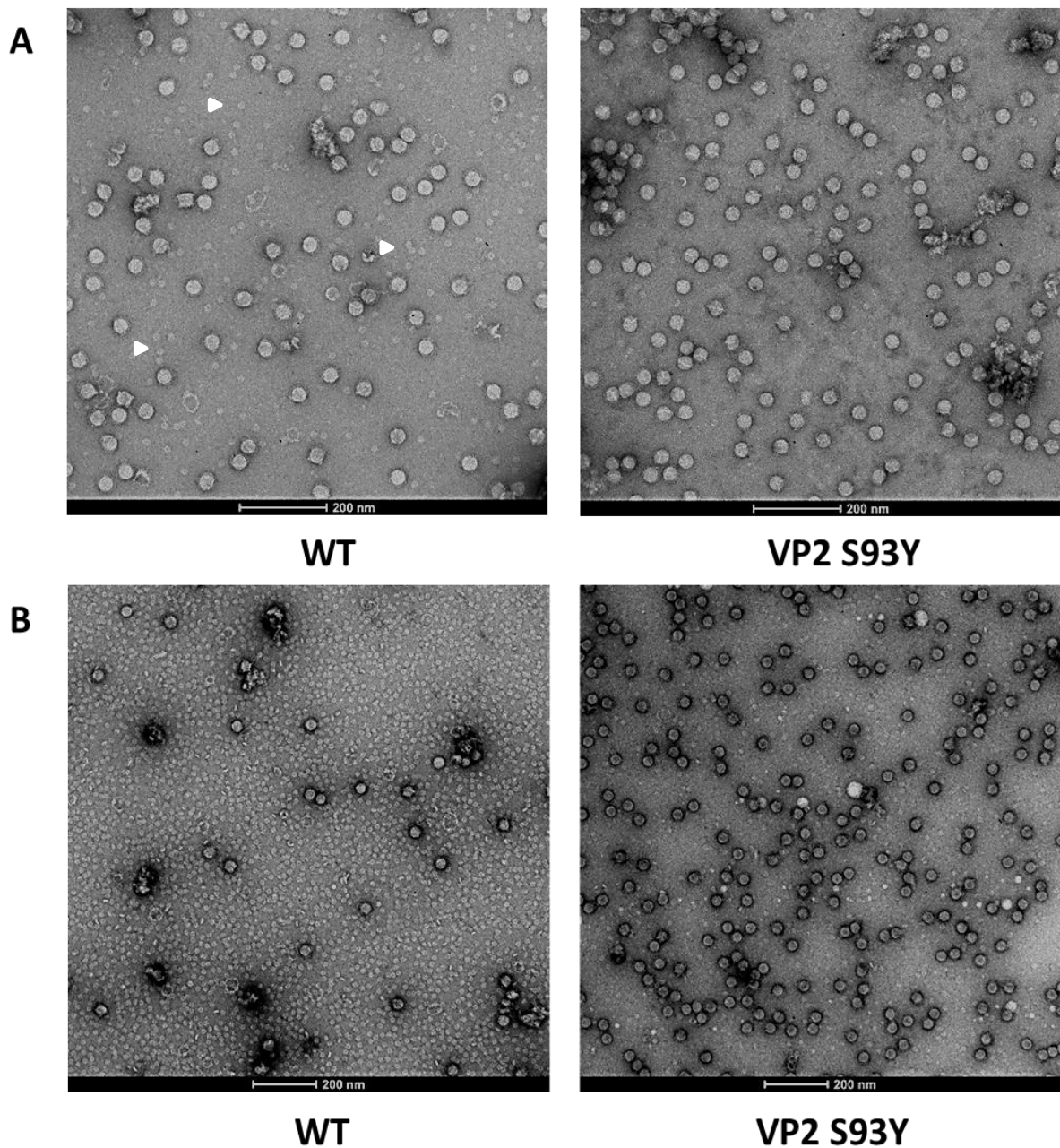


Figure 6-15: EM analysis of inactivated type O capsids after storage at 4°C. Purified inactivated virion capsids of the FMDV O1M wildtype and the corresponding VP2 S93Y mutant were analysed soon after purification (A) or after 10 days storage at 4°C (B). Almost 80% of the wildtype inactivated capsids were found to be dissociated into pentamers, whilst inactivated mutant capsids remained intact. The dissociated pentamers are highlighted by white arrows in panel A.

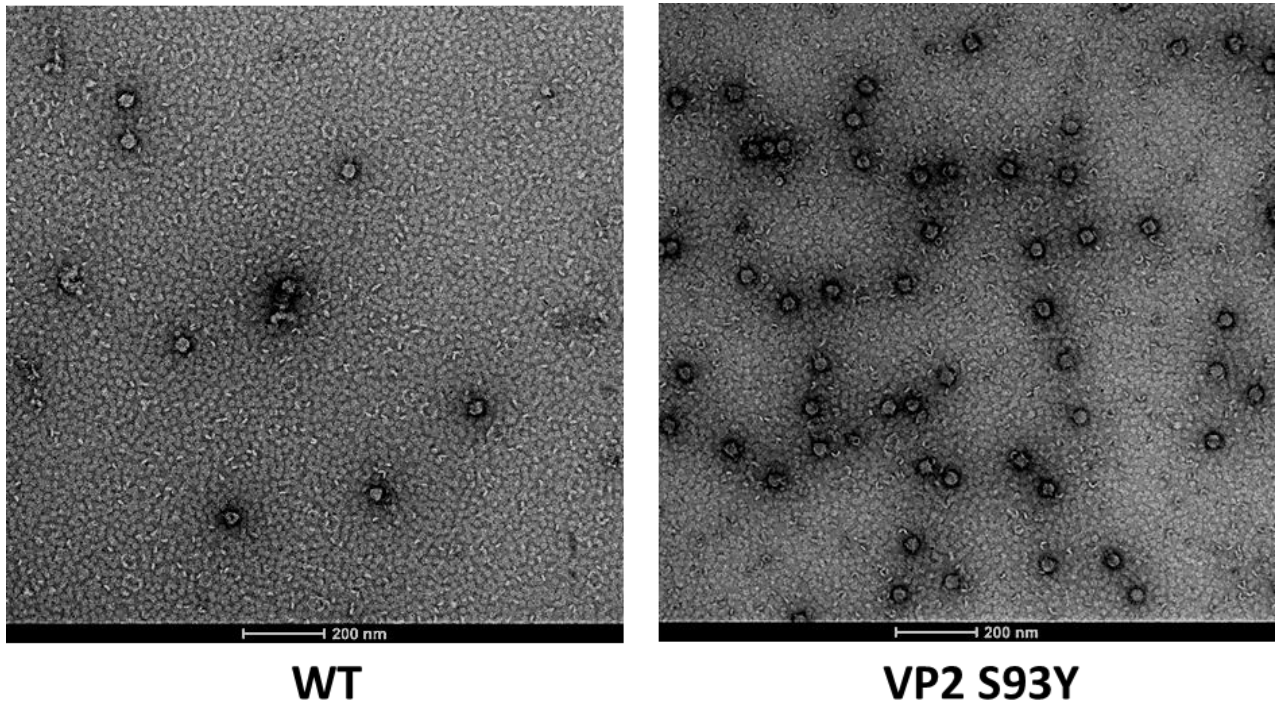


Figure 6-16: Stability of inactivated type O capsids after heat treatment. Purified inactivated capsids of O1M wildtype and VP2 S93Y mutant were incubated at 37°C for 20 minutes and the capsid intactness was analysed by EM. Only about 5% of capsids remained intact for the wildtype after heat treatment, whereas mutant capsids were not as susceptible to heating and 60% of them remained intact.

Similar results were obtained with inactivated SAT2 serotype. The effect of chemical inactivation on the thermo-stability of inactivated SAT2 was assessed by thermofluor assay as described previously. The inactivated viruses were stored for 10 days at 4°C followed by thermofluor assay. No significant changes were detected in the thermo-stability of the mutant and a similar dissociation peak was detected pre- and post-storage at 54°C, whereas with the inactivated wildtype capsids, the fluorescence peak post-storage was much smaller compared to the freshly purified capsids, despite keeping the concentration of protein the same in both experiments (Figure 6-17). Consistent with the EM experiments performed on inactivated O1M (section 6.4.3), the thermofluor data with inactivated SAT2 suggest that most of the wildtype capsids are dissociated (a small peak was observed which could be from the remaining intact capsids).

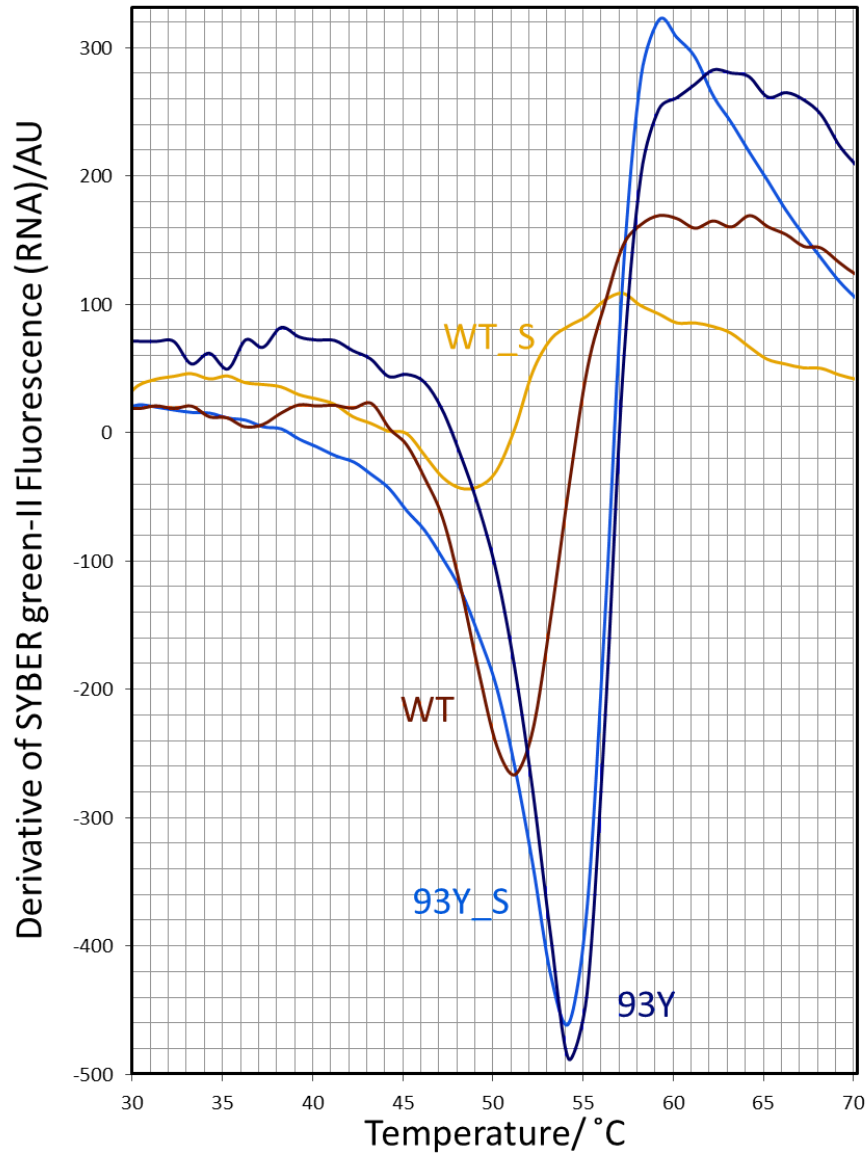


Figure 6-17: Thermo-stability of inactivated SAT2 post storage. Thermofluor analysis post-storage for 10 days at 4°C showed no difference in the stability of the mutant capsids. However, most of the wildtype capsids were found to be dissociated. Stored samples are indicated by WT_S and 93Y_S.

In addition, a shift of 2.4°C was observed with wildtype capsids from the stored sample. The capsids dissociated at 48.8°C compared to 51.2°C for a freshly purified sample. The reason for such a change in stability is unclear. It is conceivable that the capsids are in some intermediate state with weakened inter-pentameric interactions. A summary of all mutants and their stability is shown in Table 6-2.

Table 6-2: Summary table of the mutants generated and experimentally tested during this project (For more detailed list see appendix).

O Serotype								
No.	Name	Mutation	$\Delta\Delta G$ kcal/mol	Production System			Thermo- stability	pH- Stability*
				Infectious	rVV	rBVES		
1	l	VP2 S93H	- 7.7	yes	yes	-	S	U
2	m	VP2 S93Y	- 11.8	yes	yes	-	S	S
3	i	VP2 S93F	- 13.8	yes	yes	yes	S	S
4	q	VP2 S93W	- 9.5	yes	yes	-	S	S
5	s	VP2 Y98F	-8.0	yes	yes	-	S	-
5	h1	VP2 S93V	-7.1	-	-	-	-	-
6	h2	VP2 S93L	-1.9	-	-	-	-	-
7	h3	VP2 S93I	-7.2	-	-	-	-	-
8	h4	VP2 S93M	-2.8	-	-	-	-	-
9	p1	VP2 S97Q	- 5.5	yes	yes	-	S	-
10	p2	VP2 S97I	- 6.8	-	yes	-	-	-
11	p3	VP2 S97M	+ 1.9	yes	yes	-	U	U
12	o	VP2 V90N	- 3.5	-	yes	-	-	-
SAT Serotype								
1	l	VP2 S93H	-5.3	yes	-	-	S	-
2	m	VP2 S93Y	-12.2	yes	-	-	S	-
3	i	VP2 S93F	-13.3	-	-	-	-	-
4	s	VP2 H98F	-5.6	-	-	-	-	-
5	q	VP2 S93W	-21.2	-	-	-	-	-

rVV = recombinant empty capsids produced with vaccinia expression system.

rBVES = recombinant empty capsids produced with baculovirus insect cell expression system.

