

Faculty of Medicine and Health Sciences

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Applications of Phage Peptide Libraries for Epitope Discovery and Identification of Novel Anti-Virals

By

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Abstract

Advances in phage display technology revolutionised our understanding of molecular interactions. The aim of using phage display is to screen a peptide library for the identification of rare ligand-bound variants with enhanced specific interaction. One of the most common challenges in dealing with an exhaustive peptide library is the selection of target-unrelated peptides. Here we improved methodical approaches that reduce phage binding to the substrate surrounding the target protein. With this we were able to improve the quality of peptide interactions selected from phage libraries. The main purpose of this study is investigate the range of applications of phage-displayed peptide libraries in the context of identifying peptides that interact with virus proteins and antibodies. Using our refined methods, we were able to select inhibitory peptides to Hepatitis C Virus (HCV) that blockade its entry into the host, along with mapping antigenic determinants of several monoclonal antibodies such as anti non-primate hepacivirus (NPHV) antibodies and human HC33.4 anti-HCV antibody. Epitope mapping proved easier than discovering potent viral therapeutics. However, lead sequences were identified that neutralise HCV entry in *in vitro* models. The presence of thousands of HCV quasispecies leads to another difficulty of discovering pangenotypic inhibitors. Robust alignment and significant binding were demonstrated with peptides selected with both NPHV and HC33.4 human antibodies, while the selection failed with other target proteins. There might be no hot spot residues presented in the libraries used against these proteins. Future investigations should focus on developing the suggested HCV-selected peptides to enhance their affinity. All in all, phage

display technology has been successfully developed to improve the performance of peptide libraries in a way that can avoid undesirable sequences during library sorting and enhancing the chance to find favourable ligands.

Declaration

I hereby declare that this thesis, including technical writing and experiments design (unless otherwise stated), is entirely my own work and has not been submitted anywhere for any other degree.

Abdulrahim Refdan Hakami

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Abbreviations

ALT	Alanine aminotransferase
BSA	Bovine serum albumin
DAA	Direct-acting anti-viral
DLA	Double-layer agar
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
ELISA	Enzyme-linked immunosorbent assay
epPCR	Error-prone PCR
FBS	Foetal Bovine Serum
GAG	Group antigen
GGT	Gamma-glutamyl transferase
GNA	Galanthus nivalis agglutinin
HCV	Hepatitis C Virus
HCVpp	Hepatitis C Virus pseudotyped particles
HCVcc	Hepatitis C Virus cell culture system
IFN	Interferon
IPTG	Isopropyl β -D-1-thiogalactopyranoside
LB	Lysogeny broth
NEB	New England BioLabs
PCR	Polymerase chain reaction
PDPL	Phage display peptide library
PEG	Polyethylene glycol
PFU	Plaque forming unit
RBV	Ribavirin
RIBA	Recombinant immunoblot assay
SAROTUP	Scanner and Reporter of Target-Unrelated Peptides
SOC	Super optimal broth with catabolic repressor
SR-BI	Scavenger receptor class B type I
SVR	Sustained virological response
TBS-T	Tris-buffered saline with Tween 20
VSV-G	Vesicular stomatitis virus G glycoprotein
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

1 Introduction

1.1 Phage Displayed Peptide Libraries

Phage displayed random peptide libraries are methodological approaches that allow selection of peptide ligands that bind to various target proteins. As its name suggests, random peptide sequences, and even other small molecules, are displayed on one of the filamentous bacteriophage coat proteins (Beech et al., 2015). The technique revolutionised medicine, molecular biology and other fields, and became a routine approach in different scientific fields (Bratkovic, 2010). Displaying proteins on phage particles has provided solutions to combat microbial infections and led to innovative applications, including developing new diagnostic reagents (Tan and Ho, 2014). Isolation of specific phage-displayed peptides requires an iterative biopanning procedure (affinity selection) whereby libraries of different phage-displayed peptides are allowed to interact with a protein of interest multiple times, using well established protocol (Scott and Smith, 1990). As the diversity of an enriched pool decreases, the specificity of each round of biopanning increases. Commonly, successful enrichment can be achieved following three rounds of selection. However, a myriad of interfering factors limits the effectiveness of the phage display approach. For instance, when creating a new random peptide library, stochastic reduction in sequence diversity and selection during phage amplification in *Escherichia coli* can affect selection quality. Moreover, distribution of amino acid bias has been observed in later rounds of selection (Zanconato et al., 2011). Although this methodology is very powerful, there are important steps that are critical to success, and it is possible that standard biopanning protocols are not optimised for every target protein.

1.1.1 M13 phage structure and life cycle

M13, f1 and fd filamentous bacteriophages are cylindrical needle-like structures that belong to *Inoviridae* family. They form one class of single stranded DNA (ssDNA) viruses called Ff class that are more stable than tailed phage lambda (λ) and T7. Table 1-1 summarises phage proteins and their main functions. M13 phage infects *E. coli* by interacting with the host F pilus tip using pIII protein. The bacterial Tol system translocates M13 phage into the host cytoplasm in a set of poorly understood events, but it is believed that the three Tol proteins (TolQ, TolR and TolA) are required (Click and Webster, 1997). Another suggestion implicates the c-terminal domain of TolA as a coreceptor for fd phage (Riechmann and Holliger, 1997).

The ssDNA (+ strand) enters into the cytoplasm upon infection. RNA polymerase initiates synthesis of the negative strand at the origin of replication. The pII protein initiates synthesis of the positive strand, creating a nick in the positive strand of the double stranded DNA replicative form (RF), which serves as a template for phage production. Genome replication is mediated by proteins pII, pV and pX that remained in the cytoplasm. Proteins pIII and pVI are essential for the structural stability of phage particles and for termination of assembly (Rakonjac et al., 2011). At the host membrane, phage particles are assembled as virions extrude out of the bacterium (Marvin et al., 2006).

Table 1-1 A synopsis of phage proteins and their functions (Sidhu, 2005)

Protein name and number of amino acids	M. wt (Dalton)	Function
pI (348)	39,502	Morphogenetic protein
pII (410)	46,137	Replication protein
pIII (406)	42,522	Minor coat protein / host cell binding / terminates viral assembly
pIV (405)	43,476	Morphogenetic protein
pV (87)	9,682	ssDNA binding protein
pVI (112)	12,342	Minor coat protein
pVII (33)	3,599	Minor coat protein
pVIII (50)	5,235	Major coat protein
pIX (32)	3,650	Minor coat protein
pX (111)	12,672	Replication protein
pXI (108)	12,424	Morphogenetic protein

1.2 Types of Phage Libraries

1.2.1 Peptide libraries

Several M13 phage-display vectors have been modified (Figure 1-1). In recent years, generating diverse and large peptide libraries on bacteriophage proteins became possible. The concept is based on inserting random nucleotides within a phage genome between the signal peptidase sequence and the sequence of the surface protein, resulting in a constructed displayed peptide (Fagerlund et al., 2014). It has been observed that mutant phage coat protein enhances peptide display compared to the wild type phage, improving the quality of peptide library (Sidhu, 2001, Sidhu et al., 2007). With each library type comes advantages and drawbacks. Peptides displayed on pVIII are more likely to lose sequence diversity compared to pIII, along with the avidity effect and low

peptide binders associated with type 8 multivalent libraries. The cut-off length of peptides on pVIII is 6—7 residues (Iannolo et al., 1995). The low copy number of displayed peptides in type 3 library encourages selection of high affinity peptides. While the technique is limited by displaying peptide sequences containing natural amino acids, chemical modification allowed the incorporation of synthetic chemical compounds which extends the scope of this application by including a range of functional groups (Chung et al., 2014).

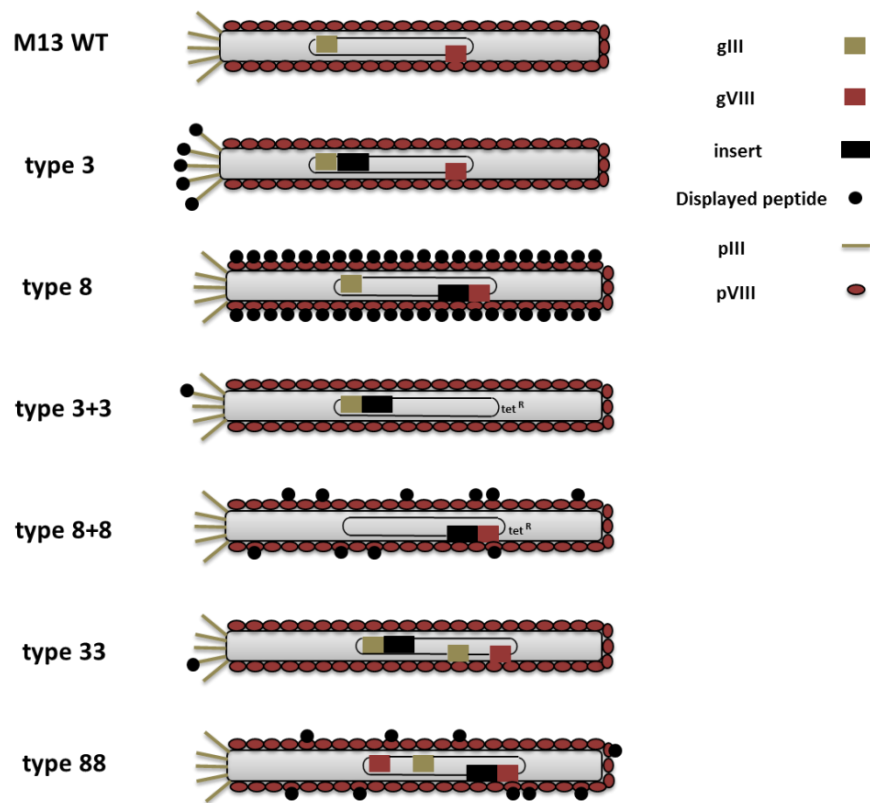


Figure 1-1 Schematic showing different phage display vectors and peptide location. Adapted from (Armstrong et al., 1996)

These vectors have been used as scaffolds for recombinant peptide libraries. There are 2700 copies of the major coat protein VIII and 5 copies of pIII (type 3 library) which is the recombinant pentavalent phage vector used in this study. Phage-borne peptides (black) are accessible for binding to target proteins during affinity selection (biopanning).

Phagemids are another genetically stable vector used for phage-displayed libraries. The vector contains the origin of replication of a plasmid, a selectable marker and a packaging sequence. Filamentous phage particles are produced from phagemids by co-infection with an engineered helper phage such as M13KO7 that lacks the packaging signal. Phagemids allow obtaining higher diversity, and can accommodate large DNA fragments (Rakonjac et al., 1997, Qi et al., 2012).

In addition to the linear (unconstrained) libraries, another structural framework was considered to improve cross-reactivity with peptides. A cyclic disulfide-constrained library was constructed by including fixed cysteine amino acids flanking random peptide sequences. Cysteine-constrained libraries were used against antibodies, proteins and carbohydrates (Bonnycastle et al., 1996). Conformational constrained libraries proved successful with anti-HIV-1 monoclonal antibody 2F5 (Palacios-Rodriguez et al., 2011), and with platelet glycoproteins, discovering cyclic peptides with dissociation constants in the nanomolar range, while reduced forms of the peptides performed with less binding affinity (O'Neil et al., 1992). However, unconstrained libraries have the potential of producing lead peptides too depending on the target protein used.

1.2.2 Antibody libraries

Phage library technology allows the display of large number of different antibodies, which can be selected for affinity to a specific antigen without the

use of experimental animal models (Winter et al., 1994). Antibody phage display allows *in vitro* affinity selection, permits functional and genetic analysis of monoclonal antibodies and helps understanding immunological interactions (Hoogenboom et al., 1998). In the past two decades, complete antibody variable domains were successfully displayed on the phage surface (McCafferty et al., 1990). Efficient methods were developed to construct and generate human single chain variable fragment (scFv) antibody libraries (Pansri et al., 2009). The library is prepared by ligating the variable heavy and light PCR products into a phage vector. Phage display becomes a robust technology for the discovery of therapeutic antibodies (Frenzel et al., 2016). A novel strategy was developed to detect the interaction between fluorescently-labelled soluble antigen and phage-displayed antibody fragments (Lagerkvist et al., 2001). However, the technique is demanding, difficult to characterise and might not reflect *in vivo* antibodies (Persson, 2009). The library might not recover all antigen-specific antibodies (Hammers and Stanley, 2014).

1.3 Phage Display as a Successful Technical Platform for Therapeutic Discovery

Filamentous fusion phage was first established by George Smith in 1985 as a novel approach for ligand discovery (Smith, 1985). Without the diversity the technique provides, studying novel molecular interactions is not practically feasible. This technology requires modifications and optimisation to successfully identify specific protein-ligand interactions. The versatility of phage display technology allows researchers to investigate the interaction

between a target protein and the best-performing peptides in a random library. The technology was used for discovery of cancer-specific peptides (Lee et al., 2015), ocular inflammatory disorders (Zhang et al., 2005), inhibitors and biopharmaceuticals, enzyme substrates, identifying protein-protein interaction, screening for receptor agonists, immunotherapy (Bazan et al., 2012), construction of gene delivery vehicles, vaccine design (Larralde et al., 2007, Hashiguchi et al., 2010), ligands for brain receptors (Li et al., 2012) and brain metastatic breast cancer (Bratkovic, 2010, Fu et al., 2014). As to neurological disorders, discovery of anti- β -amyloid antibodies using phage display methodology could provide a vaccination against Alzheimer disease (Solomon, 2007). Moreover, synthetic phage has also been used for tissue regeneration and proposes its use in cell therapy (Yoo et al., 2014). Phage display has become a cornerstone for the study of molecular interactions. While peptide libraries displayed on bacteriophage proteins can identify novel therapeutics, another outstanding discovery is that bacteriophages themselves reverse the aggregation of misfolded proteins in the brain, provides a novel approach in treating neurodegenerative diseases such as Alzheimer and Parkinson disease (Messing, 2016).

In addition to the discovery of antimicrobial peptides and receptor agonists, use of a phage display approach has become a platform for the identification of toxin neutralisers (Bernedo-Navarro and Yano, 2016). Another success was the identification of a peptide capable of binding myoglobin (a biomarker of myocardial infarction) which can be a cost-effective candidate over antibodies (Padmanaban et al., 2014). A potent anti-inflammatory short peptide that mimics

transforming growth factor (TGF)- β 1 through whole cell binding has been identified. The peptide reduced neutrophil migration, modulated IL-10 and tumour necrosis factor (TNF- α) cytokines, leading to regulatory T cells differentiation (Vaz et al., 2015). Exciting applications of this technology have been implemented successfully. The following examples are ground-breaking discoveries by utilising phage display as a platform.

1.3.1 Adalimumab (Humira)

The first fully human immunoglobulin for the treatment of rheumatoid arthritis approved as a therapeutic antibody was adalimumab (Humira; Abbott). This monoclonal anti-TNF- α antibody was approved by FDA in December 2002 followed by European approval in May 2003. Adalimumab was primarily produced by phage display technology with indistinguishable structure from the naturally occurring human IgG1 (Rau, 2002, Bain and Brazil, 2003). It was reasonably well-tolerated with no immediate hypersensitivity reaction (Papadakis et al., 2005). However, upper respiratory tract infection was the most reported adverse event (Weisman et al., 2003). This development greatly reinforces the importance of phage libraries in drug discovery.

1.3.2 Mimetics of erythropoietin

Combinatorial libraries containing large number of peptides constitute new methods for ligand identification and drug discovery. This novel approach was able to identify ligands of erythropoietin receptors that stimulate erythrocyte proliferation. In the living body, erythropoietin hormone induces erythropoiesis

(the production of red blood cells). The signalling pathway activated by selected agonists was identical to that activated by the natural ligand, despite that the representative agonist structure was unrelated to the primary erythropoietin sequence (Wrighton et al., 1996). The agonistic peptide has progressed to phase III clinical trials (Macdougall, 2008).¹ The initial discovery of an erythropoietin-mimetic peptide with an erythropoietic response *in vivo* proves that phage display technology facilitates selection of higher affinity agonists. This is one of the most prominent discoveries of a peptide agonist by phage library screening.

1.4 Applications of Phage Displayed Libraries

1.4.1 Ligands for inorganic surfaces

A new research area has been opened to discover inorganic and metal binding peptides (Frascone et al., 2013). An interesting application was the use of phage viruses in battery technology. A versatile approach using self-assembled M13 phage for positioning electrodes provided advanced battery designs with full electrochemical functionality (Nam et al., 2008). This is a unique ability developed by a biological system to grow nanostructures with a high degree of precision. Phage-directed synthesis of nanostructured metal oxides has been reported to fabricate lithium-oxygen battery electrodes (Oh et al., 2014). The approach suggests that M13 phage can be a model system to study the interaction between organic and inorganic materials. Furthermore, the diverse peptide sequences permit selection of specific ligands to polystyrene plates (Adey et al.,

¹ See Hematide[®] and CNTO drugs in Table 1-2.

1995, Gebhardt et al., 1996, Hu et al., 2016). Such polystyrene-adherent peptides were used in clinical applications as cell-repellent coatings (Kenan et al., 2006) and as immobilised ligands for immunoassays and serodiagnostic tests (Feng et al., 2009).

1.4.2 Haematological applications

There is a growing interest for the use of phage antibody libraries. Specific antibody fragments were specific to blood group antigens including ABO and Rh system by screening intact red blood cells. The selection offers an alternative application to hybridoma technology (Marks et al., 1993). Moreover, a panel of antibodies were selected against foetal red blood cells from phage display of antibodies. Specificity to cell surface antigens was confirmed by immunohistochemistry and flow cytometry. This application can provide a non-invasive prenatal genetic diagnosis as well as possible therapeutics for erythroleukaemia (Huie et al., 2001). In another application, scFv clones were isolated from phage antibody library that inhibit ICOS-Fc and B7RP-1-Fc interaction. B7RP-1 is a costimulatory molecule necessary for T cells activation. The successful inhibition can be applied to prevent autoimmune diseases and allograft rejection (Maeda et al., 2009).

Phage libraries enabled a remarkable discovery of specific peptides to haematopoietic stem cells *in vivo*. The same specific heptapeptides that home to bone marrow were isolated in another biopanning and confirmed by immunofluorescence assay. This was the first application of phage display

technology to stem cells and bone marrow that played a role in stem cell homing (Nowakowski et al., 2004).²

1.4.3 Use of filamentous phage as drug delivery system

Filamentous bacteriophages have long been used in microbial therapy. Until recently, they were used in vaccination, drug development (Sidhu, 2000) and as gene delivery vehicles (Karimi et al., 2016), where a drug can be chemically conjugated with the phage particle. This novel application was demonstrated using *Staphylococcus aureus* as a model target and chloramphenicol as a model drug (Yacoby et al., 2006).

In another genetically-modified filamentous bacteriophage, a new method has shown that phage nanoparticles serve as a specific anti-cancer therapy. The drug-delivery platform maximises anti-cancer antibodies at the tumour site. This new model overcomes severe toxicity and indiscriminate drug distribution brought by the systemic administration of chemotherapy (Bar et al., 2008). In addition, specific peptides fused to doxorubicin (anti-cancer drug) were able to target human breast cancer. This strategy relieves toxicity and opens the door to develop targeted chemotherapy approaches (Arap et al., 1998). While drug delivery to the lesion or tumour is a challenge in pharmacology research, diagnosis and treatment of malignant neoplasms became possible with this phage application (Nemudraya et al., 2016).

² Haematopoietic homing is the migration of stem cells to different organs such as spleen or to the tissue of their origin (bone marrow).

Novel methods for using phage particles as a multifunctional scaffold have been reported. For instance, M13 phage conjugated with dye molecules can target pathogenic strains of bacteria, which can be useful for imaging bacterial infections in living hosts (Bardhan et al., 2014). This shows that M13 phage is a successful carrier vector of a highly tuneable material for diagnostics and research applications. Moreover, specific peptide motifs have the potential for *in vivo* delivery. A mutant capsid protein of the human parvovirus adeno-associated virus type 2 (AAV-2) displaying NGR-motif was capable of binding CD13 which is expressed in angiogenic vasculature and other tumour cells. The tumour-targeting NGRAHA sequence, previously selected by *in vivo* phage selection, proved effective (Grifman et al., 2001).

1.4.4 Epitope discovery

Protein antigenic determinants are either linear or discontinuous regions of proteins recognised by antibodies. Antibodies recognising linear epitopes can also interact with the same denatured protein as in western blot. However, antibodies recognising linear epitopes cannot recognise cryptic epitopes which occupy domains inaccessible to antibodies. Conformational epitopes consist of parts of peptides juxtaposed by the protein folding to its original conformation (Barlow et al., 1986, Ferns et al., 1991). Mapping linear epitopes may be an easy process by using overlapping peptides of the original target or by combinatorial peptide libraries. This operation may not be of use to map conformational

epitopes. Instead, an indirect investigation based on competition binding could prove useful (Conway et al., 2003).

In the last century, site-directed mutagenesis was a useful method for defining epitopes (Benjamin and Perdue, 1996). However, manipulation of high number of antigen variants is exceedingly laborious. Phage display provided scientists with a robust technique to study protein-protein interaction (Kay et al., 2001). Identification of the actual epitope is now feasible with the combinatorial diversity of phage display approach.

Irrespective of whether a promising candidate is discovered or not, high affinity mimotopes can be selected against antibodies with favourable interaction kinetics. Future applications of selected peptides are limited by the organism elusive nature (Saphire et al., 2007). Nonetheless, initial sequence identification at high affinity is usually achieved once an established selection method is applied.

Identification of neutralising epitopes shared by virus strains would be necessary to identify broadly active inhibitors and diagnostic assays. A hybridoma clone secreting the anti- human immunodeficiency virus (HIV) monoclonal antibody 2F5 was established to neutralise HIV entry. Epitope mapping of this antibody by a hexapeptide repertoire of phage display was defined, which showed the motif ELDKWA on the virus gp41 ectodomain. However, sequence variability

of amino acids is a major obstacle to vaccine design (Muster et al., 1993, Purtscher et al., 1994).

Mapping epitopes on the Hepatitis C Virus (HCV) E1 and E2 envelope glycoproteins was previously performed using D32.10 monoclonal antibody raised against the virus. Using phage display, three HCV domains were mapped (Petit et al., 2003). The technique has also been able to determine the antigenic determinants of HCV core and non-structural (NS) proteins (Rechkina et al., 2006).

Table 1-2 Approved peptide-based therapeutics or under clinical trials (Hamzeh-Mivehroud et al., 2013)

Product	Manufacturer	Indication	Phase
Fuzeon [®] (enfuvirtide)	Roche, Trimeris	HIV	Approved
Byetta [®] (exenatide)	Amylin Pharms, Eli Lilly	Type 2 diabetes	Approved
Victoza [®] (liraglutide)	Novo Nordisk		
Sarenin [®] (saralasin acetate)	Norwich-Eaton Pharms, Procter & Gamble	Hypertension	Approved
Natrecor [®] (nesirtide)	Scios	Congestive heart failure	Approved
Prialt [®] (ziconotide acetate)	Elan Pharms	Severe chronic pain	Approved
Stilamin [®] (somatostatin acetate)	Merck-Serono	Acute variceal bleeding	Approved
Bigonist [®] (buserelin)	Sanofi-Aventis	Advanced prostate cancer	Approved
Firmagon [®] (degarelix acetate)	Ferring Pharms		
Velcade [®] (bortezomib)	Millennium Pharms, Janssen-Cilag	Multiple myeloma	Approved
Hematide [®] (Peginesatide)	Affymax, Takeda	Chronic kidney disease associated anaemia	III
Xerecept [®] (corticotropin acetate)	Celtic Pharma	Peritumoural brain oedema	III
Exubra [®]	Inhale, Pfizer, Aventis Pharmaceuticals	Diabetes type I and II	III
Iseganan	IntraBiotics	Diabetes type II	II/III
Thymogen [®] (ogluferanide disodium)	Altika, Cytran, Implicit Bioscience	Immune system related diseases	II
Onalta [®] (edotreotide)	Molecular Insight Pharms	Neuroendocrine tumours	II
CNTO 530/CNTO 528	Ortho Biotech	Chronic kidney disease associated anaemia	I
Apan [®]	Praecis	Alzheimer's disease	I
Oratomin [®]	NOBEX	Osteoporosis	I

1.5 Peptides as Potential Therapeutics

Phage display enables a high-throughput discovery of bioactive peptides. Following this initial investigation, advances in drug development enables the discovery of biologically active and marketable peptides. In addition, optimal substrate sequences were identified for proteases and other enzymes (Lowman, 1997). Due to low manufacturing cost, peptide drug candidates sometimes supersede antibodies. Peptides have several advantageous characteristics such as their greater stability at room temperature, excellent target specificity, better tissue penetration and low toxicity, but suffer from poor oral bioavailability and metabolic stability (Ladner et al., 2004, Craik et al., 2013). Table 1-2 summarises some of the peptide-based drugs.

1.5.1 Enfuvirtide

The best known clinically approved peptide inhibitor of a viral fusion process is enfuvirtide, which was discovered to have antiviral activity by binding to HIV-1 gp41, following the serendipitous observation in which HIV replication was reduced in HeLa cells following incubation with gp41-derived peptides. The peptide was subjected to clinical development to determine the best route of administration, pharmacokinetics and efficacy (Cooper and Lange, 2004).

After discovering the first approved HIV-1 fusion inhibitor, another gp41-derived peptide called sifuvirtide was designed. It has shown potent antiviral

activity and lower cytotoxicity, even to enfuvirtide-resistant strains (He et al., 2008, Wang et al., 2009). Another 34-amino-acid peptide (SC34EK) derived from the same HIV gp41 fusion protein was developed by stabilising its α -helicity (Naito et al., 2009).

1.5.2 Potential anti-microbial and anti-viral peptides

Medically important viruses cause major public health concern. Identification of inhibitors is highly desirable. High-throughput screening of phage libraries was able to identify inhibitory peptides of Japanese encephalitis virus (JEV) that interfere with viral attachment. Investigators used domain III of JEV envelope glycoprotein (E DIII) as bait (Zu et al., 2014). This interesting finding leads us to screen a phage-displayed peptide library using HCV envelope glycoprotein as thoroughly clarified in chapter 3.

An earlier study isolated phage clones against a malaria parasite by screening a disulfide-constrained hexapeptide library. Peptides were reactive to *Plasmodium vivax* and shared homology with merozoite surface protein. When these phagotopes were injected in animal models, a strong immune response was produced against phage particles with comparable avidities of immune sera, suggesting that phagotopes could be a vaccine component (Demangel et al., 1996).

Moreover, phage peptide library was a successful approach in suppressing bacterial infections (Cao et al., 2000). The emergence of microbial resistance leads to the quest for novel peptide therapeutics that can supersede antibiotics. A non-cytotoxic antimicrobial peptide (AMP), discovered using a phage-displayed peptide library, was active against Gram-negative bacteria, resulting in a significant bactericidal activity against *Pseudomonas aeruginosa* and *E. coli* (Sainath Rao et al., 2013). AMPs play a significant role in immunity too (Wiesner and Vilcinskas, 2010).

1.5.3 Peptide optimisation

Peptides can be used as intermediates for drug development by modifying backbone structure. Peptide potency can be restored by ‘hydrocarbon stapling’ to generate rigid peptide structures. This chemical optimisation can generate protease-resistant peptides of rigid mimics useful in vaccine development. In a promising example, stabilised α -helices of the membrane proximal external region of HIV gp41 were generated. These peptides were protease resistant and highly reactive to 4E10 and 10E8 HIV antibodies (Bird et al., 2014).

The same peptide stapling strategy (hydrocarbon-stapled peptides) was used to improve the structure of a receptor-derived HCV inhibitor and to overcome the poor proteolytic stability (Cui et al., 2013). Their peptide was CD81-derived inhibitor. In this project, one of the objectives is to identify HCV inhibitory peptides from random libraries followed by random mutagenesis as a starting point in the lead optimisation process. Another strategy for peptide development

following lead identification is the selective substitution of the amino acid residues and modulation of the physicochemical properties. This strategy has led to an enhanced potency of a cytomegalovirus inhibitor by 27-fold relative to its unmodified lead (Ogilvie et al., 1997). Mirror-image phage display (or retro-inverso peptides) is an elegant method where L-amino acids are replaced by D-amino acids to ensure proteolytic resistant ligands that mimic the parent peptide (Mason, 2010).

1.6 Using Phage Displayed Libraries in the Context of Emerging Hepacivirus Species

Hepacivirus genus belongs to the *Flaviviridae* family. These are enveloped, positive-sense RNA, single-stranded viruses (Tanaka et al., 2014). HCV and GB virus B (GBV-B) belong to this genus (Simons et al., 1995), but GBV-B is more related to rodent hepacivirus (Drexler et al., 2013). Emerging non-primate hepacivirus species were detected in horses, dogs and bats (Quan et al., 2013, Reuter et al., 2014, Pfaender et al., 2015, Gather et al., 2016). Other flavi-like viruses were discovered in invertebrates (Shi et al., 2016). Direct cross-species transmission is a plausible explanation (Pybus and Theze, 2016). Horizontal and vertical transmission of non-primate hepacivirus has occurred in horses (Gather et al., 2016).

HCV is the best studied virus of all hepacivirus species. It was discovered as an agent of non-A, non-B hepatitis (Choo et al., 1989). Its envelope glycoprotein was a key target for vaccine design. It has been hypothesised that the structure

of HCV envelope and other flaviviruses are analogous. An early study suggested that Tick Borne Encephalitis virus envelope E glycoprotein could be a possible model for HCV envelope (Yagnik et al., 2000).

The overall genome organisation of HCV and NPHV is similar, consisting of encoding core region, E1 and E2 proteins, p7, NS2 through NS-5B. Phylogenetic analysis of NPHV genomes from horse sera showed corresponding nucleotide regions in HCV and GBV-B. They are genetically related viruses, but there was a sequence diversity in the structural and non-structural regions of NPHV variants in a comparative sequence analysis (Burbelo et al., 2012).

There is a growing need to investigate these hepacivirus species to develop new therapeutics. In addition, no vaccine has been developed to HCV. A vaccine needs to induce broadly protective immunity, but this has been challenged (Drummer, 2014). Phage-displayed peptide libraries were successfully used to induce mimotope-specific antibody response (Larralde et al., 2007, Hashiguchi et al., 2010). We successfully selected highly specific mimotopes against HC33.4 antibody which adopt different conformations upon binding to anti-HCV antibodies. Immunising animal model with these mimotopes can generate antibody response.

1.7 Scope of the Study

The recent discoveries of new hepacivirus species requires research investigation towards more understanding of their molecular interactions. The study explored the potential of phage-displayed peptide libraries in discovering ligands with specific and desired properties towards immunoglobulins including anti-non-primate hepacivirus antibodies and large proteins such as the innate pattern recognition receptor L-ficolin. The technique was optimised to achieve better peptide selection followed by detailed investigation and analysis of the selected peptides including binding and inhibition studies, sequencing analysis and peptide alignments.

2 Optimisation of Biopanning Methodologies and Phage Binding Assays

2.1 Introduction and Aim

In previous studies using routine biopanning experiments with direct target coating, phage particles were found to interact with the polystyrene used as a solid substrate for the assay. One study suggested that tyrosine and tryptophan residues were specifically selected in those peptides enriched by such unwanted interactions (Adey et al., 1995). Repetitive serine and valine were also recovered from peptide selection experiments using polystyrene surfaces. The peptide ligands SVSVGMKPSRP and WHWNAWNWSSQQ are the most common polystyrene binding sequences selected during biopanning of phage-displayed peptide libraries. These target-unrelated peptides (TUPs) were selected using various receptors, proteins and antibodies (Huang et al., 2010, Sookrung et al., 2011, Huang et al., 2012, Stewart et al., 2012, Gutes et al., 2013). To address this, different experimental conditions should be investigated in detail to choose the best blocking reagent, washing buffer and the optimum elution method.

Assay conditions might be implicated in such non-specific binding. As this can negatively impact all phage selection experiments, this chapter aims at understanding the nature of phage non-specific interaction and resolving the background noise of affinity selection (Figure 2-1). The peptide selection followed a systematic methodical approach (Figure 2-2). In addition, this Chapter aims at investigating fast propagation phage by sequencing Shine-Dalgarno (SD) region (gIIp) of phage clones selected in this study and proposes alternative protocols to avoid this biological restriction.

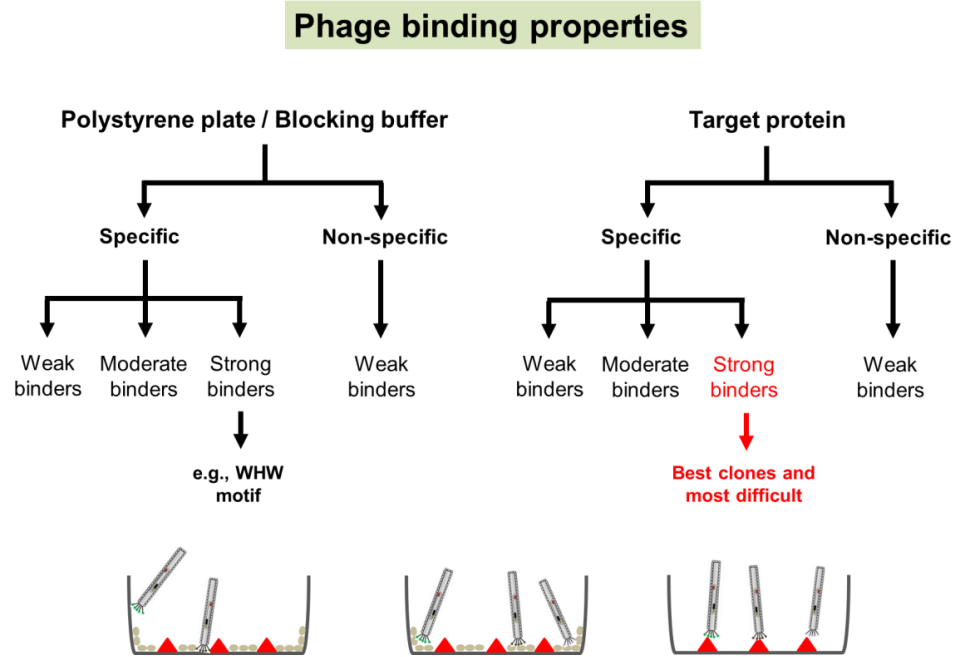


Figure 2-1 Potential phage binding characteristics observed during affinity selection of random-peptide phage displayed libraries

With billions of random sequences within a recombinant phage library, specific peptides against various target proteins can be present. For instance, sequences rich in aromatic residues such as tryptophan and histidine in a specific pattern favours plastic surface binding. The aim is to select possible ligands that cross-react with the protein of interest. Absence of favourable peptides within the library would fail the selection process.

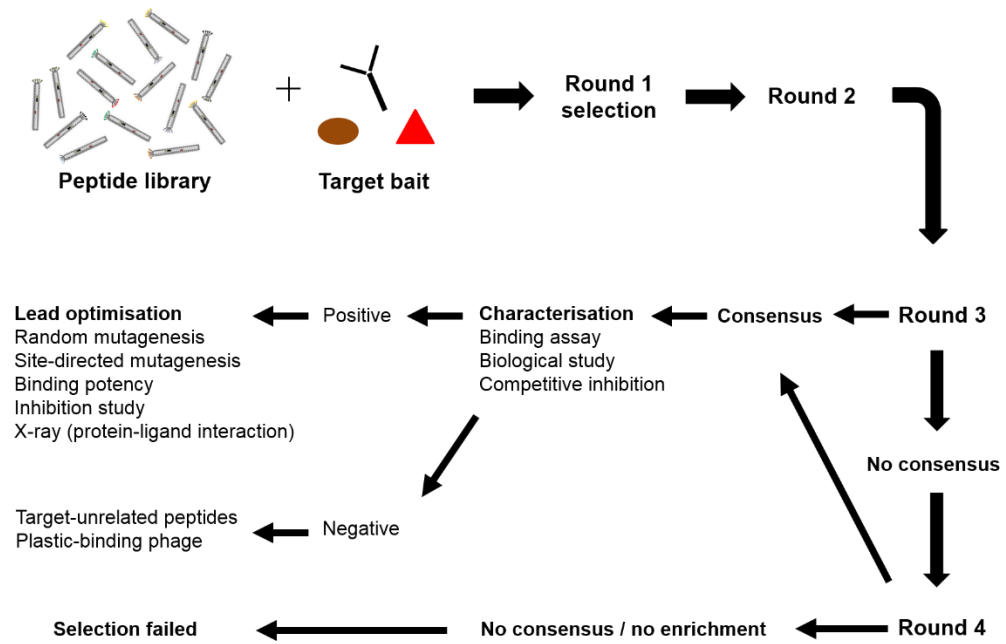


Figure 2-2 Overview of phage library screening and iterative selection

A diverse peptide library is initially screened against the protein of interest. Reactive peptides are recovered and reacted again with the same target. Presence of amino acid consensus might indicate specific binding. Further characterisations of the peptide lead can reveal the specificity of interaction. Binding potency and inhibition profile of the lead sequences can be increased by random mutagenesis. Absence of conserved motifs and enrichment indicates that no promising hit was selected.

2.2 Materials and Methodologies

2.2.1 Affinity selection (biopanning)

A 12-mer random phage library with a diversity of 1×10^9 peptide sequences (NEB, #E8110) was screened against several target proteins including HCV envelope glycoproteins, antibodies and bovine serum albumin (BSA) as a control bait (Figure 2-2). After coating $10\mu\text{g.mL}^{-1}$ of target bait in a polystyrene plate overnight, the plate was blocked for 3hr at room temperature and washed 4 times. A concentration of 1×10^{11} phage clones was added in the first round of peptide selection and incubated for 1hr. The wells were washed 10 times, and bound phage particles were eluted and amplified to prepare another sub-library for the second round. Enrichment of phage pools at the second and third round were monitored.