*pH stability at pH 6.5 compared to wildtype.

S = Stable compared to wildtype.

U = Unstable compared to wildtype.

6.5 Conclusion

Increasing the thermo-stability of FMDV capsids could lead to the generation of stable vaccines with longer shelf-life, reducing production, inactivation and quality testing costs. Thermo-stable vaccines would be less dependent on cold chains, which are very difficult to maintain especially in developing countries where FMD is endemic. In addition, other possible advantages of stable vaccines include increased stability following chemical inactivation and enhancement of the immune-response.

We have successfully shown that introducing mutations at the pentamer interface of FMDV capsids, especially hydrophobic substitutions in the 2-fold region on the α -helix, can increase the thermo-stability of the resulting capsids in two different serotypes, O1M and SAT2. These mutations were rationally designed using ‘*in-silico*’ MD simulation and the predicted increases in thermostability correspond well with the values obtained on reduction to practice. The thermo-stability of the capsids was assessed by three independent methods and showed that many of the targeted mutations were indeed more stable as predicted using the MD simulation and $\Delta\Delta G$ calculations. Wildtype O1M dissociated at 52°C compared to 53.5°C for VP2 S93Y and 54°C for S93F. When analysed alongside the O1M viruses by our collaborators at The Pirbright Institute the SAT2 wildtype dissociated at 51°C and VP2 S93Y at 54°C. The difference in the stability of the wild type and VP2 S93Y SAT2 viruses was slightly larger when assessed by our collaborators at ARC-OVI; the wildtype capsids dissociated at 47°C and VP2 S93Y at 53°C. These differences could be due to different experimental conditions, including the presence of stabilizing factors such as sucrose in the virus preparations. Thus there are differences in the absolute values obtained due to slight differences in experimental procedure but the overall trend observed remains the identical. Sucrose especially has a stabilizing effect on the capsids. As shown in Figure 6-9, the

thermo-stability of the inactivated SAT2 VP2 S93Y increased by 8°C when the thermofluor assay was performed with 40% sucrose in buffer. As the thermofluor assay is very sensitive, care should be taken to ensure that samples are kept cooled before the assays and the buffers, assay volumes and types of plastic used should be standardized as much as possible. Nevertheless, the thermofluor results showed an excellent agreement with the predicted stability by MD simulations and $\Delta\Delta G$ calculations for both O1M and SAT2 serotypes (as discussed in Chapter 2).

The thermo-stability results were similar with the inactivated SAT2 viruses when performed at ARC-OVI to those observed by us at STRUBI. However, the thermo-stability was considerably affected with the inactivated O1M serotype, with wildtype capsids dissociating at 45°C and VP2 S93Y at 46.5°C compared to 52°C and 53.5°C respectively for the infectious viruses. A significant difference in capsid stability was observed with small changes in pH and an optimal pH of 8.0 was found to increase the stability of both, wildtype and mutant capsids. In addition, the mutant capsids were also found to be pH stable compared to the wildtype. The thermo-stability increases up to pH 8.0 whilst pH values above 8.0 had an adverse effect, making both types of capsids more unstable. Despite such differences, mutants designed at and near the 2-fold axis relating two FMDV pentamers were found consistently more thermo- and pH stable compared to wildtype capsids. Furthermore, the EM analysis showed that the inactivated mutant capsids were more resistant to dissociation during storage.

The EM analysis provided striking visual verification of the capsids of the inactivated O1M and SAT2 viruses and complemented the thermofluor results. Due to strict disease regulations on the movement of FMDV within the UK, and the lack of suitable facilities at

collaborating institutes, EM analysis could only be performed on inactivated (BEI-treated) viruses. However, these restrictions allowed the EM analysis to provide an insight into the effect of BEI inactivation on the intactness of the FMDV capsids. As the BEI treatment requires two consecutive incubations at 37°C, the EM analysis also confirmed the increased stability of both the SAT2 and O1M VP2 S93Y mutants under conditions similar to those used in vaccine production. EM analysis revealed that more than 80% of the O1M wildtype capsids had dissociated into pentamers after 10 days storage at 4°C, in comparison the VP2 S93Y capsids remained intact. Furthermore, when inactivated capsids were incubated at 37°C for 20 mins, almost all wildtype capsids dissociated whereas approximately 60% of the mutant capsids still remained intact. Similar results were obtained with the SAT2 serotype after storage using a thermofluor assay. Thermo-stability of inactivated mutant capsids remained the same after storage, whereas most wildtype capsids were dissociated.

The mutations also increased the thermo-stability of the recombinant empty capsids which are otherwise extremely unstable and very difficult to produce and purify. The thermo-stability of the recombinant capsids was assessed by incubation at different temperatures followed by sucrose gradient sedimentation. Wildtype O1M capsids, as expected, were very unstable and broke apart when incubated at 45°C for 1hr whereas mutant empty capsids containing VP2 S93H and S93F mutations remained intact. When heated to 56°C for 2hrs, VP2 S93H also broke apart; VP2 S93Y remained partially stable (about 75% of the capsids remained intact) whilst VP2 S93F remained almost completely intact. EM analysis further confirmed the intactness of VP2 S93F capsids after heat treatment. Once again, these data are in complete agreement with the predicted $\Delta\Delta G$ values from the MD simulations.

The covalent mutation, VP2 S93C, in FMDV A22 and O1M was also found to be thermostable at 56°C. Furthermore, the A22 serotype capsids were stable even at pH 5.5. However, the yields were very low with O1M compared to the A22 serotype. The yields of wildtype O1M and SAT2 recombinant capsids were also very low (as discussed in Chapter 3). The exact reasons for such behaviour are unknown and more work is required to improve yields and purification of O1M and SAT2 empty capsids.

Chapter 7 : *In-vivo* experiments with stabilised FMDV capsids

7.1 Introduction

As discussed in Chapter 1, foot-and-mouth disease (FMD) is highly contagious and the most economically devastating disease of livestock. The disease has an international impact on the trade of animals and animal products which can affect many developing countries. It is also capable of causing costly outbreaks in FMD-free countries, such as the 2001 outbreak in the United Kingdom which resulted in economic losses of between 5.8 and 6.3 billion pounds (Grubman and Baxt 2004).

FMDV is endemic in many parts of the world and controlling the spread of the disease is a very complex problem, compounded by its antigenic diversity. FMDV is a highly variable virus and has been classified into seven different serotypes, each of which contains subtypes that contribute to the pool of antigenic variants. As expected, there is a limited or no cross-protection between serotypes. Therefore, vaccination against one serotype does not confer protection against other serotypes or even against all subtypes within the same serotype.

Several measures have been implemented to control the occurrence and reduce the spread of FMD, such as regular vaccinations in endemic areas, movement restrictions of animals and culling of infected animals. In FMD-free countries, such as the United Kingdom where the disease usually only results from the importation of live animals infected with FMDV, the control policy is based primarily upon selective culling of the infected animals, and any other animals which are believed to have been exposed and have a high risk of developing the disease, together with emergency vaccination and movement restriction of animals and animal products.

In this chapter, I will briefly describe FMD vaccine production and formulation and testing of vaccine potency. All animal experiments reported in this chapter were performed by either our industrial collaborators at MSD Animal Health using guinea pigs or at The Pirbright Institute using cattle. I will briefly describe the methods followed by the results and then discuss the use of stabilised inactivated virus and recombinant capsid vaccines that exhibit improved properties.

7.2 FMD Vaccines

Current FMD vaccines are made from whole-viruses which are produced by infecting baby hamster kidney-21 (BHK-21) cells in roller bottles or suspension cultures with infectious FMDV under strict bio-containment environments. The viruses are then chemically inactivated with aziridines such as BEI (binaryethyleneimine). The inactivated viruses are concentrated by precipitation using ammonium sulphate or polyethylene glycol (PEG) and purified by ultrafiltration and/or chromatographic methods. Inactivated viruses are then formulated into vaccines by mixing with suitable adjuvants. The adjuvants enhance the stability of the capsids which in turn increases the immune response. In addition, adjuvants also themselves stimulate the immune response. Generally, aluminium hydroxide gel ($\text{Al}[\text{OH}]_3$) or an oil-water emulsion such as Montanide ISA206 (SEPPIC) are used as adjuvants in FMD vaccine formulation. Oil-emulsion adjuvants in the form of water-in-oil (W/O) or water-in-oil-in-water (W/O/W), that have been shown to stabilise the FMD vaccine and produce longer-lasting immunity, are more widely used in formulations (Cloete et al. 2008). Typical FMD vaccines contain more than one FMDV serotype (*i.e.* they are multivalent). The most common vaccines are trivalent and contain isolates of serotype O, A and Asia-1, or O, A and C. In Africa, bivalent or trivalent SAT vaccines are used containing SAT1 and SAT2 or SAT1, SAT2 and SAT3 serotypes respectively (Parida 2009).

The concentration of each isolate (antigen payload) in the vaccine generally varies from 5-20µg and depends on the serotype. A report on a potency of A and SAT2 vaccines showed that when similar antigen payloads were used, SAT2-vaccinated animals were not protected upon challenge with an infectious virus, whereas all serotype A-vaccinated animals were. Similarly O serotype vaccines require higher antigen payloads in comparison to serotypes A, C and ASIA1 in order to achieve equivalent neutralising immune responses (Oh et al. 2012; Parida 2009). Such variations in antigenicity and potency of vaccines containing different serotypes against challenge can be attributed to the instability of O and SAT capsids. The intactness of the FMDV capsids (146S particle) is crucial to the immunogenicity of FMD vaccines and any degradation results in a significant reduction in immune response (Doel and Chong 1982; Hegde et al. 2009).

7.3 FMDV vaccine potency

The potency of FMD vaccines is measured in PD₅₀ (protective dose where 50% cattle are protected). A vaccine with PD₅₀ of 6 means when it is used at 1/6th of the recommended dose, the vaccine will protect 50% of cattle against challenge with a live virus appropriate to the virus type used for inoculation. The minimum potency for FMDV vaccines should be 3. Higher potency vaccines with PD₅₀ values up to 10 are used for vaccine bank storage. The potency testing of FMD vaccines is carried out according to the OIE (Office International des Epizooties) Manual (OIE 2008). A minimum of 16 sero-negative cattle no less than 6 months of age are used with at least 2 animals in a control group. The vaccines are administered intramuscularly, intralingually or according to manufacturer's recommendation with a full vaccine dose. Animals can be divided into several groups and different doses per group with different volumes can also be used. These and the non-vaccinated animals are challenged 21 days post-vaccination with a live virus of the same type with an ID₅₀ of 10⁴ (50% infectious

dose) in two different sites on the upper surface of the tongue. The animals are examined 7-8 days post challenge for any clinical signs of FMD such as feet lesions. The vaccine is considered satisfactory if it protects at least 12 animals out of 16 (Kitching et al. 2007; OIE 2008; Parida 2009).

7.4 Limitation with current inactivated vaccines.

As discussed in chapter 1, current chemically inactivated vaccines have been very useful in managing and reducing the impact of the FMD outbreaks. Vaccination against FMDV is a key countermeasure available to FMD-free and endemically infected countries. Therefore, a number of FMD vaccine banks have been established. These contain frozen inactivated virus that can be formulated to provide vaccine for deployment. However, the inactivated vaccines are very unstable, especially for O and SAT serotypes, and require expensive cold chain setups for storage and transport (Parida 2009). This is a major problem in tropical and developing countries. Even with cold-chains O serotype vaccines have very limited biological half-life, typically only of few months and require frequent immunisation of animals (every 4 to 6 months). The situation is even worse with SAT serotypes. Inactivated SAT serotype viruses are barely stable and rapidly dissociate into pentamers, severely decreasing the potency of the vaccines even when administered multiple times and with high antigen payload (Oh et al. 2012). Therefore, stabilised vaccines are required to effectively control the disease on a global scale.

7.5 Guinea pig and cattle vaccination with stabilised FMDV vaccines

The guinea pig trials with stabilised FMDV vaccines were performed by our industrial partners MSD Animal Health (MSD). Inactivated SAT2 wildtype and stabilised mutant VP2 S93Y were sent from ARC-OVI. The samples were formulated as vaccines using oil based

ISA206 (SEPPIC) adjuvant at MSD and stored for 1 month at 4°C. 10µg aliquots of these vaccines were then used to immunise two groups of 10 guinea pigs. One group was administered with the wildtype vaccine while the other group received the stabilised vaccine. Serum samples were tested on day 0, followed by 1 and 6 months post vaccination and virus neutralising antibody titres (VNT) were assessed. In a similar experiment vaccines formulated from stabilised recombinant O1M empty capsids VP2 S93F capsids were used to immunise guinea pigs while the control group of 4 guinea pigs received O1M commercial vaccine.