2.2.2 Elution method

To investigate the effect of elution step on specific phage selection, two methods were used: chemical versus bacterial elution. Elution buffer (0.2M Glycine-HCl, pH 2.2, 1mg.mL^{-1} BSA) was compared with another bacterial elution. Following chemical elution by acid treatment, we hypothesised that few strong binders resist the chemical elution and remain bound to the target protein (Figure 2-3). To liberate them, 100 μl of an early-log phase *E. coli* ER2738 culture was immediately added after recovering phage eluate, and kept at 37°C for 30 minutes with shaking, then transferred to the original bacterial culture for 4hr, followed by polyethylene glycol (PEG) precipitation. Another 100 μl of early-

log phase culture was added into an empty well as a negative control. The bacterial elution method developed here was compared with another protocol (Lunder et al., 2008) in order to successfully select phage at earlier rounds and to avoid propagation advantage clones (Figure 2-3). Whichever of the two methods is most useful, both elution methods were compared in Chapters 3 and 4.

2.2.3 Negative selection

Another approach was adopted to enhance the success rate of peptide selection in which the sub-library of the first round is incubated in a mock-well, and then the same library is transferred in a positive selection with the target of interest (Figure 2-3).³ This process aims at reducing sequences with higher affinity to the plate and can be used concurrently with the bacterial elution strategy.

³ It is not recommended to perform negative selection using the original diverse library before the first round. There are nearly 10 copies of each peptide sequence in the complex library with roughly 1.3 billion sequences. Valuable sequences might get lost in the negative selection even if they are weak-plastic binders. Amplified, yet less diverse, library following the first round (the sub-library) would have tens of thousands of copies of each sequence. In this case, the loss of few copies of a crucial sequence in the negative selection will not impact the whole selection process when performing positive selection with the target.

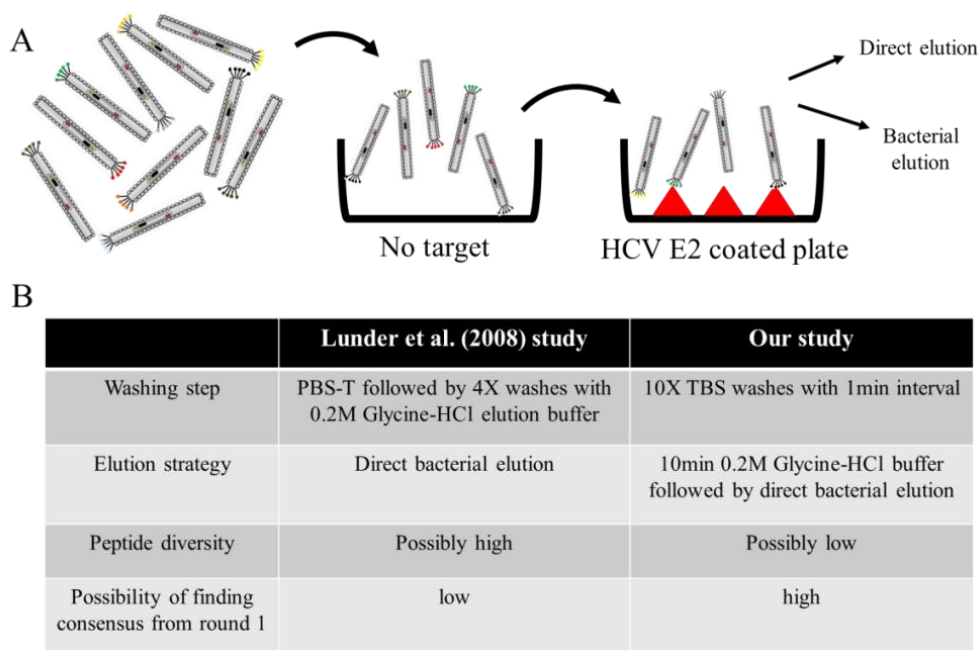


Figure 2-3 Negative selection and bacterial elution protocols

Amplified phage pool from the first round can be added in a mock-well then transferred to the protein of interest in a positive selection (A). Washing conditions and elution method were modified to reduce peptide diversity in earlier rounds and compromise propagation-related selection (B).

2.2.4 Phage titration and plaque assay

Preparation of library phage possessing an α -complemented β -Galactosidase gene (9 μ l) were serially diluted in lysogeny broth (LB) and used to infect 200 μ l *E. coli* ER2738 mid-log phase cultures (~ 0.5 OD_{600nm}). Bijou tubes (Thermo Scientific) were pre-incubated at 42°C. Four millilitres of semi-solid agar (0.75% LB agar; Sigma, 2897) was added into each tube. Then, 2% X-gal (Invitrogen) and 100mM IPTG (Thermo Scientific) were added into each tube (30 μ l each). Plates were incubated at 37°C overnight. Phage preparations were

enumerated in the double-layer agar (DLA) by counting blue plaques. Phage titres (infectious phage/ml) were calculated as:

(Number of plaques x volume [0.009ml] x 1/dilution factor).

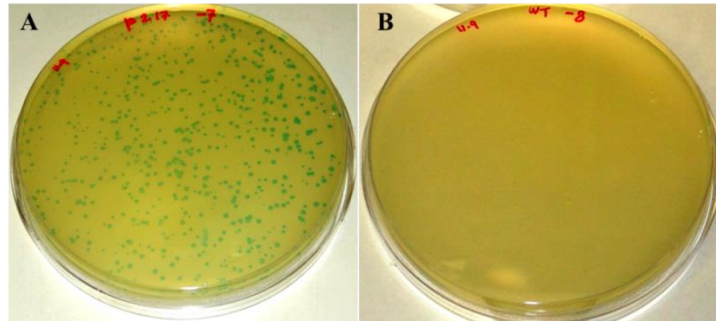


Figure 2-4 Bacteriophage plaques on the double-layer agar plate

Presence of blue plaques indicates recombinant M13KE phage (A) compared to the ubiquitous wild type M13 phage and KO7 helper phage (B). Note: Wild type M13KE phage clones do not display peptides, yet called wild type, and they would show blue plaques. Phage libraries might contain low number of wild type M13KE phage and can be non-specifically selected. Therefore, plaque assay does not distinguish between wild type M13KE and other phage clones.

2.2.5 Phage amplification and purification

Phage pools were propagated and purified according to Phage Display Peptide Library Kit (NEB Ph.D.TM-12, #E8110). Briefly, 1:100 dilution of an overnight *E. coli* K12 ER2738 culture was incubated with 25µg.mL⁻¹ of tetracycline at 37°C with good aeration to prepare an early-log phase, then 5µl phage stock were added in each tube (or a pipette tip is inoculated after being stabbed in a blue plaque). After amplifying phage clones in *E. coli* for 4.5—5 hr, the culture was centrifuged for 10 minutes at 12,000g at 4°C. The supernatant was mixed with 1/6 volume of 20% PEG/2.5M NaCl ⁽⁴⁾ to precipitate the phage at 4°C for at least 2hr or preferably overnight. Following the centrifugation at 12,000g for 15 minutes at 4°C, the white phage pellet was suspended in Tris-buffered saline (TBS; 50mM Tris-HCl, 150mM NaCl, pH7.5), spun at high speed (14,000RPM), mixed with 1/6 volume of 20% PEG/2.5M NaCl, incubated on ice for 15—60 minutes and centrifuged at 14,000RPM for 10 minutes at 4°C. The pellet was suspended in 200µl TBS and incubated at -20°C.

2.2.6 NanoDrop (Spectrophotometry)

To determine the bacterial log-phase during amplification, bacterial cell suspensions were monitored using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific).

⁴ To prepare this reagent, add 20g of PEG (M.wt 8000; Sigma) and 14.61g of NaCl and made up a final volume of 100ml water, mix and autoclave.

2.2.7 Phage binding assay

Binding of selected phage clones to their target proteins was tested using F96 MaxiSorp polystyrene plate (Nunc, #442404). The plate was coated with the target protein overnight at 4°C in carbonate-bicarbonate buffer (Sigma, C3041). Each well was blocked with 5% skimmed milk for 3hr at room temperature. The plate was washed 3 times with TBS-T buffer with 0.05% Tween 20. For each phage clone, a concentration of 1×10^{11} particles was added and incubated for 1hr at room temperature. After the washing step, phage binding to the target protein was assessed using anti-fd bacteriophage antibody (Sigma, B7786) and detected by alkaline phosphatase-conjugated anti-rabbit antibody (Sigma, A2556). P-nitrophenyl phosphate (PNPP; Sigma, N2770) was added, and the absorbance was measured at 405nm. The blocking effect, buffers and assay conditions were investigated.

2.2.8 GNA-capture enzyme linked immunosorbent assay

Galanthus nivalis agglutinin (GNA; Sigma) at a concentration of $5\mu\text{g.mL}^{-1}$ was coated on each well overnight. After blocking with 5% milk/TBST, a 1:5 dilution of HCV E1/E2-transfected HEK 293T cell lysates was added (see cell lysis, section 3.4.4). Then, the procedure was completed as described in section 2.2.7.

2.2.9 PCR, primer designing and DNA sequencing analysis

Polymerase chain reaction (PCR) was performed using the standard primer gIIIs forward 5'-CTTTAGTGGTACCTTTC-3' and -96 reverse primer 5'-

CCCTCATAGTTAGCGTAACG-3' (Eurofins MWG Operon).⁵ PCR-products were diluted 1:10 and sent with 3.2pmol.μl⁻¹ of -96 primer for sequencing analysis by Source BioScience, Nottingham. Chromatogram files in 'abi' format were analysed by Chromas Software, Technelysium (Figure 2-5 & Figure 2-10).

2.2.10 Preparation of PCR Master Mix reaction and cycling parameter

PCR reactions were performed with the NEB Q5 polymerase. For each reaction, the master mix contained 5μl 5X NEB Q5[®] reaction buffer (NEB, #B90275), 0.5μM primer gIII_s (5'-CTTTAGTGGTACCTTTC-3'), 0.5μM, primer -96 (5'-CCCTCATAGTTAGCGTAACG-3'), 200μM of each dNTP, 0.02U DNA polymerase (NEB Q5[®], host start #M0493), 1μl of template (phage preparations, diluted 1:10—1:100) and 15.75μl nuclease-free water in a total reaction volume of 25μl. For thermal cycling an initial denaturation step of 98°C for 30 s was performed, followed by 39 cycles of: 98°C for 10 s; 53°C for 15 s; and 72°C for 10 s. A final extension step of 72°C for 1 minute was performed.

⁵ No band was seen using the PCR JumpStart[™] Kit (Sigma).

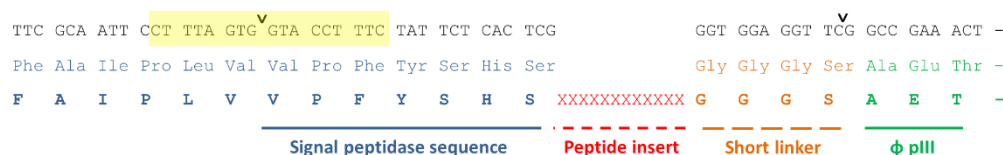


Figure 2-5 Phage restriction site

Signal peptidease (VPFYSHS) precedes the randomised sequence (in red). Acc65I restriction site (G^VGTACC) is located within the forward gIIIs primer binding site (yellow highlight), whereas EagI (C^VGGCCG) site starts at nucleotide 1631 in the glycine-rich linker site (brown).

The two primers (-96 and gIIIs) have already been designed to amplify peptides-encoding nucleotides within phage g3p. It was demonstrated that mutations in gene II was linked with fast propagation of M13 clones. To exclude the possibility of selecting phage due to faster propagation, the designed forward primer, (5'-TGTTGATGAAAGCTGGCTACA-3'; nucleotides 6629—6649), and the reverse primer, (5'-GGCCGGAGACAGTCAAATCACC-3'; nucleotides 6977—6998) (Nguyen et al., 2014), were used to amplify the Shine-Dalgarno (SD) sequence in the untranslated region (UTR) of phage gene II (nucleotides 6784—6821). The forward primer designing was performed according to version 4 of primer3web (Koressaar and Remm, 2007, Untergasser et al., 2012).

2.2.11 Multiple peptide alignment and SAROTUP database

Phage peptides were individually aligned with the natural ligand of the protein of interest using ClustalW server (<http://www.pedb.org/scripts/clustalw.php>),

followed by manual alignment. The peptide search tools SAROTUP (Scanner And Reporter Of Target-Unrelated Peptides) and MimoDB BLAST were used.

2.2.12 Statistical analyses

All statistical analyses and figures were generated using GraphPad Prism 6 Software. Depending on the result, paired t-test or One-Way Analysis of Variance (ANOVA) were used. The Bonferroni method was used to account for repeated measures from the same datasets. The threshold for significant differences between data sets was set to $p < 0.05$.

2.3 Results

A soluble construct of the HCV glycoprotein E2 (aa 410—644) was the first target bait to screen phage-displayed peptide libraries. Irrespective of successful enrichment from a peptide library, detailed analysis of the preliminary results would determine the quality of peptide sorting. Phage enrichment, binding assays and multiple peptide alignment were used as measures of successful enrichment from a peptide library.



Figure 2-6 PCR gel electrophoresis

Recombinant gene III PCR product shows a DNA size of approximately 181 bp. Two phage clones (lanes 2 and 6) contained no randomised nucleotide insert: wild-type M13KE gives a band of 132 bp. Eight out of ten phage clones possessed a recombinant insert and were selected for sequence analysis. DNA ladder in lane 1 is GeneRuler™ (Thermo Scientific, SM0333). Lane 11 is a negative control.

2.3.1 Enrichment ratio and interpretation

Performing affinity selection with the HCV E2 glycoprotein as a target bait, the selected phage were enumerated after each round of selection. Monitoring phage pool enrichment between rounds of peptide selection is the first indication of binding specificity and successful selection, but should not be the only indication. Clear enrichment of a phage pool binding to the immobilised HCV E2 proteins following iterative rounds of selection was observed (Figure 2-7). However, sequencing and binding analysis revealed that the observed enrichment was due to selected peptides that interact with the plastic surface

used to immobilise the target protein (Table 2-1 & Figure 2-11). The specificity of these interactions was determined using individual phage clones binding to the plastic plate in the presence or absence of the original target protein. Comparison of the peptide-encoding DNA sequences identified notorious consensus known to bind the plate substrate.

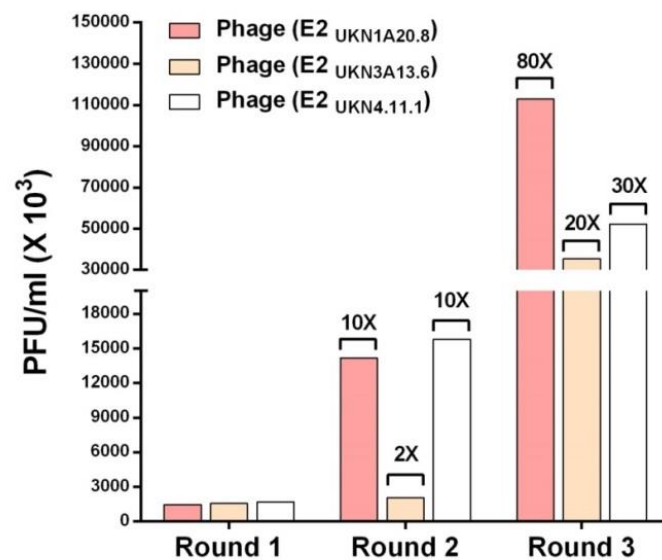


Figure 2-7 Titration of phage pools following iterative rounds of biopanning using HCV E2 (aa 410—644) as target

Using three different genotypes of HCV E2 glycoprotein, the level of enrichment was calculated. The figures above the bars are indicative fold enrichment in phage numbers relative to round one.

2.3.2 Titration of Anti-fd antibody to determine appropriate working concentration

Following peptide selection experiments, individual phage candidates were analysed using a previously reported ELISA binding assay using the original target protein from the biopanning experiment as bait. Optimising this assay helped distinguish specific signal from the noise inherent in the assay. To compare the binding of different peptides to E2, a primary antibody that interacts with phage particles (anti-fd antibody, Sigma, B7786) is usually used at a dilution of 1:1000, which gives a final working concentration of $3\mu\text{g.mL}^{-1}$. To optimise this critical method, a dose-response curve of anti-fd binding to immobilised M13 phage was performed. This showed that a dilution of 1:2000 gave a signal very similar to that of the antibody diluted 1:1000, without losing assay sensitivity. The dataset suggests using two-fold less primary antibody concentration than recommended by the manufacturer (Figure 2-8). A sharp decline in signal was observed between 10^8 and 10^9 phage particles. This demonstrated that using the primary antibody at 1:2000 ($\sim 1.5\mu\text{g.mL}^{-1}$) did not affect assay sensitivity. Another application of this experimental setup is in its ability to predict phage count using 1:2000 dilution tested against ten-fold coated-phage. The plate reader does not read any optical density more than 3.5, which limits interpretation of signals at high antibody concentration.

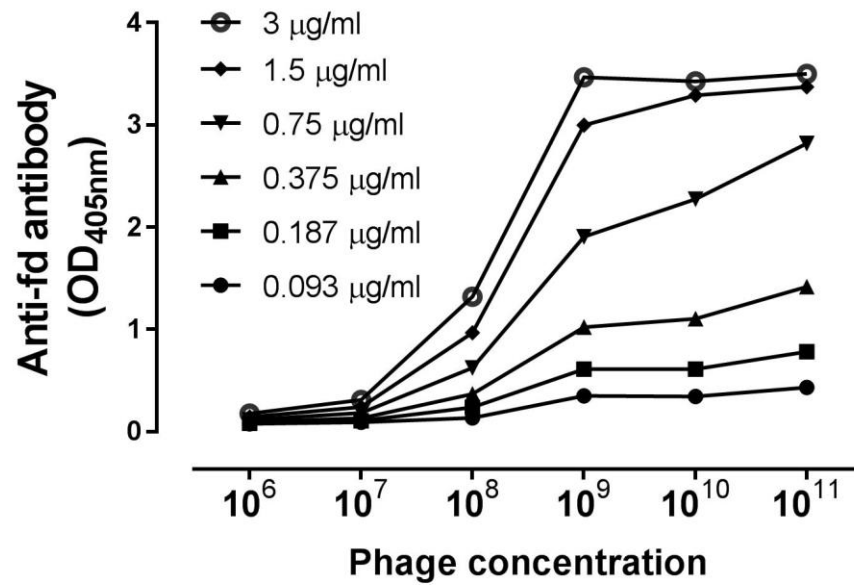


Figure 2-8 Phage ELISA performed with different concentration of Anti-fd antibody

In this experiment, a two-fold serial dilutions of anti-fd antibody was tested against phage coated at a microplate at six different concentrations. Starting at 1×10^{11} phage particles, six serial $\log_{10}$ dilutions were prepared. The absorbance at OD_{405nm} was read after 30 minutes.