The cattle experiments were performed at The Pirbright Institute under strict disease security regulations (SAPO-4). Two groups of four 100 to 150 kg Holstein Friesian calves were vaccinated either with recombinant A22 wildtype or A22 VP2 H93C empty capsids. Each animal was inoculated by intramuscular injection with 12µg of purified capsids formulated in a similar manner as described above using oil adjuvant (Seppic 206B). A booster dose containing the same amount of antigen was administered 3 weeks after the first inoculation. 34 weeks post immunisation, all eight vaccinated animals and two non-vaccinated control animals were challenged intra-dermolingually with 10^5 ID₅₀ of cattle adapted FMDV A22 virus. Animals were examined clinically for FMDV related lesions and blood samples were analysed by virus neutralisation antibody titre (VNT) assays from the day of challenge until day 9.

7.6 Results and discussion

In order to characterise their protective immunities, the inactivated viruses and recombinant empty capsids were formulated into vaccines in a similar way to that of traditional vaccines and were tested in guinea pig and cattle trials respectively.

7.6.1 Immunisation with stabilised inactivated SAT2 serotype virus

To characterise the immunogenicity of the stabilised inactivated SAT2 virus, two groups of 10 guinea pigs were immunised with 10µg of purified antigen formulated in oil adjuvant. Prior to the immunisation experiment, the vaccines were either stored for 1 month or 6 months at 4°C. One group received the SAT2 wildtype vaccine and the other received the SAT2 VP2 S93Y vaccine, after which the virus neutralising antibody titres were assessed. No FMDV neutralising antibodies were detected on the day of inoculation. However, at 1 month post-immunisation, significantly higher neutralising antibody titres (VNTs) were observed with the stabilised SAT2 S93Y antigen compared to the animals immunised with wildtype SAT2 antigen (Figure 7-1). The mean antibody titre of stabilised vaccine was greater than 6 ($\log_2$) which is considered protective (Barnett et al. 2003). In contrast, the mean antibody titre induced by the wildtype vaccine was lower than 3 ($\log_2$). When assayed at 6 months post-immunisation, the antibodies were maintained at high titres, greater than 8 ($\log_2$) with stabilised capsids, whilst the mean titre obtained with the wildtype only increased by 30% to 4 ($\log_2$), which is still lower than is considered potent. Thus, the SAT2 stabilised vaccine performed markedly better than the wildtype vaccine.

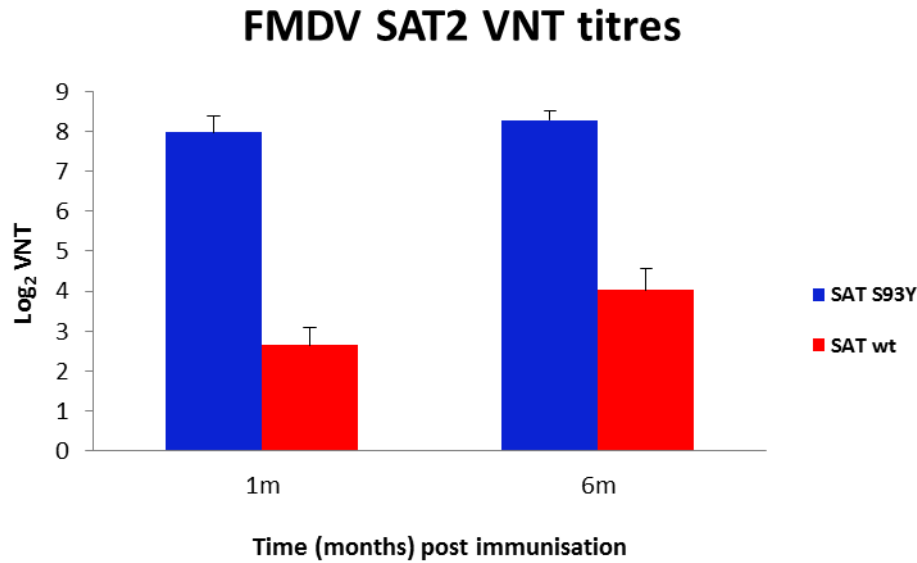


Figure 7-1: Guinea pig immunisation with wildtype and stabilised SAT2 vaccines. Two groups of 10 guinea pigs were immunised with vaccines formulated with either SAT2 wildtype or stabilised mutant VP2 S93Y. The animals were sampled at 1 and 6 months post immunisation. Significantly higher neutralising antibody titres were induced in animals immunised with stabilised vaccines compared to the wildtype vaccine. The mean neutralising antibody titre remained greater than 6 (log₂) for stabilised vaccine whereas for wildtype the titre remained lower than 4 (log₂). These experiments were performed by MSD Animal Health, Cologne, Germany.

Similarly, MSD is currently performing guinea pig experiments with the stabilised O1M vaccines for the inactivated O1M VP2 S93Y sent from The Pirbright Institute. In addition, to characterising the effect of storage on the stability of the SAT2 antigen, aliquots of vaccines formulated with wildtype and VP2 S93Y antigen were stored for 6 months at 4°C before being used to inoculate two groups of ten guinea pigs. Initial blood samples, taken at 12 days post-inoculation have been analysed and neutralising antibody titres (above background) were only detected in the animals immunised with the stabilised SAT2 antigen. We expect the neutralising antibody titres to be induced at higher levels when samples are collected after 1 month post-immunisation.

7.6.2 Immunisation with stabilised recombinant capsids

The characterisation of protective immune response generated from the stabilised recombinant empty capsids was also performed by MSD. Two groups of 10 guinea pigs were immunised with vaccines formulated with empty capsids of O1M serotype. The yield of recombinant wildtype 146S particle of O1M was extremely low, possibly due to the pH of the culture medium. The pH of SF9 culture medium is 6, a pH at which the wildtype capsids are generally dissociated (see Chapter 6 for more details). Empty capsids containing the VP2 S93F mutation were stable. Therefore, one group received vaccine formulated from the VP2 S93F empty capsids and another group received an O1M standard monovalent vaccine. The blood samples were collected and the neutralising antibody titres were analysed 1 month post-immunisation (Figure 7-2). The overall neutralising antibody titres for both vaccines were low ($>3 \log_2$). However, the titres of recombinant empty capsids are in the same range as that of the regular O1M vaccine.

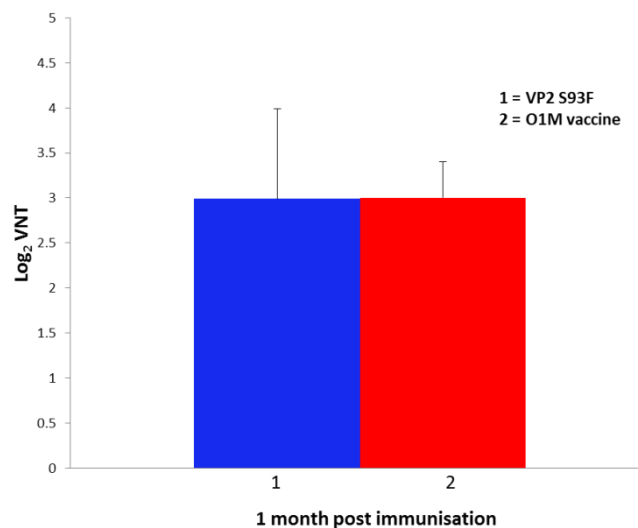


Figure 7-2: Guinea pig immunisation with standard inactivated O1M and stabilised recombinant O1M empty capsid vaccines. The guinea pig immunisation with the stabilised O1M recombinant empty particle vaccines showed the overall neutralising antibody titre to be lower. However, the titres were in the same range as that of a standard O1M inactivated vaccines. These experiments were performed at MSD Animal Health.

7.6.3 Cattle immunisation with stabilised recombinant empty capsids

Cattle immunisation experiments were performed at The Pirbright Institute. Two groups of 4 cattle were immunised, each with 12µg of purified recombinant empty capsid (A22 serotype) vaccine formulated in oil adjuvant. One group received the A22 wildtype empty capsid vaccine and the other received the stabilised A22 VP2 H93C empty capsid vaccine. All animals received a booster dose after 3 weeks. Significant increases in virus neutralization antibody titres were observed in both groups post booster dose, greater than 6 (Log₂). The antibodies were maintained at high titres up to 22 weeks post-immunisation (Figure 7-3). The titres reduced to pre-boost levels after 34 weeks in both groups of animals.

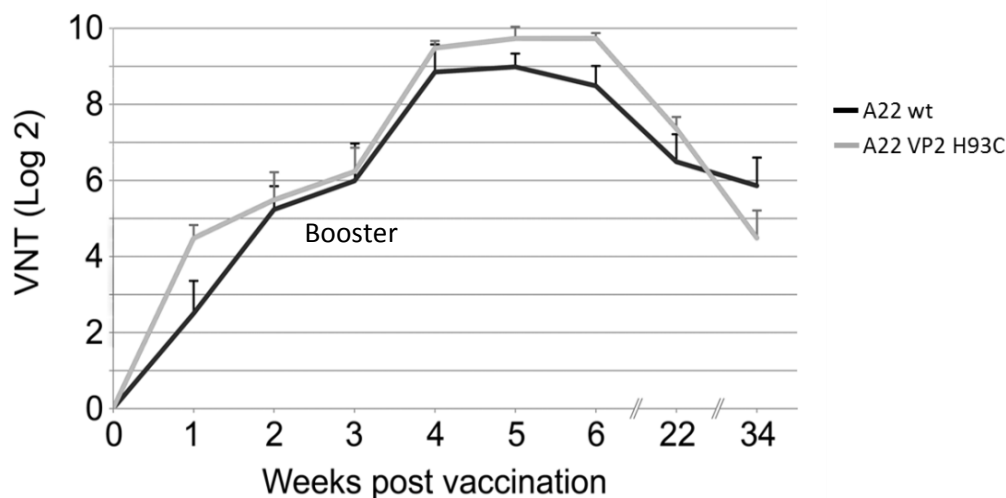


Figure 7-3: Cattle immunisation with vaccines formulated from recombinant empty capsids of A22 serotype. Two groups of 4 cattle were immunised either with A22 wildtype or with A22 VP2 S93C recombinant empty capsids. All animals were immunised at week 0 and a booster dose was given at week 3. Blood samples were collected and virus neutralising antibody analysed at weekly intervals until 6 weeks and then on week 22 and week 34. There was a significant increase in neutralising antibody titre post booster dose. The titres reduced to pre-boost levels after 34 weeks in both groups. These experiments were performed at The Pirbright Institute.

At 34 weeks post-immunisation, all vaccinated animals and two non-vaccinated control animals were needle challenged by inoculating with live FMDV A22 virus onto the upper surface of the tongue. Both control animals showed full clinical signs including lesions on all four feet. Three out of four animals immunised with mutant empty capsids were fully protected, whereas two out of four animals immunised with wildtype capsids were protected. Thus, the recombinant empty particle vaccines showed a good induction of neutralising antibody responses in cattle and conferred protection from live virus challenge.

7.7 Conclusion

Vaccination against FMDV has greatly reduced the spread of the disease and can control the disease. However, current vaccines are very unstable and have a very limited shelf-life, especially in climates with elevated temperatures in which it is difficult to maintain cold-chains. Even then many FMDV field strains, such as O and the three SAT serotype viruses, are extremely unstable and readily dissociate into pentamers. Therefore, the corresponding vaccines are poor at generating and maintaining protective immunity and animals require frequent immunisation. Even when administered multiple times, the instability of the SAT serotype viruses only yields vaccines of low protective capacity. As a result, to date, it has proved impossible to effectively control the disease on a global scale. Thus, stabilisation of FMDV capsids is required to produce more stable and better vaccines which can relax the requirement for strict cold-chains and induce stronger and long lasting immune responses.

We have successfully increased the thermal and pH stability of the FMDV capsids by rationally engineering stabilising mutations at the 2-fold axis on the α -helix of adjacent FMDV pentamers. The effects of the mutations were screened by MD simulations and $\Delta\Delta G$ calculations and using biochemical and biophysical experiments we have shown that

increasing the stability of the capsids at the 2-fold interface, between pentamers, results in a significant increase in the neutralising antibody titre at 1 and 6 months post-immunisation of guinea pigs. In addition, virus neutralizing antibodies were induced in guinea pigs inoculated with stabilized capsids after 6 months storage, whereas no specific neutralizing antibodies were detected in guinea pigs immunized with wild type antigen that had been stored under the same conditions. Thus, the shelf-life of vaccines has also been significantly improved. Further analysis of the stored stabilised capsid vaccine at a later time point will confirm these observations. Besides this, experiments are underway with vaccines formulated with the stabilised O1M serotype particles.

In addition, we have demonstrated the production and application of safe and effective recombinant FMDV empty particle vaccines that do not require extensive bio-containment regulations during manufacturing. The stabilised recombinant empty particles vaccines were shown to induce equivalent neutralising antibody titres to that of standard inactivated FMDV vaccines. The enhanced stability of these empty capsids will reduce losses during production, storage and transport of the vaccines. Finally, the absence of non-structural proteins in recombinant empty capsid vaccines will allow the development of diagnostic tests to discriminate between infected and vaccinated animals (DIVA).

Chapter 8 : Conclusion and Future Work

8.1 Problems with current vaccines against FMDV

The development of vaccines is undoubtedly one of the most important advances in the history of medicine. Since the time of Edward Jenner and his smallpox vaccination procedure (1796), vaccines have effectively controlled and contained epidemics for an increasing number of infectious diseases. Routine immunisation with modern vaccines led to the complete eradication of smallpox and a near elimination of the polio and measles from major parts of the world (Rappuoli 2011). Vaccinations today have reduced number infectious diseases affecting humans and animals to an all-time low, saving millions of lives. However, despite of these accomplishments, there is a clear need for the development of novel vaccines against challenging pathogens such as rhinovirus, meningococcus B, human immunodeficiency virus (HIV), tuberculosis and malaria for which there are no effective vaccines available (Harrison 2011; O'Hagan 2001). In addition, many vaccines, especially those used for livestock, are of poor quality and require further improvements (Paton and Taylor 2011).

Viral diseases of livestock are of major importance in developing as well as developed countries. Although many veterinary diseases are mainly restricted to the developing world, wealthier countries are not completely immune to the threats posed by such outbreaks which is evident by the massive impact on the economy from the 2001 foot-and-mouth disease (FMD) outbreak in the UK and the 2010 outbreak in Japan (Muroga et al. 2012; Thompson et al. 2002). There is also an increasing threat from bluetongue disease, expanding its range in Europe (Claude et al. 2008; Greenwood et al. 2011). A major success in the area of livestock vaccines has been the eradication of rinderpest (RP). RP was an infectious disease of cattle and other cloven hooved animals and caused by a virus closely related to measles virus

(Roeder and Rich 2009). The disease was eradicated due to a successful vaccine campaign using attenuated vaccines.

Here, we have used a structure-based rational engineering approach to design improved vaccines against foot-and-mouth disease virus (FMDV) which is endemic in many parts of the world (see Figure 1-5) and affects both national and international trade with damaging consequences for local farmers. Although, vaccines are available and there are numerous vaccination programmes around the world, frequent vaccination is required as current vaccines are not stable and perform poorly in providing immunity against challenge with infectious virus. In contrast to rinderpest, there are seven different serotypes of FMDV: O, A, ASIA1, C and SAT-1, -2 and -3 and several subtypes within serotypes, resulting in a large pool of virulent viruses that are circulating in the field and vaccines against one strain unusually give only limited protection against other.

A major factor affecting the potency of FMDV vaccines is the intactness of the viral capsids which readily dissociate into pentameric subunits at slightly elevated temperatures or pH values below 7.0. Attempts to produce alternative vaccines using antigenic peptides or protomers have shown that intact virus particles stimulate the best immune response (Grubman and Baxt 2004). The seven different serotypes of FMDV differ significantly in their capsid stability. Serotypes A and Asia-1 are relatively more stable compared to other serotypes, whilst O and SAT (SAT-1, -2 and -3) are extremely sensitive to heat and pH (Doel and Baccarini 1981). The problem is exacerbated by the chemical inactivation used to manufacture vaccines which makes the capsids even more unstable (refer chapters 3 and 6 for more details). Despite using cold-chains, which are expensive and very difficult to maintain, the vaccines have very low protective capacities and immune responses are short lived, even

when administered multiple times per year. Therefore, vaccines with improved stability which give better protection against current and emerging FMDVs are essential for disease control and to establish and maintain an FMD-free status in many regions of the world.

We have used structure-based knowledge, *in silico* design and MD simulation methods to increase the stability of the viral capsids without affecting the antigenic properties of the virus, as demonstrated by structural analysis and preliminary trials in animals.

8.2 Summary of Results

8.2.1 MD simulations and capsid stabilities

We have devised a strategy for performing restrained atomistic molecular dynamics (MD) simulations on a subset of the complete virion, enabling the efficient and reliable calculation of binding free energy for the interactions between two adjacent pentameric building blocks of the FMDV capsid. For two of the least stable serotypes, O and SAT2, a panel of mutations have been identified and modelled on existing three-dimensional structures. These models were then subjected to 1ns MD simulation from which changes in the free energy of binding were calculated. This method facilitated rapid screening and ranking of a number of stabilising mutations. Therefore, the mutations were divided into three generations where each generation were designed based on results from the preceding generation.

Simulation results showed that a single point mutation promoting the hydrophobic stacking of the aromatic side chains at the 2-fold axis at position 93 of VP2 between adjacent pentamers resulted in significantly stronger binding of the pentamers. This resulted in enhanced stability of the capsids. Thus, the VP2 S93H mutation at the 2-fold interface showed greater stability than the wildtype, with a $\Delta\Delta G$ of -7.7 kcal/mol for O1M and -5.3 kcal/mol for SAT2. The

degree of stabilisation improved with the increase in hydrophobicity for each serotype tested, for example, VP2 S93H ($\Delta\Delta G = -7.7$ kcal/mol) < VP2 S93Y ($\Delta\Delta G = -11.8$ kcal/mol) < VP2 S93F ($\Delta\Delta G = -13.8$ kcal/mol). Similarly, when the homologous 2-fold histidine residue of a stable A-serotype, A22 Iraq, was substituted with phenylalanine, (VP2 H93F), the stability of the interface was increased ($\Delta\Delta G -10.3$ kcal/mol). Furthermore, similar substitution with other hydrophobic residues also increased the stability of the interface (eg: chapter 2, Table 2.1). However, the highest stabilisation was observed with aromatic stacking interactions. Several other mutations were also sampled near and around the 2-fold helix. An extensive list of mutations and their corresponding changes in binding free energy is presented in Appendix IV. Thus, the MD simulation and binding free energy method that we developed was used as an easy way to validate the effect of the mutations on the stability of FMDV capsids. Such screening of stabilising mutants would otherwise have been very time consuming and expensive, requiring strict bio-containment facilities. Additionally, three alternative approaches which introduced covalent mutations into recombinant empty capsids were proposed; these consisted of (a) a single disulphide bond at the 2-fold axis at VP2 position 93, (b) double disulphide bonds near the 2-fold axis at VP2 positions 90 and 97 (covering each end of the α -helix at the 2-fold axis), and (b) a triple disulphide bond mutant combining (a) and (b).

8.2.2 Expression and purification of stabilised capsids

Based on the results from the MD simulations, a ranked list of mutations was used to direct the production of mutated infectious copy viruses and recombinant empty capsids, in mammalian and (for the empty capsids) insect cell expression systems. No significant differences were observed in the assembly of infectious wildtype and stabilized mutant viruses. However, the plaque sizes were different, with mutants producing very small

plaques, possibly because of the more stable capsids, requiring longer to dissociate and release the genomic material.

The recombinant capsid proteins of the stable A22 virus also self-assembled into empty virus like particles which were essentially identical to those of inactivated virus (as judged by electron microscopy and X-ray crystallography). In contrast the expression of wildtype empty capsids of unstable serotypes, such as O and SAT2, was much reduced in the insect cell expression system. However production could be rescued by the insertion of stabilising mutations (confirmed by the antibodies that selectively bind intact 146S particles). One possible reason for reduced yields of the recombinant wildtype capsids could be the acidic culture medium used for the insect cell expression. The optimal pH for insect cell culture is between 6.2 and 6.5. The FMDV capsids at such pH are very unstable. In addition, the insect cell expression takes up to 5 days at 27°C which is likely to be inherently unfavourable for the unstable wildtype capsids.

Both the inactivated viruses and empty capsids were successfully purified using sucrose gradient sedimentations. However, chemical inactivation of the infectious virions had a severe impact on the stability of their capsids. Although bands were observed in sucrose gradients, electron microscopy (EM) analysis showed that inactivated wildtype capsids dissociated soon after purification. However, inactivation had far less effect on the stabilized capsids which remained essentially completely intact.

The single cysteine mutation at position 93 of VP2 successfully formed a disulphide bond on the 2-fold axis in recombinant empty capsids of A22 serotype. However, this strategy was not successful with other serotypes which either produced very low yield (detected by Western

blot) or only formed aggregates. Similarly, mutations that facilitated the formation of double or triple disulphide bonds along the 2-fold helix were not successful. Such mutants did not form any capsids, but sometimes produced protein aggregates. Moreover, we would expect that such mutations, leading to covalent links between pentamers would only work for the empty capsids, as the di-sulphide bonds would permanently fix the capsids of the infectious virions preventing them from uncoating upon infection, producing essentially dead viruses.

8.2.3 Structure determination of stabilised inactivated and recombinant FMDV capsids using *in-situ* data collection and molecular replacement methods.

X-ray crystallography can provide vital information at the atomic level for biological macromolecules. However, it requires the formation of highly ordered diffraction quality crystals, a process that can be a major bottleneck in the structure determination process. We have successfully identified the crystallisation conditions for serotypes A, O and SAT2 of FMDV using a gradual salting-out and sitting drop vapour diffusion crystallisation method. The crystals were obtained in three different types of salt conditions consisting of 1.5M ammonium acetate, 1.5M ammonium sulphate and 1M lithium sulphate respectively. Optimization of salt concentration and pH around the initial hit conditions enabled us to get crystals with suitable dimensions for data collection.

All FMDV crystals diffracted to high resolution sufficient to solve the atomic structures, with the exception of those of SAT2 which require further optimisation. The recombinant A22 wildtype crystals diffracting to 2.1Å and the mutants VP2 S93F and VP2 S93C diffracting to 2.4 and 2.9Å resolution respectively. The crystals of inactivated O1M VP2 S93Y and recombinant wildtype diffracted to 3.5Å and 3.0Å respectively. The inactivated SAT2 wildtype and VP2 S93Y material was very limited and the concentration of the wildtype SAT2 was very low, hence it was not used in crystallisation trials. The mutant, VP2 S93Y,

produced micro-crystals with average dimensions of $20 \times 20 \times 5 \mu\text{m}^3$ with 5 different crystallisation conditions. Crystal optimization will be performed when more material becomes available.

The diffraction datasets were collected using a recently developed *in-situ* data collection method at the Diamond I24 micro-focus beamline (Axford et al. 2012). The *in-situ* technology is a quick and easy method for collecting room temperature data, avoiding the increased mosaicity often associated with cryo-crystallography. It also provides a contained environment facilitating safe data collection and avoiding the hazards and deleterious effects of crystal mounting. A sufficiently complete data set can often be collected from a single 96-well, diffraction-optimised plate. This method facilitated data collection directly from crystallisation plates, making it a high-throughput method for virus structure determination, which would not have been possible with traditional methods that involve mounting individual crystals that are extremely fragile, into capillaries.

The structures of recombinant FMDV A22 were solved by molecular replacement and the phases improved by real space NCS averaging. The models were refined at 2.1Å (wildtype), 2.4 Å (VP2 H93F) and 2.9Å (VP H93C). There were no significant changes in the atomic structure of the recombinant A22 wildtype empty capsids, which were almost identical to the A22 virus and its natural empty capsid structures. Therefore, it is highly likely that the antigenicity was preserved in the recombinant capsids. The only observable difference involved the VP3 GH-loop which was more exposed on the particle surface in the recombinant capsids; however, this loop is flexible and such an exposed state has been previously observed with another A-serotype, A10. As predicted from the MD simulations, the mutant VP2 S93F formed a hydrophobic stacking interaction of phenylalanine side-chains and the mutant VP2 S93C formed the expected disulphide bond at the 2-fold axis between

two pentamers. Interestingly, differences were observed on the interior of empty particles containing the VP2 S93C mutation. The VP4 chain of the VP2 S93C empty capsid was completely disordered and the density for only 5 residues was observed. In addition, the N-terminus of VP1 (residues 1 to 12) and residues 34-36 in VP2 were also found to be disordered. Nevertheless, the disordered regions were located on the interior of the capsids and were unlikely to affect the antigenic properties of the mutant capsids. In contrast, the VP4 chain of the VP2 S93F mutant was better ordered and its conformation was identical to both the recombinant wildtype as well as that of the A22 virus structure. The crystals of the O1M wildtype and VP2 S93Y inactivated viruses also diffracted to high-resolution; however, the analysis of the diffraction data suggested merohedral twinning. Such twinning has been observed previously with another O-serotype virus (Lea and Stuart 1995) and work is in progress to de-twin the diffraction intensities and to obtain good estimates of electron density map for the structural studies. Similarly, crystallisation conditions for the inactivated SAT2 mutant were also identified and work is under progress to optimize the conditions to grow bigger and improved diffraction quality crystals.