2.3.3 Peptide sequences

Biopanning using the HCV E2 envelope glycoprotein representing three different genotypes was performed at three iterative rounds with each target. Sequencing of the enriched phage pools revealed a consensus sequence among the peptides selected with different targets. The selected sequences contained a high proportion of aromatic amino acids, especially tryptophan, with frequent histidine at the second position of the N-terminus (Table 2-1). This consensus is an indication of enrichment after 3 rounds of selection as in Figure 2-7. The specificity was investigated with multiple peptide alignment and ELISA under different conditions (Figure 2-11).

Table 2-1 Peptide sequences selected using conventional Tween 20-containing phage library affinity selection methods against three target proteins

Target Protein	Peptide Sequences and Frequency
HCV E2 (UKN1A20.8)	WHWNAWNWSSQQ (4X)
	WHFSWWKNTAVT (2X)
	WHWQWWLQTDAT (2X)
	YHTQTTPVLNL (1X)
	FHWTWQFPYTST (1X)
	QTADGMRRASLM (1X)
HCV E2 (UKN3A13.6)	LDTNKLLYTETS (1X)
	AYALYPTRDFYN (1X)
	IPIRASPVTHLA (1X)
	CHYWWCALYSLE (1X)
	QEFSQLNWLMS (1X)
	HETLQFALKDHM (1X)
	YHWPNNWLPSTA (1X)
HCV E2 (4.11.1)	WHWSFFASPLPA (4X)
	WHWNAWNWSSQQ (1X)

2.3.4 Sequencing analysis of gII UTR reveals no mutation or deletion

Sequence variation in the M13 gII is known to influence the rate of virus replication. As it was possible that selection of non-specific peptides with a fast propagation phenotype would bias the enriched phage pools, the sequences of gII in selected clones were investigated. Selected members of enriched phage pools were PCR-amplified (Figure 2-9) using already designed primers (section 2.2.9). Gene II untranslated region (UTR) of different phage selected against various target proteins throughout this project was sequenced. No mutations were observed in all the phage analysed.

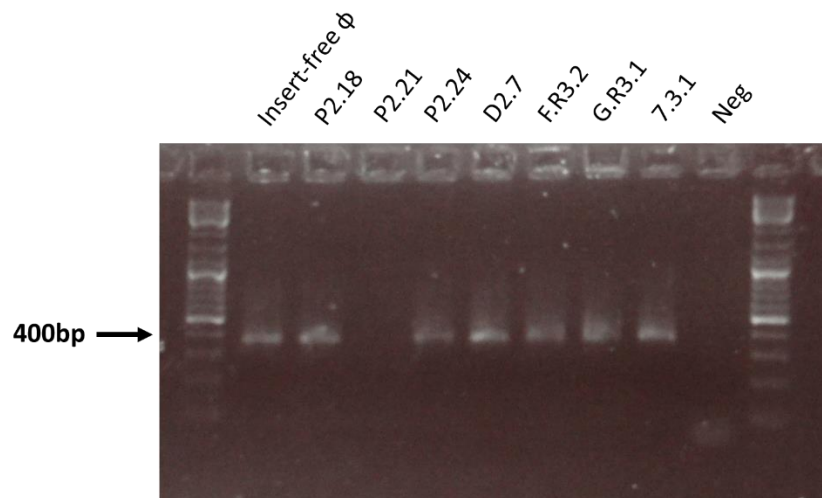


Figure 2-9 PCR gel electrophoresis with gII-specific primers

The forward primer and the reverse primer were used to amplify 370 nucleotides within phage gene II in which mutations between nucleotides 6784—6821 lead to fast propagation.

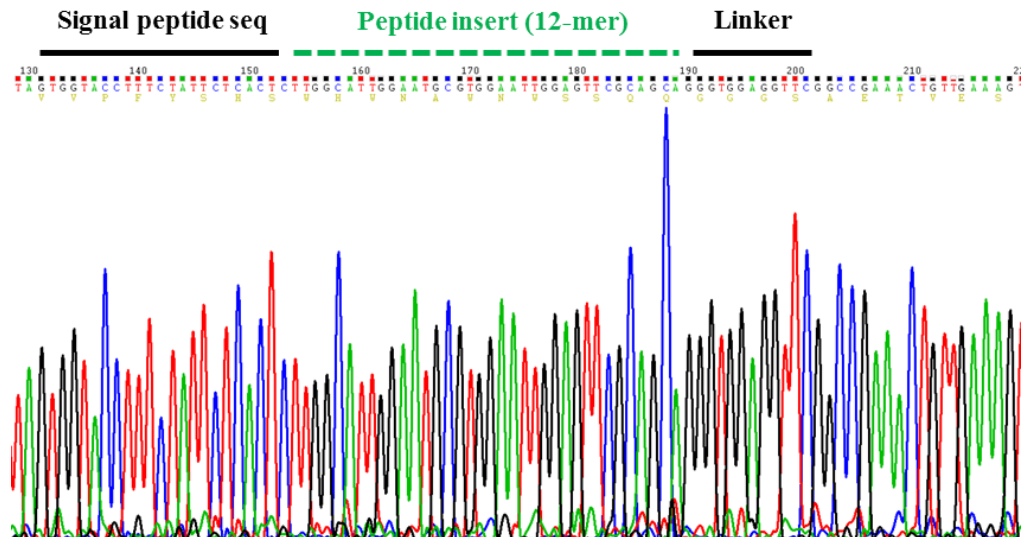


Figure 2-10 Example of chromatogram generated from sequence analysis of gIII recombinant inserts

The 36-nucleotide insert encoding a 12-mer peptide sequence follows the nucleotides encoding the signal peptide cleavage site (VPFYSHS), and preceded by a glycine-rich linker (GGGS). The mature N-terminus of phage pIII protein (AETVES...) follows this spacer.

2.3.5 Background noise is problematic in phage ELISA

Preliminary data showed that phage particles bind non-specifically to polystyrene surfaces. Selected phage members were amplified and tested against coated HCV envelope protein and against a blocked, uncoated plate. It was found that for all clones the background signal was equivalent to the signal in the presence of the target. As a consequence, further optimisation of the selection protocol was determined to be necessary.

2.3.6 Optimisation of phage binding assays for improving enrichment of selected peptides

Preliminary data suggested that reagents and assay conditions play significant roles in the non-specific phage binding. Understanding the binding characteristics governed by these conditions improves peptide selection. Also, a rapid method was developed to differentiate between polystyrene-specific peptides and other sequences.

Eight different binding protocols were used (Protocols A—H). Each protocol represented a specific combination of diluent and washing step. Protocol A used TBS as a diluent, whereas protocol B included Tween 20 in the buffer. Skimmed milk-TBS was used as a diluent in protocol C whereas milk-Tween was considered protocol D. TBS-Tween was used as a washing buffer for these four protocols. Protocols E—H used the same sequential diluents as A—D but with TBS buffer used in the washing step.

Higher signal using protocol D was only observed with plastic-binding phage that displayed ligands containing aromatic amino acids such as tryptophan, especially at the N-terminus (Figure 2-11). This signal was observed after 20—30 minutes of ELISA development. Wild type phage showed low signal with protocol E, but other peptides have higher absorbance using protocol E very noticeably. This implicated peptides as another factor along with Tween 20 in

the non-specific phage interaction to polystyrene substrates. The wild type signal in comparison with other phage-displayed peptides in Figure 2-14 matched this finding. Protocols D and E provided the best resolution between specific and non-specific binding to differentiate the binding of different peptide sequences.

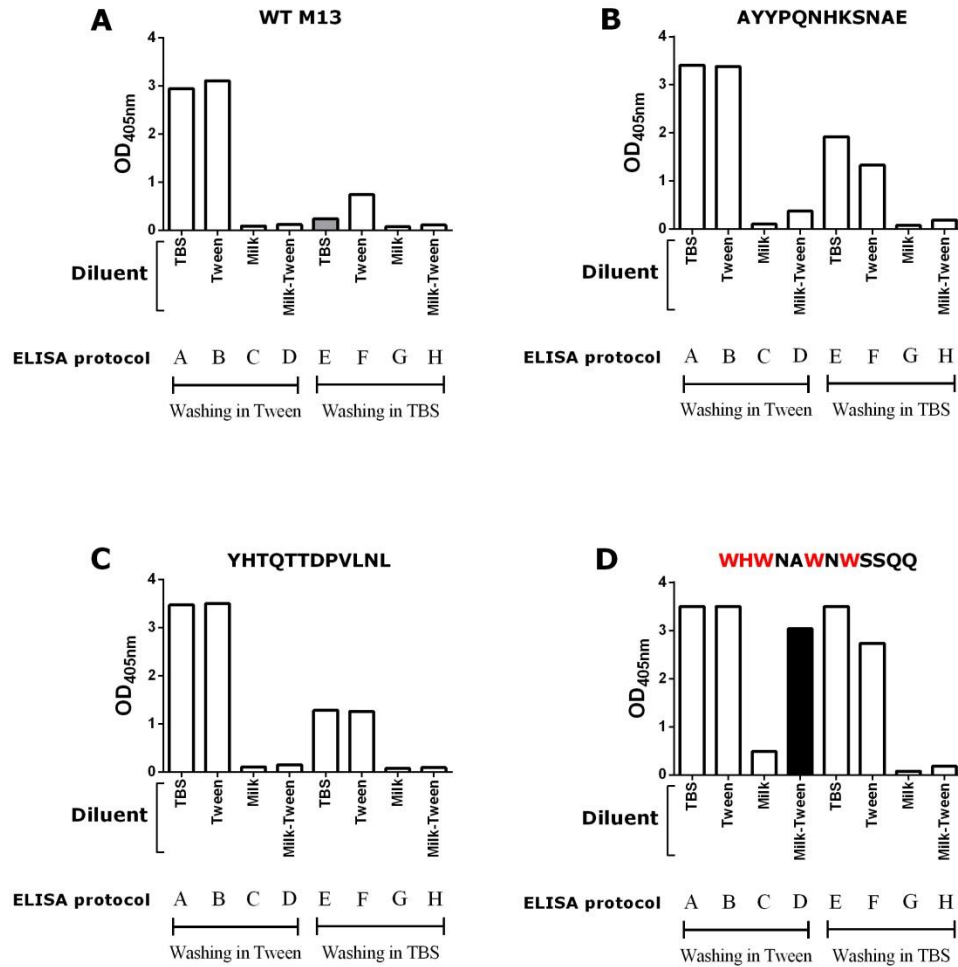


Figure 2-11 Different ELISA protocols used to optimise phage binding

A wild type phage with no displayed peptide (A), two phage-displayed peptides (B and C) and a plastic-binding phage clone that displays a WHW motif (D) were tested under eight distinct ELISA protocols designed based on the diluent used and the presence or absence of Tween 20 in the washing buffer.

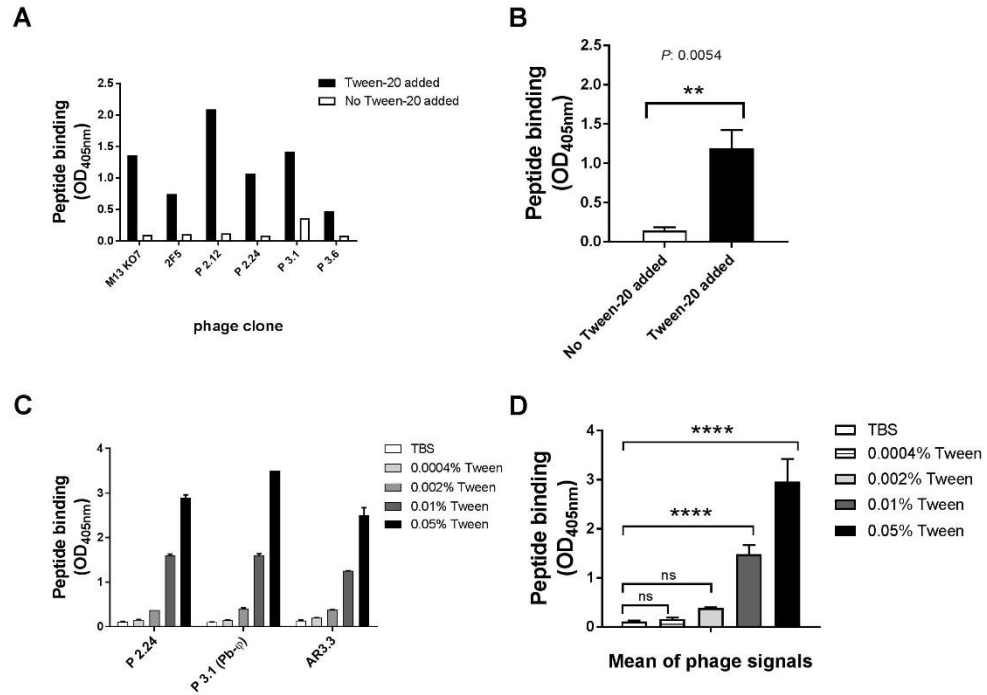


Figure 2-12 Dose-dependent effect of Tween 20

(A) Six different phage clones showed non-specific interaction when Tween 20 was used at 0.05% ($n=1$). (B) The same six phage clones were tested in an independent ELISA, and the mean of all optical densities generated in the presence (black bar) and absence (open bar) of Tween 20 was used to calculate the statistical significance ($n=6$). Comparison was made using paired t-test ($**p < 0.01$). (C) One phage clone (P2.24) from the first dataset in figure A was tested with two different phage clones to investigate the effect of different concentrations of the non-ionic detergent ($n=2$). (D) The mean of all optical densities of the three clones in figure C under each concentration was used to calculate the statistical significance ($n=2$). Comparison was made using one-way ANOVA ($****p < 0.0001$). Error bars represent $\pm$ SD of the mean.

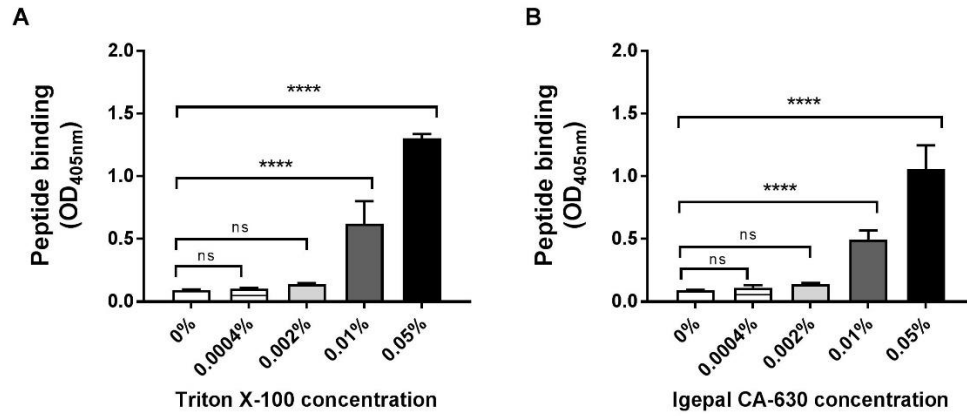


Figure 2-13 Dose-dependent effect of Triton X-100 and Igepal CA-630

Phage binding under Triton X-100 (A) and Igepal CA-630 detergents (B) was tested using five-fold serial dilution. Comparisons were made using one-way ANOVA (**** $p < 0.0001$). Error bars represent $\pm$ SD of the mean ($n=2$). NS: not significant.

With increasing concentrations of Triton X-100 and Igepal CA-630 detergents, an increase in phage binding was observed. It is obvious that non-ionic detergents cause non-specific binding to polystyrene surfaces. While the micellar behaviour of non-ionic detergents vary due to several factors (Horiuchi and Winter, 2015, Pal, 2015), the critical micelle concentration (CMC) of Tween 20 is 0.06mM (0.0074%), 0.08mM for Igepal CA-630 (0.005%) and 0.2—0.9mM for Triton X-100 (0.02%) at 20—25°C (Martinez-Sernandez et al., 2016). The highest non-ionic concentration used in ELISA was 0.05% (v/v). Despite that Tween 20 and Igepal CA-630 concentrations were used above their CMC, non-specific binding was observed with Triton X-100—that has a higher CMC value—at 0.01% which is under CMC (Figure 2-13).

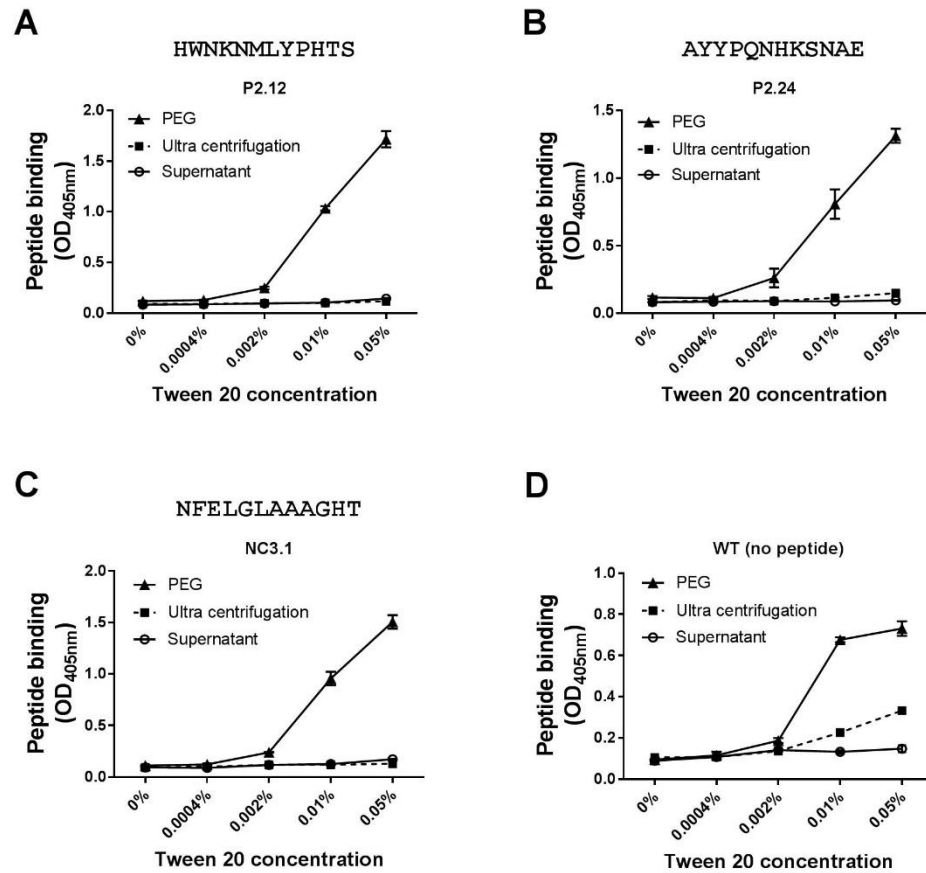


Figure 2-14 Comparison between ELISA signals using different phage precipitation methods

Phage clone P2.12 (A), P2.24 (B), a negative control phage (C) and a wild type phage (D) were tested using five-fold serial dilution of Tween 20. Comparison between ELISA signals obtained using bacteriophage prepared as un-purified supernatant (open circles), following ultracentrifugation (closed squares), or with PEG precipitation (closed triangles). Error bars represent $\pm$ SD of the mean (n=2). PEG: polyethylene glycol.