8.2.4 Stability of the designed FMDV particles

We have shown that the mutants predicted by MD simulation to enhance particle stability are indeed significantly more stable than wildtype FMDV particles. The mutants also show a good agreement between the ranking in observed thermo-stability and in predicted binding free energy for both infectious viruses and empty capsids of three different serotypes, O1M, SAT2 and A22. The thermo-stability of designed particles was assessed using three independent methods. Thermo-stability of infectious and inactivated O1M and SAT2 was assessed by detecting the release of RNA, following the dissociation of capsids, using an RNA specific fluorescent dye. Wildtype O1M dissociated at 52°C compared to 53.5°C for

VP2 S93Y ($\Delta\Delta G_{mut-wt} = -11.8$ kcal/mol) and 54°C for VP2 S93F ($\Delta\Delta G_{mut-wt} = -13.8$ kcal/mol). When analysed alongside the O1M viruses, the SAT2 wildtype dissociated at 47°C, VP2 S93H ($\Delta\Delta G_{mut-wt} = -5.3$ kcal/mol) dissociated at 51°C and VP2 S93Y at 54°C ($\Delta\Delta G_{mut-wt} = -12.2$ kcal/mol). Interestingly, one of our aims was to design O and SAT2 serotype virus particles with the same stability as that of stable A serotype virus. In the same thermofluor assay, A24 cruiserio wildtype virus dissociated at 55°C. Therefore, our MD simulations have led to the successful achievement of this.

Similarly, thermo-stability of the recombinant empty capsids was also increased when the same mutations (that worked in the infectious virions) were applied. The thermo-stability of the empty capsids was assessed by sucrose density sedimentation. Wildtype and mutant capsids were incubated at different temperatures followed by sucrose gradient sedimentation. The wildtype O1M capsids were very unstable and broke apart when incubated at 45°C for 1hr, whereas, mutant empty capsids containing VP2 S93H or VP2 S93F mutations remained intact. When heated to 56°C for 2hrs, the capsids of VP2 S93H also broke apart, whilst about 75% of the VP2 S93Y capsids and the majority of VP2 S93F capsids remained intact. These results correlate with the predicted $\Delta\Delta G_{mut-wt}$ values for these mutants: VP2 S93H: -7.7; S93Y: -11.8; and S93F: -13.8 kcal/mol.

The effect of pH on the thermo-stability of the capsids was also investigated. It was observed that small changes in pH had a significant effect on the capsid stability and an optimal pH of 8.0 was found to increase the stability of both wildtype and mutant capsids. However, higher pH values (such as pH 8.5 or 9.0) had adverse effects, making the capsids more unstable. Furthermore, the mutants were also found to be more pH stable than the wildtype. In

addition, EM and thermofluor analysis showed that the inactivated mutant capsids were more resistant to dissociation during storage.

EM analysis provided visual verification of the integrity of the inactivated viruses of O1M and SAT2 and complemented the thermofluor results. The analysis revealed that more than 80% of the O1M wildtype capsids had dissociated into pentamers after 10 days storage at 4°C, in contrast the VP2 S93Y capsids remained intact. Furthermore, when inactivated capsids were incubated at 37°C for 20 mins, almost all wildtype capsids dissociated whereas approximately 60% of the mutant capsids still remained intact. Similar results were obtained with SAT2 using a thermofluor assay. Thermo-stability of inactivated mutant capsids remained the same after storage, whereas most wildtype capsids dissociated when analysed after 10 days storage at 4°C. Finally, the covalent mutant, VP2 S93C in A22 serotype was also found to be thermo-stable at 56°C and similar to other mutants, stable up to pH 5.5.

8.2.5 Immunisation with stabilised capsids

Protective immunity generated from the stabilised capsids was characterised by animal trials in guinea pigs and cattle. Inactivated wildtype and stabilised SAT2 viruses were formulated as commercial vaccines and were used to immunise guinea pigs. One and six months after immunisation significantly higher neutralising antibody titres were observed in the guinea pigs immunised with the stabilised SAT2 VP2 S93Y antigen, compared to animals immunised with the wildtype SAT2 antigen. A similar experiment with stabilised O1M VP2 S93Y is in progress. Similarly, the vaccines formulated from the stabilised empty capsids, containing either VP2 S93F (for O1M serotype) or VP2 S93C (for A22 serotype) mutations were shown to induce equivalent neutralising antibody titres to that of standard inactivated FMDV vaccines.

8.3 Future directions

8.3.1 Current and Future work on stabilised FMDV capsids

Following the success of the first generation mutants, a series of second and third generation stabilising mutants were designed by combination of the above mutants and the stabilising effect validated using MD simulations and $\Delta\Delta G$ calculations (see Appendix IV). Some of these mutations have been introduced into capsids and the work is in progress for both, O1M and SAT2 serotypes in order to produce and characterise the stability of these mutants. With viruses, there is often an issue of reversion of mutation to wildtype form which is evolutionary more favourable for spreading infection. Such reversion was observed with SAT2 VP2 S93Y mutant virus. Initially the virus grew very slowly, however, after 6 passages the growth improved and the mutant virus had similar growth profile to that of parental wildtype virus. Upon sequencing the P1 region, it was revealed that at VP2 position 93 the mutant virus had CAT codon that translates to histidine (H) instead of TAT codon the original codon for tyrosine (Y). The first passaged stock for S93Y mutant virus was also sequenced and had the TAT (tyrosine) codon. Therefore, the reversion occurred during adaption to growth in culture. However, a histidine at position 93 of VP2 is also stable in this case, compared to serine (see chapter 2 and chapter 6 for details) as present in the wildtype virus.

The chances of such reversions may be reduced in the case of second and third generation mutants because of combination of several mutations. Third generation mutations might make capsids very stable, such mutants might be extremely useful for producing stable empty capsids. However they might produce an essentially dead virus unable to release its genome. Indeed some reduction in growth was already observed with O1M VP2 S93F (producing very small plaques) and SAT2 VP2 S93Y growing slower in suspension culture. Nevertheless,

such properties might be turned to an advantage if the effect is to produce a less pathogenic virus, which might then be a candidate for a new live attenuated FMDV vaccine. Such a vaccine might stimulate a significantly better immune response than the current inactivated vaccines.

8.3.2 Structure analysis of FMDV capsids

The atomic structures of four serotypes have been solved (see Figure 1-6); however, there is no structural knowledge for the remaining serotypes. As a result, *de-novo* X-ray crystallographic analysis should be performed on the remaining serotypes.

As a contribution towards this, several crystallisation conditions have already been identified for the highly unstable SAT2 serotype (see chapter 4). A batch of material containing stabilised SAT2 (VP2 S93Y) antigen is BEI inactivated at The Pirbright Institute and will be imported to Oxford to be used for structure determination. Similarly, preliminary analysis and purification conditions for ASIA-1 are also established (see chapter 3) and material is being produced for crystallographic analysis.

These structures will be directly relevant to the design of stabilizing mutations and for use in simulations, which require accurate atomic models. Structural validation may also help to extend the basic knowledge relating to the maturation of the capsid, which will be relevant to the design process. The structural validation will also be carried out for all stable mutants. Indeed, these structures can then be used in simulations rather than wildtype capsids to further refine and validate the simulations.

8.3.3 Cattle vaccination studies

Besides the structural studies, various experiments are planned to test some of these stabilised FMDV antigens as vaccine candidates in cattle trials. Two separate experiments are planned to be performed at the Pirbright Institute and at the Agriculture Research Centre, Onderstepoort Veterinary Institute (ARC-OVI), South Africa.

The first experiment will use inactivated virion capsids of the SAT2 serotype. Twenty-six cattle will be divided into five groups (four groups containing six cattle each and one control group with two cattle) and will be inoculated with the vaccines formulated with either wildtype or mutants virion capsids as follows:

Group 1: six cattle will be vaccinated with wildtype SAT-2 vaccine in commercial adjuvant.

Group 2: six cattle will receive SAT-2 93Y VP2 protein mutant antigen in commercial.

Group 3: six cattle will receive SAT-2 93H VP2 protein mutant in commercial adjuvant.

Group 4: six cattle will receive SAT-2 93H VP2 protein mutant with an alternative adjuvant.

Group 5: two cattle will be in a control group.

Sera will be collected at regular intervals and will be assessed using neutralising antibody titres. All animals will be challenged 28 days post vaccination with 1×10^4 infectious SAT2 virus particles. In a parallel experiment, cattle will be vaccinated using FMDV empty capsids of O1M serotype, wildtype or VP2 S93F mutant. A control group will receive a standard O serotype FMDV vaccine and as described above, VNT analysis and challenge experiments will be performed.

8.3.4 A new approach for making better vaccines

Conventional vaccines involve the use of either inactivated (killed) preparation (such as Salk polio vaccines), live attenuated variants of naturally occurring organisms (such as Sabin polio vaccines, and rubella, mumps and measles vaccines) or inactivated toxins. The mode of action of these vaccines is only partially understood. More recent vaccines for some diseases are now based on antigenic proteins or peptides, the so-called subunit vaccines and vaccines based on recombinant technology such as empty particle vaccines (Ellis 1999; Greenwood et al. 2011).

The use of subunit vaccines based on antigenic proteins and synthetic peptides has been extensively explored for FMDV. However, the immunogenicity produced was significantly less than for traditional inactivated vaccines (for review, see Sobrino et al. (2001)). Nevertheless, although conventional inactivated vaccines can generate strong neutralising response, the immunity is serotype specific and short lived (approximately 6 months in the case of good quality inactivated vaccines) compared to several years of protective immunity in naturally infected animals (Alexandersen et al. 2003). The shorter duration of immunity in vaccinated animals compared to naturally infected animals could be due to unstable inactivated and non-replicative viral particles that might not induce cell mediated immunity. Enhanced stability of the capsid may provide improved and durable immunity as demonstrated in the case of stabilised SAT2 vaccines (see chapter 7).

More efficient induction of immunity (humoral and cell mediated) has been reported for attenuated vaccines as compared to inactivated vaccines including some picornaviruses (Usherwood and Nash 1995). This approach has been difficult in the case of FMDV because of the high potential for variation and adaption exhibited by the virus. The attenuation

obtained by the classical method of serial passaging in non-susceptible cells was unstable, with frequent reversion to virulent forms of the virus (X. M. Cao et al. 1991). New attenuated vaccines have been designed by making chimeric viruses where either the RGD receptor binding site or the L protein gene were deleted and have been shown to induce good neutralising antibody response without producing clinical symptoms but could not provide complete protection (Mason et al. 1997; McKenna et al. 1995). It is possible that the combination of capsids stabilising mutation along with mutations in non-structural proteins such as IRES, L or 3C may produce more stable and better attenuated vaccines.

Another major problem with FMDV vaccines is serotype specificity and limited cross reactivity to viruses of the other serotypes. Rieder et al. (1994) demonstrated that protection against FMD could be achieved using a chimeric vaccines where the VP1 GH loop was substituted with that from another serotype. Structure based chimeric vaccine design has been successfully used to produce effective vaccines for many diseases including diseases caused by some picornaviruses (Rose and Evans 1991; Thomas et al. 2011). Recently Scarselli et al. (2011) used structure-based design to develop a chimeric vaccine antigen for *Neisseria meningitidis*. A single chimeric antigen protected against seven meningococcal strains (Scarselli et al. 2011). With the help of structure studies and bioinformatics, it is now possible to predict and map the B cell and T cell epitopes on the antigenic proteins. This can enable the design of vaccines that are more specific at generating a broader immune response. Such a structure based approach in combination with capsid stabilisation could be used to design stable chimeric FMDV capsids to generate cross reactive antibodies against multiple serotypes.

8.4 Final Remarks

Structural biology is playing an increasingly crucial role in the development and analysis of vaccines. Here, we have successfully used a structure based rational engineering approach to increase the stability of FMDV capsids without affecting the antigenic properties of the virus by using combined *in-silico* molecular dynamics simulations and experimental methods. X-ray crystallography was used to study the designed viruses and to show that the predicted mutations make the desired interactions. The stabilised vaccines produced a stronger immune response and the storage studies showed that stabilised vaccines are more resistant to dissociation during storage, thus increasing the shelf-life of vaccine antigen. We have also shown that increasing the thermo-stability of FMDV capsids results in a significant increase in the neutralizing antibody titers and strong protection against FMDV SAT2 virus. Finally, we have demonstrated the production and application of safe and stable empty capsid FMD vaccines that do not require extensive bio-containment regulations during manufacturing.

With this work, we have demonstrated the direct application of structural biology and structure based design that has the potential to lead directly to a new generation of efficacious vaccines that can provide hope that the disease can be brought under control. Finally, work has started to translate this technology into other serotypes and subtypes of FMDV.

Appendix - I: MD scripts

Library File for Dummy Atom

```
!!index array str
"DUM"
!entry.DUM.unit.atoms table str name str type int typex int resx
int flags int seq int elmnt dbl chg
"DU" "Du" 0 1 0 1 10 0.0
!entry.DUM.unit.atomsptinfo table str pname str ptype int ptypex int
pelmnt dbl pchg
"DU" "Du" 0 -1 0.0
!entry.DUM.unit.boundbox array dbl
-1.000000
0.0
0.0
0.0
0.0
!entry.DUM.unit.childsequence single int
2
!entry.DUM.unit.connect array int
0
0
!entry.DUM.unit.hierarchy table str abovetype int abovex str
belowtype int belowx
"U" 0 "R" 1
"R" 1 "A" 1
!entry.DUM.unit.name single str
"DUM"
!entry.DUM.unit.positions table dbl x dbl y dbl z
0.0 0.0 0.0
!entry.DUM.unit.residueconnect table int c1x int c2x int c3x int
c4x int c5x int c6x
0 0 0 0 0 0
!entry.DUM.unit.residues table str name int seq int childseq int
startatomx str restype int imagingx
"DUM" 1 2 1 "?" 0
!entry.DUM.unit.residuesPdbSequenceNumber array int
0
!entry.DUM.unit.solventcap array dbl
-1.000000
0.0
0.0
0.0
0.0
!entry.DUM.unit.velocities table dbl x dbl y dbl z
0.0 0.0 0.0
```

PARM99 modifications to support a dummy atom (based on Na+/Ne)

```

MASS
Du 2000.00

BOND

ANGL

DIHEDRAL

NONB
Du 0.0000 0.00000

```

Simulation steps and syntax

Step 1: Minimization of the solvent keeping all non-water atoms effectively fixed.

```

&cntrl
  imin = 1,maxcyc = 5000,ncyc = 3500,
  cut = 8.0,ntb = 1,
  ntp = 1000,
  ntr = 1, restraintmask = '!:WAT',
  restraint_wt = 500.0,
/

```

Step 2: A further minimization of the solvent and counter ions keeping all protein (and dummyatoms) fixed.

```

&cntrl
  imin = 1,maxcyc = 5000,ncyc = 2500,
  cut = 8.0,ntb = 1,
  ntp = 500,
  ntr = 1, restraintmask = '!:WAT, Na+',
  restraint_wt = 500.0,
/

```

Step 3: A final minimization of all atoms (except dummy atoms) under the influence of the dummy-atom derived restraints.

```

&cntrl
  imin = 1,maxcyc = 10000,ncyc = 10000,
  cut = 8.0,ntb = 1,
  ntp = 500,
  ntr = 1, restraintmask = '(:DUM >@ 10.0) | :DUM',
  restraint_wt = 50.0,
/

```

Step 4: The simulation model is heated gradually to 310K over a period of 50ps.

```

&cntrl
  imin = 0,irest = 0,ntx = 1,
  nstlim = 25000,dt = 0.002,
  ntc = 2,ntf = 2,
  cut = 8.0, ntb = 1,

```

```
ntpr = 5000, ntwx = 5000,  
ntt = 3, gamma_ln = 2.0,  
tempi = 0.0, temp0 = 310.0,  
ntr = 1, restraintmask = '(:DUM >@ 10.0) | :DUM',  
restraint_wt = 50.0,  
nmropt = 1  
/  
  
&wt TYPE = 'TEMP0', istep1 = 0, istep2 = 25000,  
value1 = 0.1, value2 = 310.0, /  
&wt TYPE = 'END' /
```

Step 5: The equilibration and production run.

```
&cntrl  
imin=0, irest=1, ntx=5,  
nstlim=775000, dt=0.002,  
ntc=2, ntf=2,  
cut=8.0, ntb=1,  
ntpr=5000, ntwx=5000,  
ntt=3, gamma_ln=2.0,  
temp0=310.0,  
ntr=1, restraintmask='(:DUM >@ 10.0) | :DUM',  
restraint_wt=50.0  
/  

```

MM-PBSA script for generating snapshots from MD trajectory

```

# Input parameters for mm_pbsa.pl
# This script generates snapshots from a trajectory file

@GENERAL
#
#
PREFIX                snapshot
PATH                  ./
#
COMPLEX                1
RECEPTOR             1
LIGAND                1
#
COMPT                  ./com.prmtop
RECPT                  ./prot1.prmtop
LIGPT                  ./prot2.prmtop
#
GC                     1
AS                     0
DC                     0
#
MM                     0
GB                     0
PB                     0
MS                     0
#
NM                     0
#
#####
@MAKECRD
BOX                    YES
NTOTAL                 51261
NSTART                 56
NSTOP                  155
NFREQ                  1
#
NUMBER_LIG_GROUPS      1
LSTART                 3617
LSTOP                  7232
NUMBER_REC_GROUPS      1
RSTART                 1
RSTOP                  3616
#
#####
@TRAJECTORY
TRAJECTORY              ./prod.mdcrd

```

MM-PBSA script calculating binding free energy from snapshots

```

# Input parameters for mm_pbsa.pl calculation of  $\Delta\Delta G$ 
#####
@GENERAL
PREFIX                snapshot
PATH                  ./
#
COMPLEX                1
RECEPTOR            1
LIGAND                1
#
COMPT                  ./com.prmtop
RECPT                  ./prot1.prmtop
LIGPT                  ./prot2.prmtop
#
GC                    0
AS                    0
DC                    0
#
MM                    1
GB                    1
PB                    1
MS                    1
#
NM                    0
#
#####
@PB
PROC                  2
REFE                  0
INDI                  1.0
EXDI                  80.0
SCALE                 2
LINIT                 1000
PRBRAD                1.4
ISTRNG                0.0
RADIOPT               0
NPOPT                 1
CAVITY_SURFTEN         0.0072
CAVITY_OFFSET          0.00
FILLRATIO              2.0
#
SURFTEN               0.0072
SURFOFF                0.00
#
#####
@MM
DIELC                  1.0
#
#####
@GB
IGB                    2
GBSA                   1
SALTCON                0.00
EXTDIEL                80.0
INTDIEL                1.0
#
SURFTEN               0.0072
SURFOFF                0.00
#
#####
@MS
PROBE                  0.0
#
#####

```

Appendix - II: EBI-PSIA output

	Structure 1		Structure 2	
Selection range	A		B	
Class	Protein		Protein	
Symmetry operation	x,y,z		,,	
Symmetry ID	1_555		0_555	
Number of atoms				
interface	327	(17.9%)	328	(18.0%)
surface	1208	(66.2%)	1209	(66.3%)
total	1824	(100.0%)	1824	(100.0%)
Number of residues				
interface	85	(36.5%)	85	(36.5%)
surface	227	(97.4%)	228	(97.9%)
total	233	(100.0%)	233	(100.0%)
Solvent-accessible area, A ²				
interface	3051.7	(21.7%)	3053.3	(21.7%)
total	14045.8	(100.0%)	14056.9	(100.0%)
Solvation energy, kcal/mol				
isolated structure	-189.4	(100.0%)	-189.2	(100.0%)
gain at complex formation	-5.0	(2.6%)	-5.1	(2.7%)
average gain	-8.1	(4.3%)	-8.2	(4.3%)
P-value	0.706		0.706	

Hydrogen bonds				Salt bridges			
		XML				XML	
##	Structure 1	Dist. [Å]	Structure 2	##	Structure 1	Dist. [Å]	Structure 2
1	A:THR3115[OG1]	3.50	B:THR2007[OG1]	1	A:LYS3193[NZ]	3.17	B:GLU2192[OE2]
2	A:THR3115[OG1]	3.38	B:THR2008[OG1]	2	A:HIS2021[NE2]	3.81	B:GLU2213[OE1]
3	A:LEU3151[N]	2.68	B:LEU2009[O]	3	A:ARG2060[NE]	3.87	B:GLU2213[OE1]
4	A:HIS3144[N]	3.33	B:HIS2021[O]	4	A:ARG2060[NH1]	2.99	B:GLU2213[OE1]
5	A:GLU3146[N]	3.06	B:THR2023[O]	5	A:LYS2063[NZ]	2.61	B:GLU3146[OE2]
6	A:ARG3120[NH2]	3.36	B:SER2024[OG]	6	A:GLU2192[OE2]	3.17	B:LYS3193[NZ]
7	A:ARG3120[NH1]	3.07	B:THR2025[O]	7	A:GLU2213[OE1]	3.87	B:ARG2060[NE]
8	A:ASP3148[N]	3.42	B:THR2025[O]	8	A:GLU2213[OE1]	2.99	B:ARG2060[NH1]
9	A:LEU3151[N]	3.25	B:GLN2027[OE1]	9	A:GLU2213[OE1]	3.81	B:HIS2021[NE2]
10	A:ASN3152[N]	2.89	B:GLN2027[OE1]	10	A:GLU3146[OE2]	2.61	B:LYS2063[NZ]
11	A:HIS2021[NE2]	3.40	B:LEU2051[O]				
12	A:GLN2057[NE2]	2.91	B:THR2053[OG1]				
13	A:GLN2057[NE2]	3.17	B:ARG2054[O]				
14	A:LYS3193[NZ]	3.17	B:GLU2192[OE2]				
15	A:ARG2060[NH1]	2.99	B:GLU2213[OE1]				
16	A:THR2008[OG1]	3.38	B:THR3115[OG1]				
17	A:THR2007[OG1]	3.50	B:THR3115[OG1]				
18	A:ARG2060[NH2]	2.88	B:ALA3140[O]				
19	A:ARG2060[NH1]	3.09	B:CYS3142[O]				
20	A:ARG2060[NH2]	3.52	B:CYS3142[O]				
21	A:THR2023[N]	3.19	B:HIS3144[O]				
22	A:LYS2063[NZ]	2.61	B:GLU3146[OE2]				
23	A:THR2025[N]	2.91	B:GLU3146[O]				
24	A:GLN2027[N]	2.88	B:ASP3148[OD1]				
25	A:THR2026[OG1]	2.78	B:ASP3148[OD2]				
26	A:GLN2027[NE2]	3.20	B:ASP3148[O]				
27	A:GLN2027[NE2]	3.02	B:ASN3152[O]				
28	A:LEU2009[O]	2.68	B:LEU3151[N]				
29	A:HIS2021[O]	3.33	B:HIS3144[N]				
30	A:THR2023[O]	3.06	B:GLU3146[N]				
31	A:SER2024[OG]	3.36	B:ARG3120[NH2]				
32	A:THR2025[O]	3.42	B:ASP3148[N]				
33	A:THR2025[O]	3.07	B:ARG3120[NH1]				
34	A:GLN2027[OE1]	2.89	B:ASN3152[N]				
35	A:GLN2027[OE1]	3.25	B:LEU3151[N]				
36	A:LEU2051[O]	3.40	B:HIS2021[NE2]				
37	A:THR2053[OG1]	2.91	B:GLN2057[NE2]				
38	A:ARG2054[O]	3.17	B:GLN2057[NE2]				
39	A:GLU2192[OE2]	3.17	B:LYS3193[NZ]				
40	A:GLU2213[OE1]	2.99	B:ARG2060[NH1]				
41	A:ALA3140[O]	2.88	B:ARG2060[NH2]				
42	A:CYS3142[O]	3.52	B:ARG2060[NH2]				
43	A:CYS3142[O]	3.09	B:ARG2060[NH1]				
44	A:HIS3144[O]	3.19	B:THR2023[N]				
45	A:GLU3146[OE2]	2.61	B:LYS2063[NZ]				
46	A:GLU3146[O]	2.91	B:THR2025[N]				
47	A:ASP3148[OD1]	2.88	B:GLN2027[N]				
48	A:ASP3148[OD2]	2.78	B:THR2026[OG1]				
49	A:ASP3148[O]	3.20	B:GLN2027[NE2]				
50	A:ASN3152[O]	3.02	B:GLN2027[NE2]				

Appendix - III: FMDV sequence alignment

VP1

A22/1-211 1 TTTTGESADPVTITVENYGGGTQVQRKHHTDVTFIMDRFVKIQNLNPT-HVIDLMO 55
 A10-61/1-212 1 TTTTGESADPVTITVENYGGGTQVQRKHHTDVGFIIMDRFVKINSLSPT-HVIDLMO 55
 C58-c1/1-208 1 TTTTGESADPVTITVENYGGGTQVQRKHHTDVAFLVDRFVKVTVSDNQ-HTLDVMO 55
 O1BFS/1-213 1 TTSAGESADPVTITVENYGGGTQIQRRKHHTDVSFIMDRFVKVTQONQI-NILDLMQ 55
 O1M/1-211 1 TTSAGESADPVTATVENYGGGTQVQRKHHTDVSFILDRFVKVTPKDOI-NVLDLMQ 55
 ASIA-1/1-209 1 TTTTGESADPVTITVENYGGGTQTARRLHTDVAFLVDRFVKLTQPKST-QLDLMQ 55
 SAT1/1-219 1 TTSAGEGADPVTITDASAHGGDITRTTARRHTDVTFLDRFVLVKGKTNDNKLVLPLLS 56
 SAT2/1-216 1 TTSAGEGAEVVTITDPTTHGGKVTTTPRRVHTDVAFLDRSTHV-HTNTTAFVVDLMD 55

A22/1-211 56 THQHGLVGALLRAATYYFSDLEIVVR-HDGNLTWVPNGAPEAALSNTGNPTAYLKA 110
 A10-61/1-212 56 THKHGIVGALLRAATYYFSDLEIVVR-HDGNLTWVPNGAPEAALSNTSNPTAYNKA 110
 C58-c1/1-208 56 AHKDNIVGALLRAATYYFSDLEIAVT-HTGKLTWVPNGAPVSAALNTTNPTAYHKA 110
 O1BFS/1-213 56 VFSHTLVGGLLRATYYFSDLEIAVK-HEGDLTWVPNGAPEKALDNTTNPTAYHKA 110
 O1M/1-211 56 TBSHTLVGALLRATYYFADLEAVK-HEGNLTWVPNGAPEAALDNTTNPTAYHKA 110
 ASIA-1/1-209 56 IBSHTLVGALLRSATYYFSDLEVALV-HTGPVTWVPNGAPKTALNHTNPTAYQKQ 110
 SAT1/1-219 57 TKEKSLVGALLRAATYYFSDLEVACVGTNAWVGWTPNGSPVLTEVGD-NPVVFSRR 111
 SAT2/1-216 56 TKEKALVGAILRSATYYFSDLEVACVGHKHVFWQPNGAPRTTQLGD-NPMVLSRN 110