2.3.7 Non-ionic detergents enhance non-specific selection of WHW-containing peptides

Due to the high background binding, the impact of assay conditions was investigated. Strikingly, the data presented in Figure 2-11 can be interpreted that Tween 20 plays a significant role in phage non-specific binding to plastic surfaces. This finding was confirmed by performing an assay using different concentrations of detergent, revealing a dose-dependent relationship between detergent concentration and background signal (Figure 2-12).

2.3.8 PEG is associated with detergent-mediated non-specific binding

Non-ionic detergents contain ethylene oxide (C_2H_4O) units. Polyethylene glycol (PEG) contains the same chemical backbone. This polymer is routinely used to precipitate M13 phage particles during affinity selection. To assess whether or not PEG contributes to the non-specific phage interaction observed in the previous experiments, two precipitation methods were compared: a PEG-free ultracentrifugation and a PEG-precipitated phage method. Interestingly, the non-specific signal observed with phage precipitated using PEG was clearly evident, whereas a low background signal was achieved with phage prepared without PEG precipitation. This observation was confirmed using Tween 20 in a dose-dependent manner (Figure 2-14).

2.3.9 Effect of washing stringency in ELISA experiments

We next determined how stringent the washing step must be in an ELISA experiment, or more critically in peptide selection (biopanning), to successfully enrich phage-displayed peptides. A control phage was added to a blocked plate in the absence of target protein. After 12 washing steps, phage particles in each 200µl aliquot of washing buffer were counted. The effect of Tween 20 was tested by considering two conditions. Before performing this experiment, attempts were made to equalise phage titre in both conditions to a fixed concentration i.e. 1×10^{11} p.f.u. All phage dilutions (including the first phage titre) were counted in plaque assay (Figure 2-15). Despite that phage titre in the Tween 20 condition is twice that of the TBS condition, washing efficiency of both conditions were similar. The trend suggests that 4 to 5 times washes can reduce background significantly. This also depends on how much phage was firstly added.

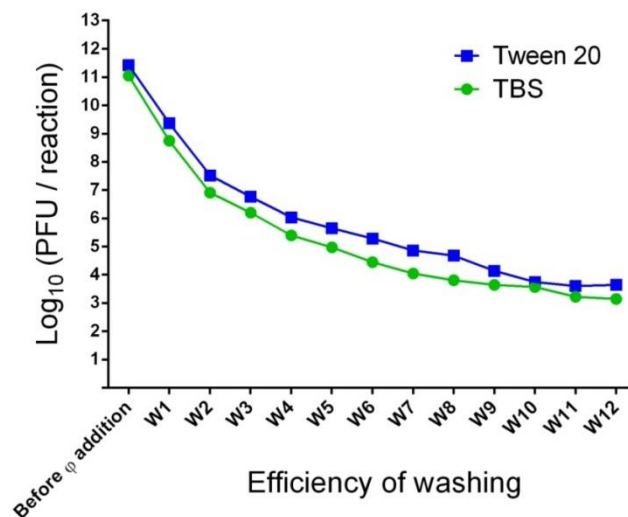


Figure 2-15 Effect of washing stringency with and without Tween 20

Phage number was counted in each washing step by plaque assay. Twelve wash cycles were considered (W1—W12).

2.3.10 Effect of washing conditions on phage non-specific binding

following chemical elution

In line with the previous experiment (Figure 2-15), a similar experiment was performed using a non-coated plate by counting eluted phage following acid elution after a number of washes (3 and 10 times). In this case, Tween 20 was implicated in non-specific phage binding (Figure 2-16), supporting our previous findings. This finding will inform the essential number of washes needed in a particular experiment at particular phage concentration.

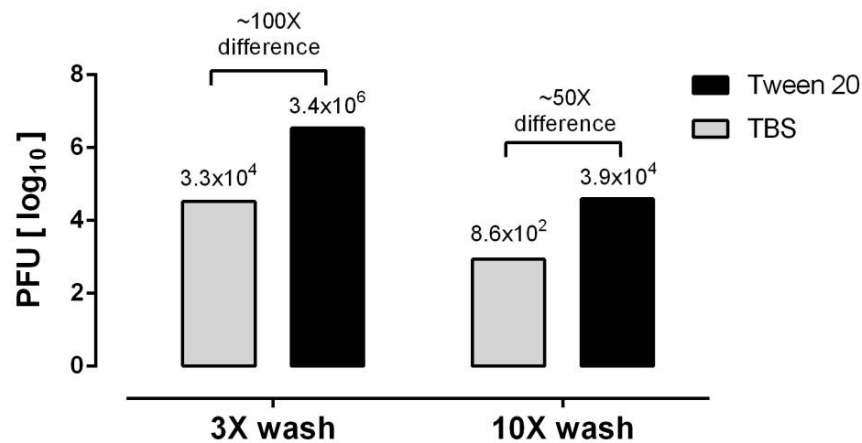


Figure 2-16 Effect of Tween 20 and washing stringency on phage non-specific binding following acid elution in a mock plate

Phage number was counted following 3 and 10 wash cycles, and the number of all phage clones recovered per eluate are shown above the bars. The starting phage concentration was 2×10^{11} . PFU: Plaque forming unit.

2.3.11 Effect of selection of blocking reagent on background binding

The primary aim of including a blocking reagent in an ELISA is to reduce non-specific binding of analytes (antibodies or antigens) to the substrate. However, some antigens have the potential to interact with the blocking buffer, causing undesirable background signals. Owing to this, three blocking buffers (skimmed milk, BSA and fish gelatin) were tested in phage binding assays under two conditions to test whether a specific combination of blocking and detergent would optimise the specific binding of phage-displayed peptides (Figure 2-17). Fish gelatin and BSA contribute to the highest background signal. Tween 20 had another added effect on the non-specific interaction. Skimmed milk was the optimum blocking reagent.

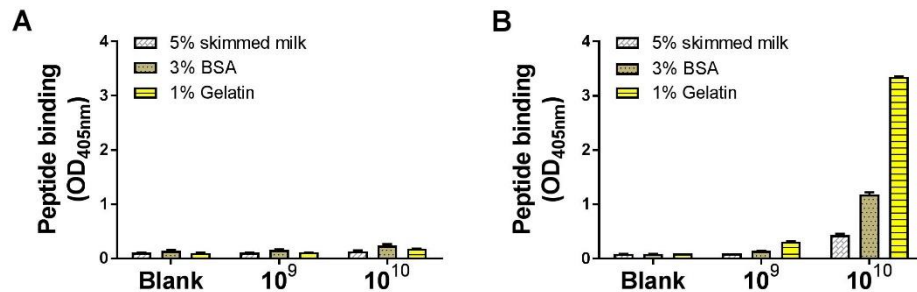


Figure 2-17 Effect of different blocking buffers and Tween 20 on non-specific binding of phage particles to polystyrene surfaces

A recombinant phage with the peptide sequence (YHTQTTPVLNL) was added into an empty plate at 10⁹ and 10¹⁰ concentration using three different blocking reagents without detergent (A) and with Tween 20 added in the wash buffer (B). Error bars represent $\pm$ SD of the mean (n=2).

2.4 Discussion and Conclusion

Understanding the binding characteristics of peptides presented on the surface of phage particles is important to optimising selection protocols. This enables selection of peptides in more precise and specific ways. With billions of diverse sequences, no single approach will be optimal for each application. Previously, the technique has been used as a platform for the discovery of novel peptide ligands. However, notorious and unrelated peptides have been published in numerous studies, undermining the technique usefulness. This has raised difficulties such as specific peptide selection failure. This has to be addressed as our first peptide selection (Table 2-1) ended in failure. As far as the specific selection approach is concerned, here I investigated phage binding characteristics under different assay conditions. These empirical improvements will, it is hoped, facilitate selection of binders with the desired affinity and specificity in each individual application.

Phage competition, infection rate and growth kinetics of different phage clones within the library have all been implicated with unsuccessful peptide selections (Derda et al., 2011). To minimise confounding effect, optimised protocols are crucial. There is the possibility the technique can succeed by implementing various optimisations.

SAROTUP is a free scanning application to help exclude previously identified motifs (Huang et al., 2010). MimoDB BLAST tool is another repository of phage peptides that have already been published. In 2010, the database collected

10,716 peptides from 571 publications. The second version in 2012 compiled 15,633 peptides (Ru et al., 2010, Huang et al., 2012). Biopanning data bank (BDB) is an update of these databases that contains more than 25,000 peptides (He et al., 2016). Selected peptides were blasted using these on-line tools to identify possible target-unrelated peptide (TUP) and to confirm that peptide candidates have not been selected by other research groups.

The phage harbouring the peptide SVSVGMKPSRP is one of the most notorious sequences selected from phage-displayed peptide libraries. This TUP was identified using enormous number of target proteins, antibodies and receptors (Huang et al., 2010, Huang et al., 2012) including HCV envelope glycoprotein E2 (Hong et al., 2010). Reproducing these findings remains largely questionable. Likewise, other TUPs were identified in our laboratory (Table 2-1). These sequences were compared with other published peptides such as WHWSFFASPLPA (Cao et al., 2012), WHWNAWNWSSQQ (Sookrung et al., 2011, Stewart et al., 2012, Gutes et al., 2013), and FHWTWQFPYTST (Stewart et al., 2012). To address this difficulty, different conditions and buffers were subjected to detailed comparisons. This included the blocking reagents, washing buffers and elution methodologies.

The selection of appropriate buffers was demonstrated to have great influence on the success of biopanning experiments (Figure 2-11). Strikingly, a link between non-specific binding of phage to polystyrene surfaces and non-ionic detergents including Tween 20, Triton X-100 and Igepal CA-630 was found

(Figure 2-13). They enhance selection of peptides containing aromatic amino acids (Table 2-1 & Figure 2-11). Understanding the mechanism in which Tween 20 facilitates this unequivocal background noise remains to be investigated. With the strong link found between non-ionic detergents (that contain ethylene oxide as does polyethylene glycol) and phage background binding (Figure 2-12), Figure 2-14 implicates PEG (used for phage precipitation) in phage non-specific binding. In an empty ELISA plate coated with skimmed milk, high signals were observed when using PEG-precipitated phage. The comparison was made using bacteriophage prepared as un-purified supernatants following centrifugation of bacterial culture and another phage preparation under high PEG-free ultracentrifugation. Despite the similar trend observed with the wild type phage, higher signals in the other preparations implicate peptides in the background noise. Phage concentration in every preparation is another factor that speeds up the absorbance during colour development.

In relation to the critical micelle concentration (CMC), Tween 20 and Igepal CA-630 non-ionic detergents were used above their CMC value (0.0074% and 0.005% respectively). However, when Triton X-100 non-ionic detergent was used below its CMC threshold, non-specific phage binding was still observed, showing a statistically significant result at 0.01% (Figure 2-13). This suggests that the existence of micelles does not cause the non-specific binding. The total number of ethylene oxide repeating units $C_{14}H_{22}O$ $(C_2H_4O)_n$ in Tween 20 ($n=20$) is twice that of Triton X-100 ($n=10$). Likewise, the optical density observed in the presence of Tween 20 is twice that of Triton X-100 at the same conditions and concentrations (Figure 2-12 & Figure 2-13). The number of ethylene oxide

repeating units in both detergents justifies the non-specific interaction than the micellisation interpretation.

Resolving the background noise, while difficult, is still possible. Conventional phage ELISA protocol proves otherwise and did not resolve the non-specific phage binding. In phage library screening, failure to identify specific ligands is a serious setback. Therefore, optimising the technique is crucial. Of all the conditions concerning non-specificity, Tween 20 may be considered the most important factor that influences selection of unwanted peptide sequences. The non-specific binding in the absence of detergents is negligible in phage binding compared to assays using non-ionic detergents. However, when blocking buffer is used as a phage diluent, binding of undesirable phage-displayed peptides is minimised, even in the presence of Tween 20. In selection experiments, billions of variants are used with limited numbers of each sequence. In this case, using blocking buffer as a diluent might absorb some of the peptides before incubation with the target. Therefore, a detergent-free TBS is recommended in future phage biopanning experiments. This effect is more pronounced in ELISA assays using bacteriophage precipitated with PEG, which is chemically analogous to the detergent, and appears to interact with Tween 20 in these assays. For this reason, Tween 20 can be used in ELISA experiments with phage particles mixed with the blocking buffer, which minimises background binding effects.

Blocking reagents are widely described to bind to polystyrene surfaces, occupying regions of the assay substrate and competing for binding with

polystyrene-specific phage-displayed peptides. This was observed with the sequence FKFPLYEHVIRG (Feng et al., 2009) and other plastic-binding peptides such as HWGMWSY and WHWRLPS (Vodnik et al., 2012).

It has been found that different preparations of commercial bovine serum albumin (BSA) work differently as a blocking agent, affecting the final result. Because of these differences, and as blocking buffers may not all be alike, three reagents were examined. Figure 2-17 shows that blocking agents (such as skimmed milk, BSA and fish gelatin) can contribute to non-specific phage binding, especially fish gelatin. BSA-related noise has previously been reported (Xiao and Isaacs, 2012). Taking results of Figure 2-17 into consideration, skimmed milk was a better blocking reagent in phage binding experiments. Tween 20 has long been used as an important detergent in reducing non-specific binding of various analytes (Kim et al., 2003, Buchwalow et al., 2011), and this advantage should not be underestimated.

Genetic differences in phage libraries have been demonstrated to affect M13 replication. When we assessed phage for propagation advantage (section 2.3.4), no deletion or mutation in gII was found in any of the selected phage (Figure 2-9). However, and given the diverse random sequences within the library, peptide structure can affect the rate of phage amplification (Derda et al., 2011). This limitation is inherent to the library approach, and there is no way of mitigating the impact on amplification of phage-displayed peptides.

Rambert and colleagues claimed that FHENWPS peptide motif targets CD23 and may decrease inflammatory markers in patients suffering from arthritis by preventing the activation of macrophages (Rambert et al., 2009), whereas another study has shown that this motif was the most frequent peptide binding to the control BSA (Pulicherla and Asokan, 2011). Sequences such as FHW/YHW at the N-terminus and YPQN/YPQ are sometimes selected by other researchers using various targets, and some peptides with these suspicious motifs can specifically bind the target bait such as the motif YHWYGYTPQNVI selected against epidermal growth factor receptor (Li et al., 2005), and VYPQNKHHTWYS and its specific binding to 3/11 antibody (Tarr et al., 2006). The presence of these repetitive motifs does not necessarily indicate unrelated peptide, but once a consensus reveals these motifs, careful investigation and characterisation should be taken into consideration before reaching a conclusion.

Phage-displayed peptides are not limited only to discovery of ligands for biological targets, but can identify ligands against inorganic surfaces (Nam et al., 2008, Oh et al., 2014). Given the high affinity of plastic-binding phage, it should be made clear that polystyrene specific-peptides, while not desirable in peptide sorting and phage binding ELISAs, have the potential of being useful in other applications. For instance, the polystyrene-specific ligand FKFPLYEHVIRG was successful in anti-HIV ELISA by fusing it genetically with a recombinant envelope, and proved sensitive and specific in diagnostic assays (Feng et al., 2009). Another important application of polystyrene binding

peptides comes with polyethylene glycol to form amphiphilic macromolecules. These amphiphiles were able to prevent mammalian and bacterial cell adhesion (biofilm formation), making cytophobic (cell-repellent) coatings. Bacterial colonisations of medical devices are not uncommon, and this application provides incentive for further pharmaceutical and medical applications (Kenan et al., 2006).

Published optimised phage protocols are very scant. Starting with billions of diverse sequences and ending up with few affinity enriched peptide candidates, the technique has to be optimised and performed accurately. Various conditions were examined to understand the enigma of non-specific binding and to establish a standardised phage binding assay. The modified bacterial elution described in Figure 2-3 might allow selection of specific and acid-resistant phage with moderate or high binding advantage to the coated protein bait. Having optimised the peptide library selection protocols, the next three Chapters aimed at contextualising the general protocols developed of this Chapter and test the feasibility of applying these optimised protocols.

3 Peptide Selection using Hepatitis C Virus Envelope Glycoproteins

3.1 Hepatitis C Virus Biology, Epidemiology and Diagnosis

Hepatitis C Virus (HCV) is one of the major causes of chronic liver disease that affects roughly 3% of the world's population (Messina et al., 2015). Aside from liver diseases, a growing evidence implicates HCV in neuropsychiatric disorders mediated by the release of cryoglobulins, inflammatory cytokines and autoantibodies that affect the nervous system (Mathew et al., 2016). More than 170 million individuals are chronically infected with HCV (Table 3-1). There are challenges in assessing virus geographic distribution that limit its control and prevention (Shepard et al., 2005). Diagnosing HCV at an early stage proves difficult due to subclinical (asymptomatic) infections.

The virus is an extreme example of genetic variability (Tarr et al., 2015), circulating as, at least, 6 antigenically distinct genotypes divided into 66 subtypes (Hedskog et al., 2015), which allows it to escape immune response and become resistant to neutralising antibodies (Bankwitz et al., 2010). Therefore, viruses exist as diverse variants known as quasispecies. The most widespread HCV is genotype 1b that showed severe clinical outcomes and poor response to treatment (Cheng et al., 2015).

Adaptive and innate immunity contribute to the protective immune response in HCV infection. The humoral innate immune response includes production of defensins, complement components and pentraxins. L and H ficolins, as well as mannose binding lectin, are synthesised by the liver as defence collagens and contribute to neutralisation of HCV infection (Tarr et al., 2012a). HCV uses

evasion mechanisms that allow persistent infection by interfering with the immune response (Chang, 1998). Mutations can cause glycan shifting on the HCV E2 glycoprotein, allow the virus to escape neutralising antibodies (Pantua et al., 2013) and impair T cell recognition (Burke and Cox, 2010). Developing novel drugs that target highly-conserved regions of HCV proteins remains a high priority, although great progress has recently been made with clinical administration of directly-acting anti-viral compounds (DAAs).

Table 3-1 The scale of HCV pandemic (Mohd Hanafiah et al., 2013)

Countries and estimated total population	Individuals with seropositivity
Asia (>3525 million)	>113.9 million (3.2%)
Asia Pacific (>180 million)	>2.4 million (1.3%)
Australasia (>24 million)	>0.6 million (2.5%)
Caribbean (>42 million)	>0.7 million (1.6%)
Europe (>740 million)	>19.1 million (2.6%)
Latin America (>517 million)	>7.6 million (1.5%)
Middle East and North Africa (>420 million)	>15 million (3.6%)
North America (>337 million)	>4.4 million (1.3%)
Oceania (>8 million)	>0.2 million (2.5%)
Sub-Saharan Africa (>775 million)	>17.8 million (2.3%)

3.1.1 Envelope glycoproteins

HCV E1 envelope glycoprotein is believed to be involved in the membrane-virus intracytoplasmic fusion (Idrees and Ashfaq, 2013), and interacts physically with E2 to bind and enter the target cell (Li et al., 2009). HCV E2 is a better studied envelope glycoprotein and has been targeted for therapeutic

interventions. E2 comprises a receptor-binding domain (residues 384—661), and the compact core has β -strands, random coils and two small α -helices (Kong et al., 2013, Khan et al., 2014). It binds to the cell surface through scavenger receptor class B type I and CD81. The variable regions of E2 play a significant role in virus assembly and infectivity (McCaffrey et al., 2011).

A conserved region, crucial for virus entry, is the amino acids 412 to 423. This linear epitope is recognised by several neutralising antibodies. Other monoclonal antibodies were generated to bind a cyclic peptide containing amino acids 412—422 with a high degree of selectivity. Their inability to interact with a more flexible conformation explains the role of viral mechanisms in neutralisation escape (Sandomenico et al., 2016). It has been found that non-neutralising antibodies against epitope II of E2 protein (aa 434—446) disrupt the neutralisation of antibodies at epitope I (aa 412—426). This leads to the hypothesis that neutralising antibodies is restrained due to interfering antibodies responsible for the persistence of HCV *in vivo* (Zhang et al., 2009). X-ray crystallographic analysis of epitope II showed that its structure exists in distinct conformations. The structural transition is mediated by the flexibility of a highly conserved glycine residue. The tertiary structure of epitope II might determine the neutralisation potential of antibodies. HCV can present a specific epitope II conformation to avoid neutralisation (Deng et al., 2014).

Immunising animals with E2 was sufficient to induce antibody response capable of neutralising diverse glycoproteins in cell culture system, but serum

lipoprotein reduced neutralisation. Therefore, recombinant envelope glycoproteins became a candidate for prophylactic vaccines (Stamatakis et al., 2007). Following animal models, the first human clinical trial using well-tolerated and safe adjuvanted E1E2 glycoproteins was conducted, with detectable serum antibodies. Subjects developed HCV-neutralising antibodies with lymphocyte proliferation response to the envelope glycoproteins (Frey et al., 2010, Wong et al., 2014).

3.1.2 HCV receptors, entry and replication

Host receptors that mediate viral attachment are CD81, scavenger receptor class B type I (SR-BI), claudin-1 (CLDN-1), occludin (OCLN), heparan sulfate (HS), low density lipoprotein receptor (LDLr) and lectins (Bartosch and Cosset, 2006, von Hahn and Rice, 2008, Lupberger et al., 2012). However, other candidates have been implicated such as Niemann-Pick C1-like 1 (NPC1L1) and CD5 (Lupberger et al., 2012, Samreen et al., 2012).

Great progress has been made towards understanding the entry mechanism of HCV. Several steps are involved in HCV entry. Firstly, the virus interacts with the indispensable cell surface host receptor (SR-BI) that enables its interaction with CD81 via E2 envelope glycoprotein. Then, the virus is guided to the tight junction (TJ) protein (CLDN-1) that interacts significantly with CD81 followed by a later entry step via OCLN. Members of the tyrosine kinase family mediate this process. Then, the virus is internalised to the host cell via clathrin-mediated endocytosis. The membrane of an early endosome maintains virions at low pH

until fusion. An unusual feature of HCV is its association with lipoproteins that relate the virus with lipid metabolism. There are nearly 300 copies of apolipoprotein E (apoE) per HCV virion that play a role in modulating virus entry and its assembly (Ogden and Tang, 2015).

HCV is believed to be fused by a cholesterol precursor localised in the bile canaliculi (NPC1L1) (Betters and Yu, 2010). Serum response factor binding protein 1 (SRFBP1) seems to be implicated in HCV life cycle in a post-fusion step (Feneant and Cocquerel, 2015). After uncoating, the viral RNA is released into the cytosol and translated at the rough endoplasmic reticulum. Host signal peptidase generates the viral structural proteins by cleaving the polyprotein. The remainder are processed by HCV cysteine protease (NS2-3) and NS3-4A complex. HCV assembly starts by accumulating viral RNA and core protein. The viral envelope protein is obtained by budding through the endoplasmic reticulum membrane (Bartenschlager et al., 2013).

3.1.3 Liver diseases

HCV infection is associated with liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC); the third cause of cancer deaths (Testino et al., 2016). Numerous studies were conducted to understand HCV tropism. Approximately, 60—80% of infected individuals develop chronic hepatitis determined by persistent viraemia (presence of HCV RNA in the blood) and abnormal level of serum enzymes. The rest clear the virus spontaneously following incubation period. One-third of chronically infected patients progress to cirrhosis and HCC

(Larrubia et al., 2008), and less than 20% of HCC occur in non-cirrhotic liver (Alkofer et al., 2011). The prevalence and incidence of liver disease is increasing, and cirrhosis-associated complications correlate highly with death. Earlier identification of individuals at high risk of liver diseases could be one of the major advances in the next decade (Fitz, 2016).

HCV genotypes and immune-mediated processes and apoptosis may play a role in the mechanism by which liver is damaged, along with changes in gene expression pattern. A strong cytopathic effect of Vero cells was observed with genotype 1b. The release of cytokines induces fibrotic responses and cause death of hepatocytes (Silberstein et al., 2016).

Distinct pathological manifestations such as inflammation, hepatic reactivation, liver steatosis and progression to liver fibrosis were linked with specific HCV genotypes (Rumi et al., 2005, Leandro et al., 2006). It has been found that HCV-related hepatocarcinogenesis is indirectly caused by elevated reactive oxygen species (Medvedev et al., 2016). Despite that oxidative stress is a by-product of many cellular processes, its increase is a biomarker for DNA damage.

Direct oncogenic effect of HCV proteins in inducing cell transformation has been suggested. Carcinogenesis might arise due to virus-mediated apoptosis, deregulation of host cell cycle, death of hepatocytes, compensatory proliferation and reinfection (Lemon and McGivern, 2012). Expression studies of HCV Core

have provided insight into the role of this structural protein in disease progression, lipid synthesis and cellular transformation. When Core protein was expressed in transgenic mice, they developed hepatic steatosis (Kao et al., 2016). However, and due to methodological limitations, the exact mechanisms by which HCV causes hepatocarcinogenesis are not fully understood (Sukowati et al., 2016).

All of these liver diseases with the incidence of HCV-induced cirrhosis and the rise in hepatocellular carcinoma ultimately necessitate liver transplantation. Unfortunately, infection of the engrafted liver is common. Up to 30% of patients ended up with cirrhosis within 5 years of transplantation. It was shown that entry inhibitors, complemented with direct-acting anti-virals (DAAs), prevent liver graft infection in animal models and delay it in clinical trials. This provides a potential synergistic use of entry inhibitors and DAAs in preventing graft infection (Felmlee et al., 2016).

3.1.4 Diagnosis of HCV

In the blood bank setting, a sensitive and specific assay is required for diagnosis of HCV. The first ELISA (enzyme-linked immunosorbent assay) generation included a purified and recombinant C100-3 (NS4)-coated wells, with part of NS4 named as 5-1-1 encompassed in RIBA (recombinant immunoblot assay). Due to the high percentage of false positivity associated with ELISA-1, a second generation ELISA was developed (Figure 3-1). It involved the recombinant core c-22-3, c33c (NS3), c100-3 (NS4) and 5-1-1 (Chien et al., 1992). A third

generation (ELISA-3) was introduced in Europe that included c-200 (c100-3 and c33c) and NS5 recombinant antigen (Nardiello et al., 1996). A fourth generation combined antibody-antigen assay (Monolisa HCV Ag-Ab Ultra; Bio-Rad Laboratories) was introduced to improve diagnosis (Schnuriger et al., 2006). To detect HCV RNA, nucleic acid amplification technology (NAT) is the most sensitive molecular assay (Marwaha and Sachdev, 2014).

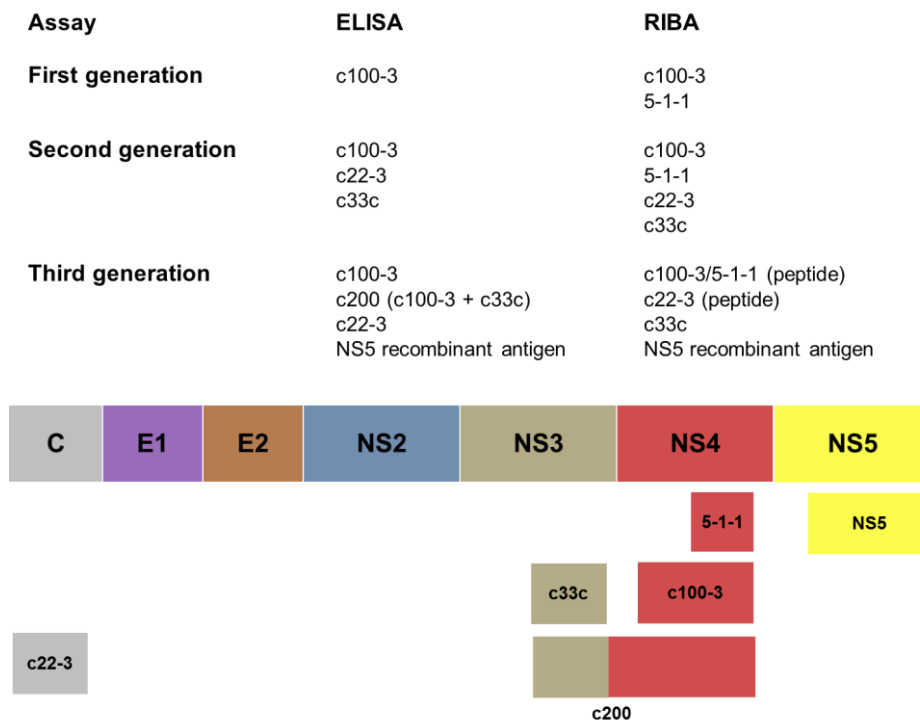


Figure 3-1 Comparison between ELISA and RIBA in HCV diagnosis. Adapted from (Al Dhahry et al., 2003)

ELISA and RIBA were developed over three generations by including recombinant antigens to enhance specificity. The coloured blocks are representing the genome of the virus.

C: Core

E: Envelope

ELISA: Enzyme-linked immunosorbent assay

NS: Non-structural

RIBA: Recombinant immunoblot assay

In addition, gamma-glutamyl transferase (GGT) and alanine aminotransferase (ALT) are initial markers for the diagnosis and follow-up of chronically-infected patients. GGT and ALT are predictable markers of HCV-related liver damage and viral hepatitis (George et al., 2009, Everhart and Wright, 2013).

3.2 HCV Treatments

As far as HCV treatment is concerned, three categories of cell culture systems helped developing the current HCV drugs. Models of virus replication steps include replicons (the first culture system), the HCVpp (pseudoparticle) technology and the HCVcc (cell-cultured derived) system which involves the complete HCV replication cycle (Bartenschlager et al., 2013). Every step of the viral life cycle becomes a potential target for developed anti-HCV agents.

Pegylated interferon and ribavirin (peg-IFN and RBV) combination was the mainstay of HCV treatment. Sustained virological response (SVR) could be achieved in 50% of genotype 1 infected patients and up to 80% with genotypes 2 and 3 (Zeuzem, 2008). However, and due to adverse events, researchers were developing other drug candidates to achieve pangenotypic active drugs with little risk for resistance and improved SVR.

IFN- α does not act directly on HCV replication, but seems to affect the translation of HCV proteins. Its potent activity is mediated by establishing a non-

virus-specific antiviral activity within the cell by inducing IFN-stimulated genes (ISGs) upon binding to IFN cell-surface-receptor subunits. This leads to receptor associated tyrosine kinase 2 (Tyk2) and Janus-activated kinase 1 (Jak1). The signal transducer and activator of transcription proteins 1 and 2 (STAT1 and STAT2) get phosphorylated by the activated kinases. Then, STAT1/2 complex is translocated to the nucleus and combines with IFN-stimulated response elements, leading to multiple ISGs expression. Moreover, a correlation was found between NS5A amino acid mutations and sensitivity to IFN- α (Enomoto et al., 1996).

Unlike IFN, RBV has a small direct effect on HCV-RNA replication. This guanosine analogue is phosphorylated inside hepatocytes to form ribavirin monophosphate (RMP), diphosphate (RDP) and triphosphate (RTP). Early chain termination followed by inhibition of replication is mediated via the misincorporation of RTP by RNA polymerases (Feld and Hoofnagle, 2005).

Lead inhibitory peptide sequences of HCV NS3-4A were developed as a result of optimisation series from combinatorial phagemid display to protein crystallography and other modifications. Following this peptide optimisation, the cell-penetrating peptides (CPPs) showed an interesting intracellular delivery and inhibited viral replication in a cellular model, albeit with high inhibitory concentration (36 μ M) compared to *in vitro* inhibition i.e. IC₅₀ of 23nM (Kugler et al., 2012).

3.2.1 Vaccine design

In animal models, broadly neutralising antibodies that protect against HCV quasispecies could be a prophylactic vaccine (Law et al., 2008). Other studies demonstrated the significance of recombinant glycoprotein vaccination (Ray et al., 2010, Stamatakis et al., 2011), the cross-reactivity of vaccine-induced T cells (Kelly et al., 2015) and the advantages of DNA vaccination (Naderi et al., 2014). However, no vaccine has been approved so far due to virus diversity, viral escape strategies and blockade of innate immunity signalling (Park and Rehmann, 2014).

3.2.2 Entry inhibitors

No HCV entry inhibitor has been brought to market so far. Peptide leads in this study (Chapter 3) were not used at high concentration to determine their IC_{50} . In future investigations, peptide synthesis considering the same GGGS linker at high concentration might show the IC_{50} if peptides adopt the same conformation of pIII-fused peptides.

Interestingly, aspirin downregulates claudin-1 via the proteosomal degradation pathway and inhibits HCV entry in both pseudoparticles system and infectious HCV. But it requires 4mM to show an IC_{50} using the *Firefly* luciferase assay (Yin and Zhang, 2016). Other HCV entry inhibitors include anti-transferrin receptor 1 antibodies (Martin and Uprichard, 2013), the antimalarial ferroquine

(Vausselin et al., 2013) and the kinase-inhibitor sorafenib (Descamps et al., 2015). Antibodies produced against the tight junction protein, claudin-1 (CLDN-1), neutralise HCV entry by inhibiting E2-CD81-CLDN association, and blocked entry of highly infectious and resistant variants (Fofana et al., 2010, Krieger et al., 2010).

LJ001 small molecule is a broad-spectrum antiviral, effective against enveloped viruses including Influenza A, Nipah, Rift Valley fever, Yellow fever, West Nile and HCV. It specifically inhibits virus-cell fusion by a lipid-based mechanism with no overt toxicity (Wolf et al., 2010).

Flavonoid derivatives are broadly active inhibitors that might be used in combination therapy. A library screening of plant-derived extracts allowed the isolation of a novel flavone capable of inhibiting entry of all HCV genotypes in a post-attachment entry step. The synthetic molecule, ladanein (BJ486K), was also effective against a variant resistant to entry inhibitors and showed a synergistic effect with cyclosporine A with enhanced antiviral potency (Haid et al., 2012).

Trachelogenin, a plant-derived lignan, showed an inhibitory effect on HCV entry through interfering with E2 and CD81 with low toxicity. It exhibited synergistic effects with interferon or telaprevir. With the cell-to-cell diminishing

effect it offers, trachelogenin is a candidate for future combination therapies (Qian et al., 2016a).

Understanding HCV entry mechanism helps finding novel inhibitors. Some viruses take advantage of cellular uptake pathways as a route of internalisation. As mentioned in section 3.1.2, HCV is internalised to the host cell via clathrin-mediated endocytosis in a low-pH dependent manner. Bafilomycin A1 neutralises the pH, leading to impaired endocytosis. Therefore, this lysosomotropic agent blocks entry of HCVpp by inhibiting endosomal acidification (Meertens et al., 2006).

3.2.3 Direct-acting anti-virals

Understanding HCV-host interaction with the advent of cell culture gave scientists insights on HCV replication that lead to the development of direct-acting anti-virals (DAAs); one of the most spectacular medical success stories. The new agents replaced interferon with cure rates of more than 90% (Gotte and Feld, 2016). There are four categories of DAAs: (i) NS3/4A inhibitors; (ii) NS5A protein inhibitors; (iii) NS5B (nucleoside-type) polymerase inhibitors; (iv) NS5B (non-nucleoside-type) polymerase inhibitors. Current DAAs for treatment of HCV are summarised in Figure 3-2. Other NS3/4A inhibitors are faldaprevir (BI-201335), vedoprevir (GS-9451), narlaprevir (SCH 900518), danoprevir, vaniprevir, sofosbuvir and deltaprevir. AZD-7295 and GSK 2336805 are other NS5A inhibitors (De Clercq, 2014). SVR has significantly increased with the introduction of various DAAs. However, selection of HCV

drug resistance in non-responders and significant increases in cost are public health implications of the widespread use of DAAs that will only be affordable by wealthy societies (Soriano et al., 2013). The WHO elimination target proposes 80% reduction incidence by 2030, but reducing HCV global incidence should be done in a cost-effective measure (Scott et al., 2016).

A remarkable SVR was observed with sofosbuvir. Patients infected with HCV genotypes 1 or 4 had achieved a rate of 90% SVR following 12 weeks of treatment and 67% with genotypes 2 or 3 (Lawitz et al., 2013). A fixed dose of ledipasvir-sofosbuvir has been approved with a high SVR rate in a single-tablet combination, well-tolerated and easy to administer, but with lower efficacy to genotype 3 compared to HCV-1 (Nkuize et al., 2016).

A renal allograft recipient with significant weight loss, whose HCV load remained high, received a new combination therapy of sofosbuvir and ledipasvir. In this case report, the patient presented undetectable viral load and his raised liver enzymes were normalised within 3 weeks with normal kidney function after 16 weeks and sustained HCV remission (Jha et al., 2016).

Another pangenotypic inhibitor is velpatasvir (GS-5816); a novel second-generation NS5A inhibitor. However, resistant-associated substitutions were present after a period of 48-week follow-up. M28, L31 and Y93 were the most prevalent substitutions (Lawitz et al., 2016).

High rates of SVR were produced in patients with recurrent genotype 1 under telaprevir-based triple therapy following liver transplantation (Forns et al., 2016). Conversely, this was a complex treatment option in a long-term follow-up analysis of telaprevir-based triple therapy in infected liver graft recipients (Klein et al., 2016).