A22/1-211 111 PFTRLALPYTAPHRVLATVYNGTSKYSAGGTG-R-----RGDLGLPLAARVAAQLP 159
 A10-61/1-212 111 PFTRLALPYTAPHRVLATVYDGTNKSAS-DS-R-----SGDLGSAARVATQLP 158
 C58-c1/1-208 111 PVTRLALPYTAPHRVLATAYTGTTTYTAS-----A-----RGDLAHLTTTHAHL 156
 O1BFS/1-213 111 PLTRLALPYTAPHRVLATVYNGECKYSRNAVPNL-----RGDLQVLAQKVARTLP 160
 O1M/1-211 111 PLTRLALPYTAPHRVLATVYNGNSKYGDGTVANV-----RGDLQVLAQKAARALP 160
 ASIA-1/1-209 111 PITRLALPYTAPHRVLSTVYNGKTTYGEESR-----RGDLAALARVSNRLP 158
 SAT1/1-219 112 GTTRFALPYTAPHRVLATVYNGDCKVKTGTAPRENIRGDLATLAARIASETH-IP 166
 SAT2/1-216 111 NVTRFAIPFTAPHRLLSTVYNGECKEYTKVTAIR---GDREVLAAQKYSAAKHS 162

A22/1-211 160 ASFNFGAIQATTIHELLVRMKRAELYCPRPLLAVERSSQDRHKQKI IAPAKQ - - 211
 A10-61/1-212 159 ASFNVGAIQAQAIHELLVRMKRAELYCPRPLLAIKVTSQDRYKQKI IAPAKQLL 212
 C58-c1/1-208 157 TSFNFGAVKAETITELLVRMKRAELYCPRPILPIQPTG-DRHKQPLVAPAKQL - 208
 O1BFS/1-213 161 TSFNVGAIKATRVTELLVRMKRAELYCPRPLLAIHPTIE-ARHKQKI VAPVKOTL 213
 O1M/1-211 161 TSFNVGAIKATRVTELLVRMKRAELYCPRPLLAIHPTDQ-ARHKQKI VAPVKQ - - 211
 ASIA-1/1-209 159 TSFNVGAVKADTITELLIRMKRAELYCPRPLALDITIQ-DRRKQEI IAPVKQ - - 209
 SAT1/1-219 167 TTFNVGMIVTQAEDVYLRMKRAELYCPRPVLTHYDHNGRDRYKTLVKPAKQ - - 219
 SAT2/1-216 163 STFNFGFVTADKPV DVVYRMKRAELYCPRALLPAYTHAGGDRFDAPIGVAKQLL 216

VP2

A10-61/1-218 1 DKKTEETTLLEDRLLTTRNGHTTSTTQSSVGVTYGYSTFEDHVAGPNTSGLETRV 55
 A22/1-218 1 DKKTEETTLLEDRLLTTRNGHTTSTTQSSVGVTYGYSTQEDHVS GPNTSGLETRV 55
 O1BFS/1-218 1 DKKTEETTLLEDRLLTTRNGHTTSTTQSSVGVTYGYATAEDFVSGPNTSGLETRV 55
 O1M/1-218 1 DKKTEETTLLEDRLLTTRNGHTTSTTQSSVGVTYGYATAEDFVSGPNTSGLETRV 55
 C58c1/1-218 1 DKKTEETTLLEDRLLTTRNGHTTSTTQSSVGVTFGYATAEDSTSGPNTSALETRV 55
 ASIA1/1-218 1 DKKTEETTLLEDRLLTTRNGHTTSTTQSSVGVTYGYAVAEDAVSGPNTSGLETRV 55
 SAT1/1-219 1 DKKTEETTLLEDRIVTTSHGTTTTSTTQSSVGVTYGYALTDKFLPGPNTNGLETRV 55
 SAT2/1-219 1 DKKTEETTLLEDRLLTTRNGHTTSTTQSSVGITYGYADSDSFRSGPNTSGLETRV 55

A10-61/1-218 56 VQAERFFK KFLFDWITDKPFGYLTKLELPIDHHGVFGHLVDSYAYMRNGWDVEVS 110
 A22/1-218 56 VQAERFFK KHLFDWITDKAFGHLELPIDHKG VYGHVDSYAYMRNGWDVEVS 110
 O1BFS/1-218 56 VQAERFFK THLFDWVTSDFSFRCHLELPIDHKG VYGSLSYAYMRNGWDVEVT 110
 O1M/1-218 56 AQAERFFK THLFDWVTSDFGRCHLELPIDHKG VYGSLSYAYMRNGWDVEVT 110
 C58c1/1-218 56 HQAERFFK MALFDWVP SQNF GHMHKVVLPHEPKGVYGLVKS YAYMRNGWDVEVT 110
 ASIA1/1-218 56 TQAERFFK KHLFDWITPDL SFGHCHYLELPSEHKG VYGLMSYAYMRNGWDVEVT 110
 SAT1/1-219 56 EQAERFFK KHLFDWITLQQFGT TYVLELPIDHKG IYGLVDSHAYIRNGWDVQVS 110
 SAT2/1-219 56 EQAERFFK EKLFDWITSDKPF GTLYVLELPIDHKG IYGLTDSYAYMRNGWDVQVS 110

A10-61/1-218 111 AVGNQFNNGCCLLVAMVPEWKAFTREKYQLTLFPHQFISPRNTMTAHITVPYLVGV 165
 A22/1-218 111 AVGNQFNNGCCLLVAMVPEWKEFTPREKYQLTLFPHQFISPRNTMTAHITVPYLVGV 165
 O1BFS/1-218 111 AVGNQFNNGCCLLVAMVPELCSIQKRELYQLTLFPHQFINPRNTMTAHITVPFVGV 165
 O1M/1-218 111 AVGNQFNNGCCLLVAMVPELCSIQKRELYQLTLFPHQFINPRNTMTAHITVPFVGV 165
 C58c1/1-218 111 AVGNQFNNGCCLLVAMVPEMGDISDREKYQLTLFPHQFINPRNTMTAHITVPYVGV 165
 ASIA1/1-218 111 AVGNQFNNGCCLLVAMVPELKDTRQKYQLTLFPHQFINPRNTMTAHITVPYVGV 165
 SAT1/1-219 111 ATATQFNNGCCLLVAMVPELCKLDDREKYQLTLFPHQFLNPRNTMTAHITVPYLVGV 165
 SAT2/1-219 111 ATSTQFNNGCCLLVAMVPELCSLKAREEYQLTLFPHQFINPRNTMTAHLQVPYLVGV 165

A10-61/1-218 166 NRYDQYK KHKPWLTVVMVLSPLTVSNTA-APQIKVYAN IAPTVHVAGELPSKE 218
 A22/1-218 166 NRYDQYK KHKPWLTVVMVLSPLTTNTVS-AGQIKVYAN IAPTHVHVAGELPSKE 218
 O1BFS/1-218 166 NRYDQYK VHKPWLTVVMVVA PLTVNTEG-APQIKVYAN IAPTNVHVAGELPSKE 218
 O1M/1-218 166 NRYDQYK VHKPWLTVVMVVA PLTVNTEG-APQIKVYAN IAPTNVHVAGELPSKE 218
 C58c1/1-218 166 NRYDQYK QHRPWLTVVMVVA PLTTNTAG-AQQIKVYAN IAPTNVHVAGELPSKE 218
 ASIA1/1-218 166 NRYDQYK LHKPWLTVVMVVA PLTVK TGG-SEQIKVYMAA IAPTVHVAGELPSKE 218
 SAT1/1-219 166 DRHDQGRHQA WTLVVMVLA PYTNDQTI GSTKA EYVNI IAPTNVYVAGEKPVKQ 219
 SAT2/1-219 166 NRHDQGRHQS WSLVVMVLT PPTTEAQMNSGTVEYVNI IAPTNVYVAGELPGKQ 219

VP3

A22/1-221 1 GIVPVACSDGYGGLVTTDPKTADPVYGMVYNPPRTNYPGRFTNLLDVAEACPTFLR56
 A10-61/1-221 1 GIFPVACADGYGGLVTTDPKTADPVYGVYNPPKTNYPGRFTNLLDVAEACPTFLR56
 OIBFS/1-220 1 GIFPVACSDGYGGLVTTDPKTADPVYGVFNPPRNQLPGRFTNLLDVAEACPTFLR56
 O1M/1-220 1 GIFPVACSDGYGGLVTTDPKTADPVYGVFNPPRNQLPGRFTNLLDVAEACPTFLR56
 Cs8-c1/1-219 1 GIFPVACSDGYGNMVTTPKTADPAYGVYNPPRTALPGRFTNLLDVAEACPTFLM56
 ASIA-1/1-219 1 GIVPVACVDGYGNMVTTPKTADPVYGVSNPPRTSFPGRFTNLLDVAEACPTFLR56
 SAT1/1-221 1 GILPVAVSDGYGGFQNTDPKTSADPVYGHVYNPARTLYPGRFTNLLDVAEACPTLLD56
 SAT2/1-222 1 GIVPVACADGYGGFQNTDPKTSADPIYGHVYNPSRNDCHGRFSNLLDVAEACPTLLD56

A22/1-221 57 FDEGKPYVVVTRTDEQRLLAKFDVSLAAKHMSNTYLSGIAQYVAQYSGTINLHFMFT112
 A10-61/1-221 57 FDDGKPYVVVTRADDTRLLAKFDVSLAAKHMSNTYLSGIAQYVTQYSGTINLHFMFT112
 OIBFS/1-220 57 FEGGVYVVTTKTDSRVLAQFDMSLAAKHMSNTFLAGLAQYVTQYSGTINLHFMFT112
 O1M/1-220 57 FEGGVYVVTTKTDSRVLAQFDMSLAAKHMSNTFLAGLAQYVTQYSGTINLHFMFT112
 Cs8-c1/1-219 57 FEN-VPYVSTRTDGQRLLAKFDVSLAAKHMSNTYLAGLAQYVTQYTGTLNLFHFMFT111
 ASIA-1/1-219 57 FGEVFPVKITVNS-GDRLLAKFDVSLAAKHMSNTYLAGLAQYVTQYSGTIMNLFHFMFT111
 SAT1/1-221 57 FNGVPYVQTQSNSSGSKVLACFDLAFGHKNMKNTYMSGLAQYFAQYSGTINLHFMFT112
 SAT2/1-222 57 FDGKPYVVVTKNN-GDKVMAAFDVAFTHKVHKNTYLAGLADYVTQYSGLNLFHFMFT111

A22/1-221 113 GSTDSKARYMVAYVPP--GVETPPDTPEKAAHCIIHA EWD TGLNSKFTFSIPYVSSAA166
 A10-61/1-221 113 GSTDSKARYMVAYIPP--GVETPPDTPEEAAHCIIHA EWD TGLNSKFTFSIPYVSSAA166
 OIBFS/1-220 113 GPTDAKARYMVAYAPP--GME-PPKTPEAAAHCIHA EWD TGLNSKFTFSIPYLSAA165
 O1M/1-220 113 GPTDAKARYMVAYAPP--GME-PPKTPEAAAHCIHA EWD TGLNSKFTFSIPYLSAA165
 Cs8-c1/1-219 112 GPTDAKARYMVAYVPP--GMD-APDNPEEAAHCIIHA EWD TGLNSKFTFSIPYISAA164
 ASIA-1/1-219 112 GPTDAKARYMVAYIPP--GMT-PPTDPERAAHCIIHSEWD TGLNSKFTFSIPYLSAA164
 SAT1/1-221 113 GPTNNKAKYMVAYIPP--GTHPLPETPEMA SHCYHA EWD TGLNSTFTFTVPYVSSAA166
 SAT2/1-222 112 GPTHKAKFMVAYVPPGI EVELPKTPEDAAHCVHSEWD TGLNSTFTFAVPYVSSG167

VP4

SAT1/1-85 1 GAGQSSPATGSQNQSGNTGSIINNNYMQQYQNSMDTQLGDNAISGGSNEGSTDIT55
 SAT2/1-85 1 GAGQSSPATGSQNQSGNTGSIINNNYMQQYQNSMDTQLGDNAISGGSNEGSTDIT55
 ASIA-1/1-85 1 GAGQSSPATGSQNQSGNTGSIINNNYMQQYQNSMDTQLGDNAISGGSNEGSTDIT55
 OIBFS/1-85 1 GAGQSSPATGSQNQSGNTGSIINNNYMQQYQNSMDTQLGDNAISGGSNEGSTDIT55
 O1M/1-85 1 GAGQSSPATGSQNQSGNTGSIINNNYMQQYQNSMDTQLGDNATSGGSGNEGSTDIT55
 Cs8-c1/1-85 1 GAGQSSPATGSQNQSGNTGSIINNNYMQQYQNSMDTQLGDNAISGGSGNEGSTDIT55
 A22/1-85 1 GAGQSSPATGSQNQSGNTGSIINNNYMQQYQNSMDTQLGDNAISGGSNEGSTDIT55
 A10-61/1-85 1 GAGQSSPATGSQNQSGNTGSIINNNYMQQYQNSMS TQLGDNTISGGSNEGSTDIT55

SAT1/1-85 56 STHTNTQNNDFWFSKLAQSAFSGLVGALLA85
 SAT2/1-85 56 STHTNTQNNDFWFSKLAQSAISGLFGALLA85
 ASIA-1/1-85 56 STHTNTQNNDFWFSRLASSAFSGLFGALLA85
 OIBFS/1-85 56 STHTNTQNNDFWFSKLASSAFSGLFGALLA85
 O1M/1-85 56 STHTNTQNNDFWFSKLASSAFSGLFGALLA85
 Cs8-c1/1-85 56 STHTNTQNNDFWFSKLASSAFSGLFGALLA85
 A22/1-85 56 STHTNTQNNDFWFSKLASSAFSGLFGALLA85
 A10-61/1-85 56 STHTNTQNNDFWFSKLASSAFTGLFGALLA85

Appendix – IV Extended list of mutations

List of Mutants O1M serotype

No.	name	Mutation	Rationale	$\Delta\Delta G$ kcal/mol	Comments	Thermoflour assay (infectious virus)	Vaccinia expression
1	l*	VP2 S93H	Histidine is found in A22 which is the most stable FMDV serotype. It can stabilise the interactions at 2-fold axis.	- 7.7	Stable in simulations and in wet lab	Tested in empty capsid	Not stable at 56 °C however, stable at 45°C.
2	m*	VP2 S93Y	Aromatic residues like tyrosine, phenylalanine and tryptophan can make pi-pi stacking interactions at 2-fold. These interactions are known to stabilise proteins and protein - DNA interactions. As there is enough space at 2-fold, these residues can be well accommodated.	- 11.8	Stable in simulations and in wet lab	stable	Stable at 56 °C
3	i**	VP2 S93F		- 13.8	Stable in simulations and in wet lab	Stable	Stable at 56 °C
4	q*	VP2 S93W		- 9.5	Stable in simulations and in wet lab	Stable	Not stable at 56 °C, lower temperatures to be tested
5	u*	VP2 S93Q	Glutamine side chain can make hydrogen bonds with its symmetry partner.	- 6.7	Stable in simulations but same as wild-type	Not stable	Not stable at 56 °C, lower temperatures to be tested
6	r*	VP2 S97V	There is a hydrophobic residue valine at position VP2 90, sitting opposite to VP2 97 serine on an adjacent protomer, using hydrophobic residues such as alanine, valine, isoleucine or methionine can make stable hydrophobic interactions instead of serine at this position.	- 5.3	Not tested in the lab.	Not tested in infectious particles	Not tested
7	p2*	VP2 S97I		- 6.8	Stable in simulation and wet lab.	Not tested in infectious particles	Some heat stability at 56 °C, lower temperatures to be tested
8	p3*	VP2 S97M		+ 1.9	Slightly unstable in simulation. Poor yield and partially stable in wet lab.	Not tested in infectious particles	Some heat stability at 56 °C, lower temperatures to be tested
9	p1*	VP2 S97Q	Glutamine side chain can make hydrogen bonds with VP2 93 serine and stabilise the interface.	- 5.5	Stable in simulation and in wet lab.	Stable	Not stable at 56 °C, lower temperatures to be tested
10	o*	VP2 V90N	Asparagine side chain can make hydrogen bond with VP2 90 serine.	- 3.5	Stable in simulation and wet lab but poor yield.	Not tested	Stable at 56 °C
11	t*	VP2 H87V	F/Y failed due to clash with 62F, so a small side chain valine was used.	- 4.1	Valine with small side chain was accommodated well and interface found to be stable.	Not tested	Not tested
12	s*	VP2 Y98F	Tyrosine is capping C-terminal end of the 2-fold	- 11.67	Stable in simulation and in	Stable	In empty capsids,

			helix, using hydrophobic phenylalanine can stabilise the helix. It also makes the interface more hydrophobic.		wet lab.		needs to be tested
13	v	VP2 S93Y, S97I	Stable single mutants were combined to make more stable interface.	- 9.4	All these mutants are stable, however no additive effect on stability observed. Needs to be tested in the lab.	Not tested	Not tested
14	d1	VP2 S93Y, V90N		- 9.35		Not tested	Not tested
15	y	VP2 S93F, H87V		- 11.04		Not tested	Not tested
16	d2	VP2 S93H, Y98F		- 10.29		Not tested	Not tested
17	d3	VP2 S93F, Y98F		- 10.62		Not tested	Not tested
18	d4	VP2 Y91F, Y98F		- 9.51		Not tested	Not tested
19	d5	VP2 T95V, Y98F		- 5.61		Not tested	Not tested
20	d6	VP2 H87V, Y98F		- 10.98		Not tested	Not tested
21	d7	VP2 H87V, S97V		+ 0.56	Similar to wild type.	Not tested	Not tested
22	d8	VP2 H87V, VP3 H141L		- 4.57		Not tested	Not tested
23	x	VP2 H87V, VP3 H141V		- 9.11	Additive effect on stabilisation was observed; need to be tested in the lab.	Not tested	Not tested
24	t1	VP2 S93H, T95V, Y98F	A triple mutant, residues observed in A22 serotype.	- 12.82	This mutant is stable and the residues are naturally occurring in A22 serotype. It can help with the yield as the residues are naturally occurring in other serotype.	Not tested	Not tested
25	t2	VP2 S93F, L94V, Y98F	A triple mutant with unstable side chain of L94 changed to V.	- 12.52		Not tested	Not tested
26	t3	VP2 S93Q, V90L, H87V	VP2 S93Q, V90L are observed in Polio virus interface, adding a third stable mutant can increase the stability.	- 9.41	Additive effect was observed with Y98F which occurs in FMDV A22, an example of chimera between A22 and Polio.	Not tested	Not tested
27	t4	VP2 S93Q, V90L, Y98F		- 11.93		Not tested	Not tested
28	t5	VP2 S93Q, V90A, Y98F		- 9.48		Not tested	Not tested
29	aa	VP2 F62Y, H87V, VP3 H141V	Helix capping mutant – stabilises the charge on 2-fold helix	- 11.97	Capping the helix, stabilises the interface	Not tested	Not tested

* These mutants and their combinations are claimed in the current patent.

** These mutants were formulated into vaccine and used for in Guinea pig trials and have shown to have improved properties compared to wild type vaccines.

List of Mutants SAT2 serotype

No.	name	Mutation	Rationale	$\Delta\Delta G$ kcal/mol	Comments	Thermoflour assay	Vaccinia expression
First Generation Mutants							
1	l*	VP2 S93H	Results in O1M showed the hydrophobic change at 2-fold was successful in stabilising the interface. Same changes can also help in SAT2. In absence of structure of SAT2, a SAT1 structure was used as a model.	-5.3	As expected hydrophobic change at 2-fold does stabilise the interface.	Stable up to 51°C	-
2	m**	VP2 S93Y		-12.2		Stable up to 53°C	In progress
3	q*	VP2 S93W		-21.9		In progress	-
4	s*	VP2 Y98F		-5.6		In progress	-
5	i*	VP2 S93F	This is a reverse approach. SAT1 already has Q at VP2 position 93 whereas SAT2 has S. Substituting Q to S change in SAT1 destabilises the interface by + 15.42 kcal/mol. So there is a probability that this change will stabilise SAT2.	N/A	This mutation cannot be simulation in SAT1 structure due to steric hindrance in the model. It is still worth to test it in SAT2.	In progress	-
6	*	VP2 S93Q		N/A	Suggested, cannot be tested directly by simulation without the structure of SAT2.	In progress	-
7		VP2 F62Y, H87M, VP3 H142V	2-fold helix capping mutants	- 8.27	Stable	In progress	-
8		VP2 F62Y, H87V, VP3 H142V		+ 7.06	unstable	-	-
9		VP2 F62Y, H87V, Y98F VP3 H142V		-4.35	Stable	In progress	-

* These mutants and their combinations are claimed in the current patent.

** These mutants were formulated into vaccine and used for in Guinea pig trials and have shown to have improved properties compared to wild type vaccines.

List of Mutants A22 serotype

No.	name	Mutation	Rationale	$\Delta\Delta G$ kcal/mol	Comments	Thermoflour assay	Vaccinia Expression
1	i*	VP2 H93F	Results in O1M and SAT2 showed the hydrophobic change at 2-fold was successful in stabilising the interface.	-10.27	Capsids can be formed and the structure is solved showing the mutation at the right position.	not tested	not tested
2	m*	VP2 H93Y		-	Suggested based on O1M and SAT2 results but not simulated due to lack of interest (A22 is already stable)	not tested	not tested
3	*	VP2 H93Q	Glutamine can form hydrogen bonding with its symmetry related partner.	-2.27	-	not tested	not tested

List of Mutants ASIA1 serotype

No.	name	Mutation	Rationale	$\Delta\Delta G$ kcal/mol	Comments	Thermoflour assay	Vaccinia Expression
1	i*	VP2 G93F	Results in O1M and SAT2 showed the hydrophobic change at 2-fold was successful in stabilising the interface. Based on this, following mutants were suggested.	-	Due to absence of structure mutants were not simulated. Work is underway for structure studies.	not tested	in progress
2	m*	VP2 G93Y		-		not tested	in progress
3	q*	VP2 G93W		-		not tested	not tested

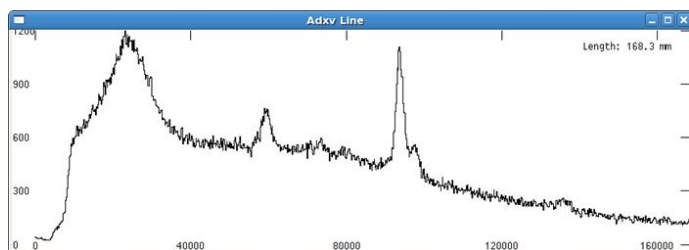
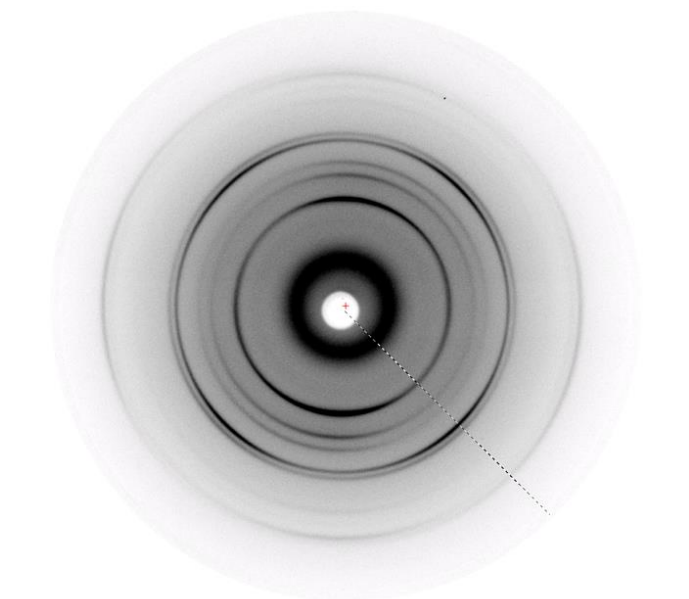
* These mutants and their combinations are claimed in the current patent.

** These mutants were formulated into vaccine and used for in Guinea pig trials and have shown to have improved properties compared to wild type vaccines.

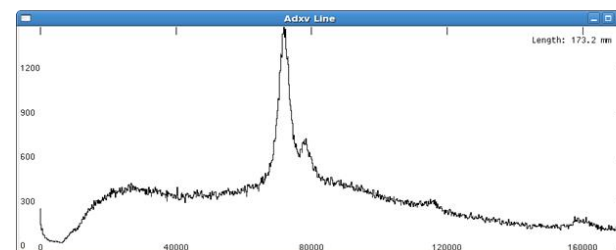
Negative control used to test simulation results – O1M.

No.	name	Mutation	Rationale	$\Delta\Delta G$ kcal/mol	Comments	Thermoflour assay	Vaccinia Expression
1		VP2 R60G	These mutations are internal controls to validate the data from simulation. VP2 arginine is making a salt bridge with glutamate on adjacent residue, destroying this interaction can make the capsids very unstable. Glutamine at VP2 57 is also making hydrogen bonds across the interface and changing it should make the particle unstable.	+ 33.7	Negative control for simulation.	Not tested	Not tested
2		VP2 R60L		+ 24.0	Negative control for simulation.	Not tested	Not tested
3		VP2 Q57E		+ 17.7	Negative control for simulation.	Not tested	Not tested
4		VP2 Q57L		+ 12.3	Negative control for simulation.	Not tested	Not tested

Appendix – V Scattering profiles



Standard Greiner sealing tape



ThermalSeal tape 50µm

Reduced background scattering with ThermalSeal, Background scattering was compared between standard Optiseal tapes and ThermalSeal tape. The background scattering was significantly reduced with the ThermalSeal tape. The images were recorded with in-house source (at 1.5418 Å), with a sample to detector distance of 250 mm. The resolution at the edge of the detector is 2.6 Å. (Data provided by Karl Harlos)

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