Simeprevir (TMC435) was developed for the treatment of HCV genotype 1 and 4. The SVR was improved in combination with peg-IFN and RBV. The safety and efficacy of simeprevir in combination with peg-IFN and RBV was examined to manage recurrent hepatitis c in liver transplants recipients in a pilot study of 12 weeks. No HCV-RNA was detected in 3 patients out of 5. The drug was somewhat effective, but further studies are needed to reach a robust conclusion (Tanaka et al., 2015).

In a double-blind study, groups of patients received peg-IFN and RBV with and without boceprevir. A high SVR rates were achieved with the group received boceprevir (Conteduca et al., 2014), suggesting that combination therapy with the new DAAs is more effective.

A rapid suppression of HCV RNA was associated with grazoprevir, a second generation NS3/4A inhibitor, in combination with RBV which was evaluated on patients infected with genotype 1 (Gane et al., 2016). Another combination therapy with elbasvir is under development for the treatment of genotypes 1 and

4. Elbasvir/grazoprevir proved effective regimen with favourable safety profile. This could lead to the first global approval for the treatment of HCV genotypes 1 and 4 (Carrion and Martin, 2016, Keating, 2016).

Eight cirrhotic patients with chronic HCV genotype 1b were enrolled in a retrospective study to evaluate the efficacy of daclatasvir and asunaprevir combination therapy. Eighty-eight percent of the patients achieved a high SVR after 12 weeks of treatment with limited adverse events (Nam et al., 2016).

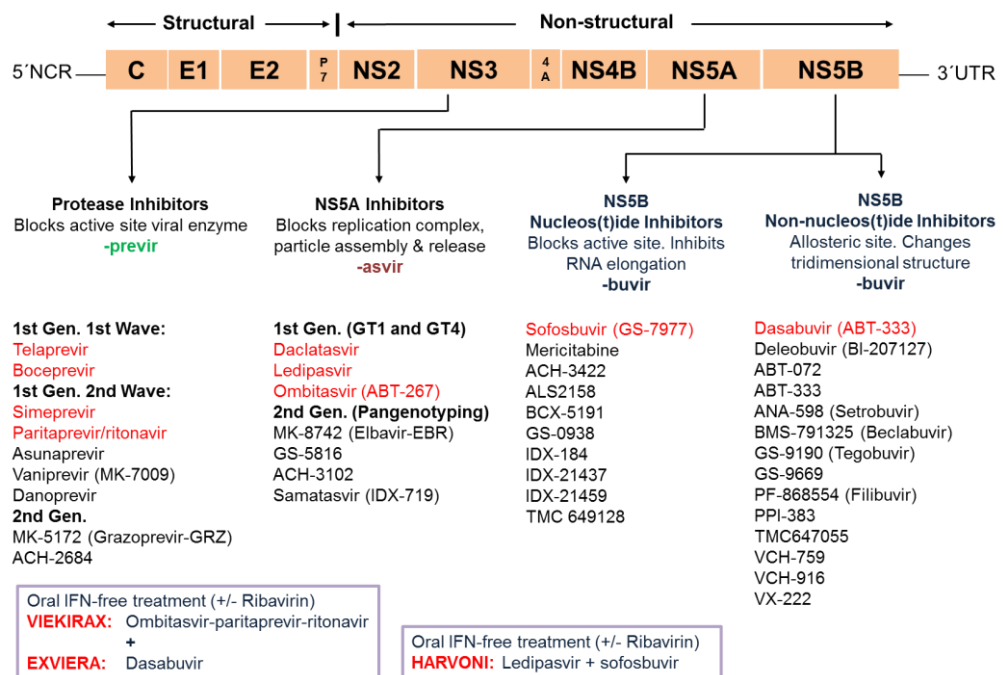


Figure 3-2 Current drugs available for HCV treatment. Adapted from (De Clercq, 2014, Perales et al., 2015)

Four categories of direct acting anti-virals were developed for the treatment of chronic hepatitis C infection: protease inhibitors (-previr), NS5A inhibitors (-asvir), NS5B nucleoside and non-nucleoside-type inhibitors (-buvir). The second generation of agents aimed at improving drug resistance profile and side effects. Drugs in red have been approved. New oral IFN-free combinations are in boxes. NS: non-structural.

3.2.4 Side effects and drug resistance

High cost of medications, adverse events, long treatment duration and viral resistance are new challenges. Patients treated with various direct-acting antivirals (DAAs) were monitored. These DAAs included ritonavir, ombitasvir, dasabuvir, sofosbuvir, ledipasvir, semiprevir, paritaprevir and asunaprevir (Figure 3-2). Common adverse events were reported such as fatigue, headache, nausea, pruritus (severe itching), insomnia, diarrhoea, asthenia (loss of energy), rash, irritability, dyspnoea (shortness of breath), cough, anaemia, haematological abnormalities, hyperbilirubinaemia and photosensitivity (e.g., with simeprevir), elevated aminotransferase levels as with paritaprevir and asunaprevir (Banerjee and Reddy, 2016). However, grazoprevir was well-tolerated. Dysgeusia (distortion of sense of taste) and anaemia were the main adverse events in patients treated with boceprevir (Conteduca et al., 2014).

Despite the development of DAA therapy, drug resistance has become a new challenge. For instance, point mutations at A156 confer resistance to all NS3 protease inhibitors, whereas resistance to most protease inhibitors was associated with R155 substitutions (Thompson et al., 2011). These mutations are attributed to the poor fidelity of HCV RNA-dependent RNA polymerase along with a replication error rate of 10^{-4} substitution per nucleotide per year. This is nearly 10-fold that of HIV reverse transcriptase. The rapid rate of HCV production is roughly 100-fold that of HIV; 10^{12} virion particles per day. This suggests that resistant variants rapidly outgrow wild-type virus (Wyles, 2013).

Sofosbuvir is a nucleotide analogue with pangenotypic activity that has relatively high barrier to resistance. However, NS5B substitutions were emerged in patients who failed sofosbuvir treatment: L159F (with or without L320F or C316N) and V321A. Amino acid changes at S282 position were linked with sofosbuvir resistance. Analyses of these substitutions indicate that these variants may affect sofosbuvir activity (Donaldson et al., 2015). Despite its activity against all HCV genotypes, genotype 3 remains a challenge with low SVR especially in patients with cirrhosis (Feld et al., 2016). This drug is contraindicated for patients with advanced renal disease (Banerjee and Reddy, 2016).

Treatment outcomes are determined by the dominance of naturally occurring resistant strains. Pre-existence of Y93H resistance-associated variants in the NS5A significantly affects SVR by daclatasvir (DCV) and asunaprevir (ASV) therapy for genotype 1b after prospectively selecting 138 patients from 152 treated with DCV and ASV (Ikeda et al., 2016). This study recommends evaluating the dominance of resistant variants for DAAs treatment.

One of the reasons behind treatment failure to prevent graft reinfection using current marketed drugs is attributed to the cell-to-cell transmission of HCV (Qian et al., 2016b). Targeting HCV entry ensures a promising approach by

identifying the best lead mimic from a random peptide library that cross-reacts the entry-dependent E2 glycoprotein.

3.3 Aim and hypothesis

HCV is a global health concern. Although new therapies have recently been introduced into the clinic with great success, it is likely that preventing escape from drug interventions will require targeting multiple steps of the HCV life cycle. Virus entry was an attractive target for intervention for other viruses, but as yet no drugs targeting HCV entry have yet been brought to market. The optimised phage selection (Chapter 2) was utilised for possible identification of inhibitors targeting E2 glycoprotein, which is essential for receptor mediated entry. Lead peptides have the potential for optimisation and development as a starting point to develop more biologically active inhibitors. HCV proteins are genetically diverse, making discovery of novel inhibitors far from feasible. Thus, a truncated form of the conserved receptor-binding domain of E2 (amino acids 410—644 on the HCV polyprotein) was expressed, purified, and used as a target for peptide library enrichment. Depending on the representation of amino acids within the library, there might be a few binding partners that form stable interaction with the virus envelope and blockade essential steps in the entry pathway.

3.4 Materials and Methodologies

3.4.1 Target protein preparation

3.4.1.1 HCV envelope glycoprotein

Well-folded HCV sE2 envelope proteins from different genotypes were used to screen a phage-displayed peptide library. These proteins were a kind gift of Dr Muhammad Hamed, which were expressed by stably transfecting *Drosophila* S2 cells (Gilmartin et al., 2012), detected by western blot, purified and reacted with known HCV monoclonal antibodies (mAbs) (Figure 3-3). The antibodies reactivity suggests that the protein is folded and mimic the authentic structure (Kong et al., 2013, Khan et al., 2014). The isolates were sE2 UKN1A20.8, UKN3A13.6 and 4.11.1. See sections 2.2.1 & 2.2.2 for more technical details on the selection technique.

3.4.2 Cell culture

Human hepatocellular cancer cells (Huh7) and human embryonic kidney cells (HEK293T cells) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with heat inactivated foetal bovine serum (FBS) and MEM non-essential amino acids (NEAA) solution grown in T225 tissue culture flasks.⁶ All of these reagents were from Gibco[®], Life Technologies.

⁶ To prepare this, 50ml of FBS and 5.5ml of NEAA were added to 500ml of DMEM.

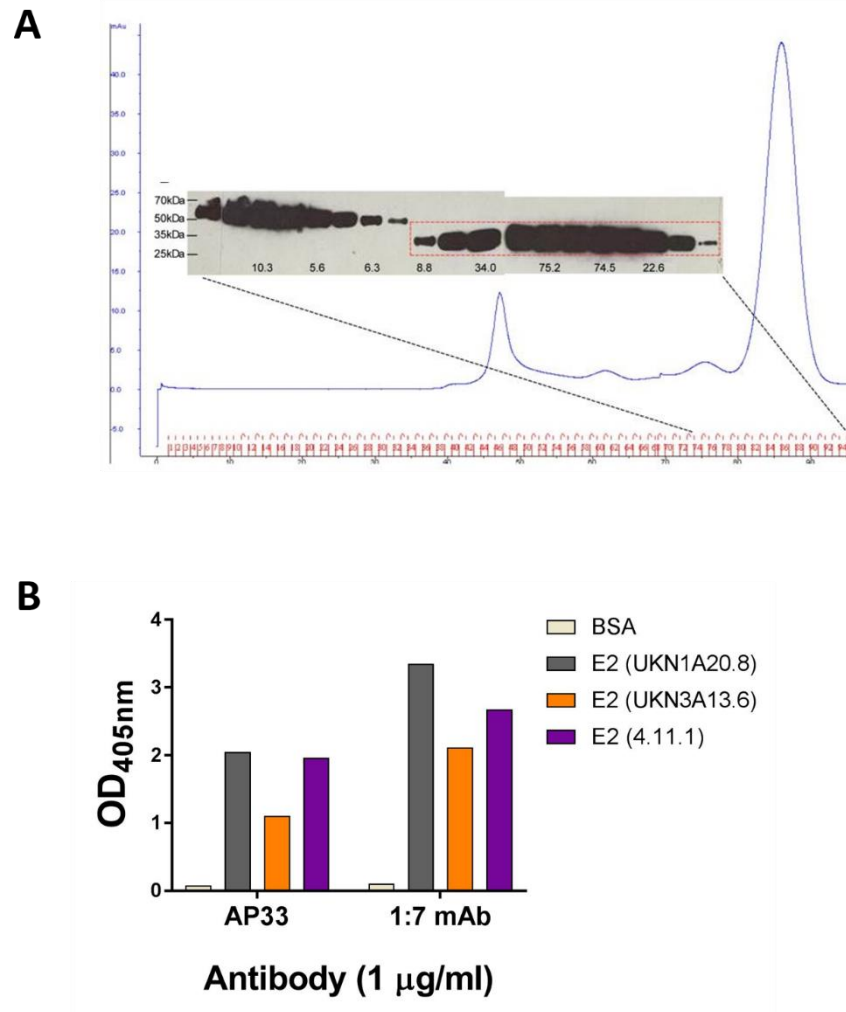


Figure 3-3 Purification and detection of HCV E2 glycoproteins

(A) Proteins in fractions 73—94 were separated by size exclusion chromatography and detected by western blot. The UV absorbance at 280nm is indicated by the blue line. The red numbers indicate 1ml elution fractions that were collected. The red rectangle indicates monomeric proteins. The amount of protein in each fraction is indicated below the dotted rectangle which was determined by BCA assay. This western blot and protein purification was performed in collaboration with Dr Muhammad Hamed. (B) AP33 antibody that recognises a linear epitope (aa 412—423) and a conformational 1:7 antibody that interacts with residues 527, 529 and 535 were tested against the soluble form of sE2 glycoproteins from genotypes 1A, 3A and 4.11.1. Bovine serum albumin was used as a control protein (n=1).

3.4.3 Transfection and production of HCV pseudoparticles

For transfection, 1.2 million HEK293T cells were seeded in 10mm tissue culture dish (Primaria) containing 7ml Opti-MEM (reduced serum medium; Invitrogen) to allow of 40—50% confluency after 24hr. HCV pseudoparticles (HCVpp) were generated by transfecting HEK293T cell line with phCMV containing the murine leukaemia virus retroviral packaging vector (MLV gag-pol), a reporter plasmid encoding firefly luciferase flanked by the MLV LTRs (long terminal repeats) and a plasmid encoding HCV envelope glycoproteins E1 and E2 (Bartosch et al., 2003). For each dish, 600µl of Opti-MEM in Eppendorf tube are supplemented with 24µl PEI (polyethylenimine) and 2µg of each plasmid.

3.4.4 Cell lysis

To harvest transfected HEK293T cells which contain HCV E1E2 protein, 1ml of lysis buffer was added into each 10mm dish (Primaria) and were gently shaken for 15 minutes, and then centrifuged for 5 minutes at 10,000g. The supernatant was recovered and stored at -20°C. Lysis buffer was prepared by diluting 0.5M iodoacetamide⁷ (Sigma, #I1149) with (1mM EDTA, 150mM NaCl, 50mM Tris-HCl and 1% Igelpal CA630 detergent)⁸ buffer 1:25 (e.g., 40µl of iodoacetamide + 960µl of lysis buffer). The cell lysate was used in GNA-capture ELISA (section 2.2.8).

⁷ This reagent should be prepared fresh.

⁸ To prepare this buffer, add 5ml of 1M Tris-HCl, 0.876g of NaCl, 200µl of 0.5M EDTA and 1ml of Igelpal detergent. Bring up volume up to 100ml with water.

3.4.5 Neutralisation assay

Ninety microlitres of the supernatant-containing HCVpp (section 3.4.3) were reacted with 10µl phage stock and added into 15,000 Huh7 cells in a white 96-well plate (Corning, 3917) with penicillin-streptomycin (Gibco®). After 3 days incubation at 37°C, cells were lysed with 50µl lysis buffer (Promega) per well for 15 minutes with gentle shaking. To detect luminescence in relative light units (RLUs), BMG Labtech FLUOstar Omega microplate reader was primed with luciferase substrate and RLUs were read. Similarly, the neutralisation assay was performed by treating cell-cultured HCV (HCVcc) with the promising phage candidates. The cell culture system is prepared by transfecting Huh7 with the full-length HCV RNA (Wakita et al., 2005). This experiment was performed at the Centre for Biomolecular Sciences, University of Nottingham, in Biological Safety Level III containment laboratory, using a Class II laminar flow hood with the help of the Virus Research Group.

3.4.6 Multiple peptide alignment and scoring system

Alignment scores between selected mimotopes and the natural ligand of the target protein were measured using BLOSUM62 substitution matrix (<http://www.expasy.org/>).

3.4.7 Random mutagenesis of lead peptides by error-prone PCR

The first step in peptide development is to randomly mutate the lead sequence. A convenient approach is by error-prone PCR where an unbalanced ratio of nucleotides and a low fidelity Taq polymerase is used with manganese to increase error rate.

Error-prone PCR reactions were performed with a low fidelity Taq polymerase (Jane Bioscience, #PP-102). For each reaction, the master mix contained 5µl 10X reaction buffer, 2pmol (g3R) forward primer (5'-TTAGTGGTACCTTTCTATTCTCACTCT-3'), 2pmol (linker) reverse primer (5'-TCGGCCGAACCTCCACC-3'), unbalanced ratio of dNTPs, 5U Taq polymerase, 1µl template (diluted 1:1000 or 2ng concentration), up to 7mM error-prone solution (manganese, Mg^{2+})⁹, 34µl PCR-grade water in a total reaction volume of 50µl. For thermal cycling, an initial denaturation step of 98°C for 30 s was performed, followed by 30 cycles of: 94°C for 10 s; 60°C for 15 s; and 72°C for 10 s. A final extension step of 72°C for 10 s was performed.

⁹ Should be added last. Oxidation of Mn^{2+} to Mn^{3+} may destroy the polymerase.

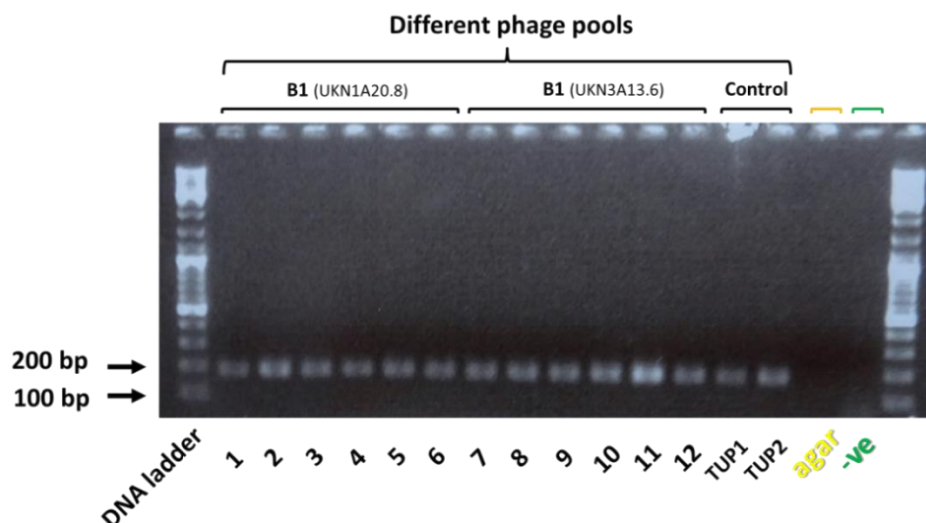


Figure 3-4 PCR gel electrophoresis

Phage clones from different pools selected against two genotypes of HCV E2 (UKN1A20.8 and UKN3A13.6) were purified and PCR-amplified. TUP: Target Un-related Peptide.

3.4.8 Purification of PCR product

Following PCR reaction (Figure 3-4), the 180bp PCR-product (-96 primer) was purified using QIAquick Purification Kit (QIAGEN, 28104). Following error-prone PCR and digestion of the 80bp, the product was purified using QIAquick Nucleotide Removal Kit (QIAGEN, 28304).

3.4.9 M13KE plasmid transformation and purification

To amplify M13KE plasmid for restriction digestion and cloning experiments, chemically competent *E. coli* (Thermo, C4040) was used to transform 1µl of the

original M13KE plasmid stock, incubated with 250µl SOC rich medium at 37°C and plated on LB agar containing 100µg.mL⁻¹ ampicillin. Colonies containing the plasmid were amplified in LB broth (containing 100µg.mL⁻¹ ampicillin) overnight. Cultures were purified using QIAGEN Plasmid Mini Kit and quantified spectrophotometrically using NanoDrop[®] software (ND-1000) (Figure 3-27).

3.4.10 Restriction digestion and ligation reaction

M13KE plasmid and purified PCR-product of phage candidates were digested with Acc65I and EagI restriction enzymes and ligated with T4 DNA ligase. The reaction contained 5µl 10X NEBuffer 3.1 (NEB, #B7203), 1µl Acc65I restriction enzyme (NEB, #R0599), 1µl EagI restriction enzyme (NEB, #R0505), 1µg DNA (M13KE plasmid or insert in separate reactions) and 42µl nuclease-free water in a total reaction of 50µl. For ligation, the mass of insert was calculated at 5:1 insert:vector ratio, where insert length is 68 nucleotides and vector length is 7222 nucleotides. The reaction contained 2µl 10X T4 DNA ligase buffer (Thermo Scientific, B69), 100ng linear M13KE vector DNA, 1µl insert DNA (diluted to the final concentration needed; i.e. 4.708ng), 1µl Weiss U T4 DNA ligase enzyme (Thermo Scientific, EL0016) and 16µl nuclease-free water in a total reaction of 20µl. The reaction was incubated for 10 minutes at 22°C and then heat inactivated for 10 minutes at 65°C and purified (section 3.4.8).

3.4.11 Preparation of electrocompetent cells

One litre of LB broth medium was inoculated with 10ml of an overnight *E. coli* ER2738 culture and incubated at 37°C with good aeration and shaking at 250rpm until culture reaches an optical density of 0.5—1.0 (OD_{600nm}). The flasks were chilled on ice for 30 minutes and centrifuged at 5000g for 15 minutes at 4°C to harvest the cells. The supernatant was discarded and 200ml of ice-cold autoclaved water were added to suspend the pellet, and then centrifuged as before. After discarding the supernatant, each pellet was suspended with 100ml of ice-cold autoclaved water. Suspensions were combined in a single bottle and centrifuged as before. The supernatant was discarded, and 50ml of ice-cold autoclaved 10% (v/v) glycerol in water was added to suspend the pellet, followed by centrifugation at 8000g for 10 minutes at 4°C. The supernatant was discarded without disturbing the pellet and suspended in 5ml of ice-cold 10% glycerol. The 5ml bacterial suspension was aliquoted in 100µl volume each and immediately frozen in dry ice-ethanol bath and stored at -80°C.

3.4.12 Electroporation and plaque assay

Following random mutagenesis (section 3.4.7) and ligation experiment (section 3.4.10), 3µl of the ligated M13KE plasmid was added into 100µl of electrocompetent *E. coli* cells (section 3.4.11). The reaction was placed in chilled 0.1cm cuvette and inserted in the chamber electrode. The pulse was delivered using BIO-RAD *E. coli* Pulser at 2.5k voltage. Immediately, 1ml SOC

outgrowth medium was added.¹⁰ The outgrowth was incubated 30—45 minutes at 37°C with shaking. Several dilutions of the outgrowth in LB medium were prepared. Ten microlitre of each dilution was tested in plaque assay as previously described (section 2.2.4).

3.5 Results

3.5.1 Affinity selection using chemical versus bacterial elution

Having investigated various methods in phage screening, and to test the hypothesis that Tween 20 favours selection of plastic-binding phage (Chapter 2), chemical elution under different conditions with the detergent was applied in peptide library selection using HCV glycoproteins from two genotypes (Table 3-2 & Table 3-3). The result confirmed the link between non-ionic detergent use and the high frequency of aromatic amino acids such as the motif WHW. Other selected candidates remained to be investigated.

¹⁰ The rapid addition of SOC medium aims to maximise the recovery of transformants. One minute delay after applying the pulse can cause three-fold drop in transformation and up to twenty-fold drop by 10min.

Table 3-2 Amino acid sequence of peptides selected following two rounds of affinity selection using HCV E2 (UKN1A20.8) as bait with the chemical elution method

Phage Name	Sequence
T2.2 _(1a)	ANNTSFFMQNNQ
T2.3 _(1a)	GGPGQSYQPLQY
T2.4 _(1a)	GLQTGNPLWSGK
T2.5 _(1a)	TTDPSMHSARYL
M2.1 _(1a)	INNNSNWNRSRP
M2.2 _(1a)	LASTPTTQGHTQ
M2.3 _(1a)	VNTYNTHPMDQD
M2.4 _(1a)	AQSNTLEPKPVR
M2.5 _(1a)	AMPRVTNPLLEM
bT2.2 _(1a)	SIAANQVHLSGN
bM2.1 _(1a)	EPMKLPPPDTRT
bM2.3 _(1a)	LMNPPPTRHLPV

Table 3-3 Chemically eluted phage-displayed peptides against HCV E2 (UKN3A13.6) after three rounds of biopanning

Two conditions were performed based on the diluent used. Tween 20 was used as a washing buffer in both conditions. Grey blocks represent aromatic residues.

Phage pool	Clone name	Peptide sequences
M.Tween.R3_(3a) (Diluent: 5% milk, Washing buffer: Tween 20)	AR3.1	HPHSGRTPLISA
	AR3.2	YPQHQHTTHRDY
	AR3.3	TPMSGFFWDTDY
	AR3.4	GMAPYAERSLTS
	AR3.5	HINPAYRNLLMQ
	AR3.6	SAWEAEFWHANF
	AR3.7	NNYATAEFGLKH
	AR3.8	NHPDHKHAVNRLT
T.Tween.R3_(3a) (Diluent: TBS, Washing buffer: Tween 20)	BR3.2	WHWSWWNPQNLT
	BR3.3	WHWSWWNPQNLT

Table 3-4 HCV-E2-selected clones using a detergent-free bacterial elution strategy following the first round of affinity selection

Pool B.1 _(1a)		Pool B.1 _(3a)	
Target: HCV E2 (UKN1A20.8)		Target: HCV E2 (UKN3A13.6)	
Φ Clones	Peptide sequences	Φ Clones	Peptide sequences
B.1.2	TQAHVAPLKNSL	B.1.1	DHPLMRYSDLAS
B.1.5	DAQAQSHYPNYK	B.1.2	NILHGLEPPLLY
B.1.6	SHSDLMSLTSQA	B.1.3	NILSHTSPLLIE
B.1.7	GHLASRDSAVPM	B.1.5	TMRHMPDAYNKE
B.1.8	MPMHSDSPRGKQ	B.1.6	YLSDQGVVAVTM
B.1.9	DLTFTVNPLSKA	B.1.8	DNLMYYNKITRL
B.1.10	GQERPANENRTE	B.1.9	EMNLMGAHNLT
B.1.13	NQSSHLITLQHS	B.1.11	WFDTNDRWYLG
B.1.16	TMEMTLDFVRTL	B.1.12	TAVGTFTYNGRM
B.1.17	NMLHTTSLASLL	B.1.13	NTNTTIAVWQVS
B.1.19	MKNNERRSTHR	B.1.14	AVSLSSLALTKK
-	-	B.1.15	DHPLMRYSDLAS
-	-	B.1.16	HKMESYPGPFPL
-	-	B.1.17	YDRIPKQIDLIS
-	-	B.1.18	RFINDARVEQSY
-	-	B.1.19	IDTSGTQAGPTT

3.5.2 Negative selection and sequence comparison

Negative selection (section 2.2.3) was applied with the third HCV E2 bait (genotype 4.11.1). It permitted comparison of enrichment and sequence homology using two different selection conditions. The first amplified phage pool was subjected to a subtractive panning cycle, in which the sub-library was incubated in the absence of the target, followed by positive selection where the library was recovered and incubated with the target protein. This method improved the observed consensus sequence, the redundancy of peptides and showed greater enrichment with the HCV E2 target, suggesting a high number of specific peptides within the second pool (Table 3-7).

Table 3-5 Peptide sequences from the mock plate using negative selection

Peptide sequences were grouped based on conserved residues by physicochemical properties. Grey blocks represent dicysteine-containing motifs. Fully conserved amino acids are indicated in red letters. Green colour indicates strong conservation. Weak conservation is shown as blue.

Phage pool	Clone name	Peptide sequence
nsR2 (negative selection from R2)	nsR2.1	VPKNGALAVTSM
	nsR2.11	EYTLGSYAFISR
	nsR2.7	HLEPIKPASTFP
	nsR2.8	DVLTADVAAARVP
	nsR2.9	VLPAPSCMSEAC
	nsR2.10	IVGISDRASEAQ
	nsR2.12	SYYTSQCTKYEC
	nsR2.2	QLSLSLRDKVYT
	nsR2.3	IATNPSLQPADP
	nsR2.5	NDSQFGDTYAPK
	nsR2.15	YVAPPARSAPLV
	nsR2.16	TMGSTTNQTFPF
	nsR2.18	SEHQWYTAAGVR
	nsR2.20	NLSRSVSTAARF
	nsR2.21	RDFSSTIWPGLQ
	nsR2.16	TMGSTTNQTFPF
nsB2 (bacterial elution from nsR2)	nsB2.2	APIINTWAMRWY
	nsB2.3	LNFSYQMANQV
	nsB2.4	EGFRPPSNGFGF

Table 3-6 Peptide sequences from the E2-coated plate using negative selection

Peptide sequences were grouped based on conserved residues by physicochemical properties. Fully conserved amino acids are indicated in red letters. Green colour indicates strong conservation. Weak conservation is shown as blue.

Phage pool	Clone name	Peptide sequence
4B2 (bacterial elution from 4R2)	4B2.1	HAPIPVSPFDLK
	4B2.4	VGMHGRANTLHD
	4B2.2	DPG PM PAT ATNR
	4B2.5	DV STLS SP AMHR
	4B2.6	AVVFKT SSDGPR
	4B2.3 previously B1.5 _(3a)	TMRHMPDAYNKE
	4B2.7	TMRHMPDAYNKE
4R2 (chemical elution from R2)	4R2.1	VSPAQHSTNRPG
	4R2.5	QHTPSSTEYAWH
	4R2.9	TVSIPTHDSTDR
	4R2.10	HVGHPAYRTSSV
	4R2.18	EFTSPVRVGGET
	4R2.2	GVES S GLTGL TA
	4R2.3	GSNILLPEK STA
	4R2.6	SEYH ASQ SGGGA
	4R2.17	GSVW APY SASFA
	4R2.11	YIL PPY SGMHEQ
	4R2.16	YSL SSPT GTVSW
	4R2.21	HPK PTE HW RALA
	4R2.22	AKI TNDQ V RVVA
	4R2.8	TMRHMPDAYNKE
	4R2.13	TMRHMPDAYNKE
	4R2.14	TMRHMPDAYNKE
	4R2.19	TMRHMPDAYNKE

Table 3-7 Enrichment of phage pools after two rounds of affinity selection with HCV E2 protein (genotype 4.11.1) as bait

This phage enrichment was calculated from the previous biopanning experiment where the phage pool from the first round was reacted against uncoated well, then the pool was recovered and reacted against the target (Table 3-5 & Table 3-6). Recovery rate from the target-coated plate was 16 times higher than the mock plate. PFU: plaque forming unit.

	Mock well	Target-coated well
PFU	4.000 X 10 ⁴ /ml (4.600 X 10 ³ /eluate)	6.555 X 10 ⁵ /ml (7.538 X 10 ⁴ /eluate)
Recovery difference	16 X	

3.5.3 Selected peptides mimic HCV receptors

An interesting finding among peptides (Table 3-4 & Table 3-6) is that they mimic HCV receptors in a non-gapped alignment. A scoring system was used to calculate the significance of alignment. No clear consensus found between the receptors and peptides selected in a non-specific fashion (Table 3-2, Table 3-3 & Table 3-5). This homology may support the claim that specific peptides were selected. Despite that these alignments can provide new insight into receptor usage during the entry cascade, they should be carefully interpreted within the limits of already published findings regarding HCV-host receptor interaction.

3.5.4 Neutralisation of HCVpp by selected peptides

Irrespective of the potential for selected peptides to mimic HCV receptors, discovering peptides that neutralise virus entry is the top priority in library screening experiment. With a highly diverse library, the assumption is that few sequences would be able to interact specifically with the viral envelope and inhibit its function. Notwithstanding this, the vast majority of peptides were non-neutralisers (Figure 3-5) and some clones increased infectivity. Few peptides were able to show an observable inhibition of infectivity.

B1.9_(1a) was a promising lead peptide identified following biopanning against HCV E2 representing genotype 1a (Figure 3-6). This lead showed an additive neutralising activity when used with another weak inhibitor (Figure 3-9). Another lead sequence was discovered that neutralised HCV genotype 3 entry (Figure 3-7), with a reproducible result at varied phage concentrations (Figure 3-8).

3.5.4.1 Calculation of molar mass of phage-displayed peptides

The average phage concentration used in neutralisation assays was about 1×10^{10} virions. The molar concentration of displayed peptides can be calculated by linking the molar mass of the peptide and its copy number with Avogadro's constant (Lisitsa, 2015). The following equation should be considered: (copy number of a peptide) x (molecular weight of peptide sequence) / (Avogadro's

number). Since there are five copies of pIII each displays a peptide sequence, the peptide copy number would be 5×10^{10} . The average molecular weight of an amino acid is 110 Dalton; therefore, the 12-mer peptide sequence would have an average molar mass of 1300 g.mol^{-1} . Based on the given values, the dodecapeptide molarity in each reaction will be calculated as follows: $(5 \times 10^{10}) \cdot (1300) / (6.022 \times 10^{23})$. From this calculation, the theoretical peptide concentration is 0.1nM. While any inhibitory effect from such a low concentration is favourable to select lead sequences, it should be noted that the same concentration of a synthetic peptide might not show the same effect in the context of phage pIII peptide presentation. In addition, higher phage concentration (say 10^{11} and higher) could form phage aggregates, and this would compromise the inhibitory effect.

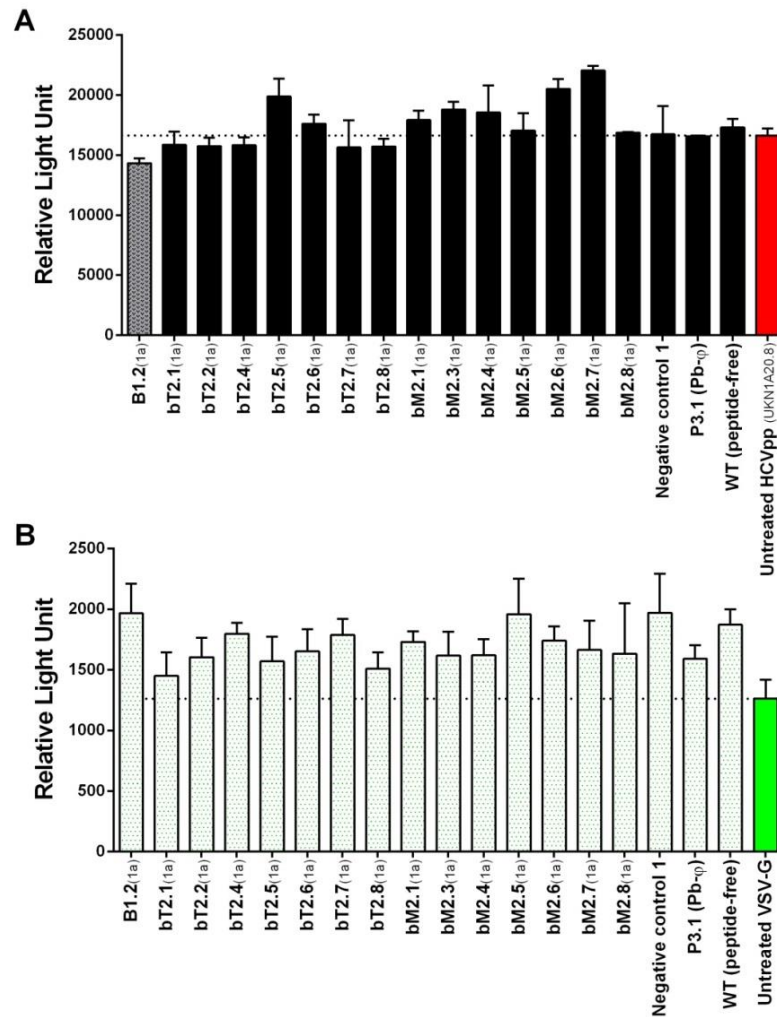


Figure 3-5 Neutralisation assay of HCV pseudoparticles treated with phage clones

(A) Ninety microlitres of supernatant-containing HCV pseudoparticles from genotype 1A (isolate UKN1A20.8) were treated with 10 μ l of each phage clone isolated from the first biopanning method. Fourteen clones were used along with two control phage clones that were selected against BSA and polystyrene. One clone (B1.2) from the optimised bacterial elution method (Table 3-4) achieved some measure of inhibition. Because of the lack of inhibition by most clones, not all phage members were sequenced. (B) The same phage clones were used to treat vesicular stomatitis virus (VSV-G) pseudoparticles as a control. No unspecific inhibition was observed. Results are expressed as relative light units. Error bars represent $\pm$ SEM (n=3). Wt: wild-type.

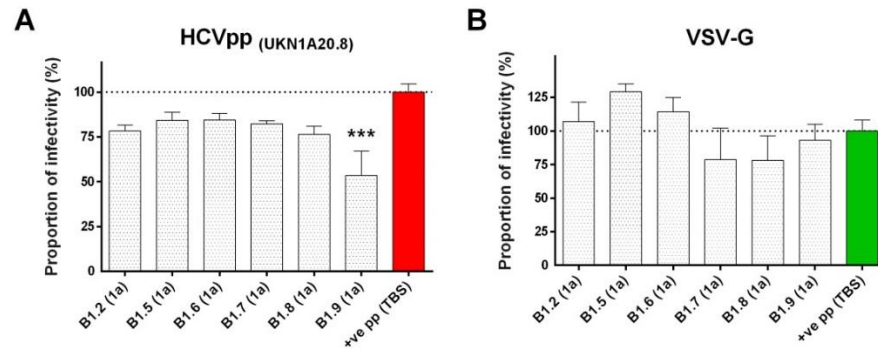


Figure 3-6 Neutralisation of HCV entry (UKN1A20.8) using phage-displayed peptides enriched following a detergent-free selection with bacterial elution strategy

(A) The inhibitory effect of six phage clones selected against HCV E2 envelope glycoprotein from genotype 1A (Table 3-4) were tested against its equivalent HCV pseudoparticles. (B) The same phage clones were tested against vesicular stomatitis virus as a control. No neutralisation observed with the control VSV-G pseudotyped particles. Results are expressed as percentage of infectivity compared to untreated pseudoparticles. Comparisons were made using one-way ANOVA (** $p < 0.001$). Error bars represent $\pm$ SEM ($n=3$).

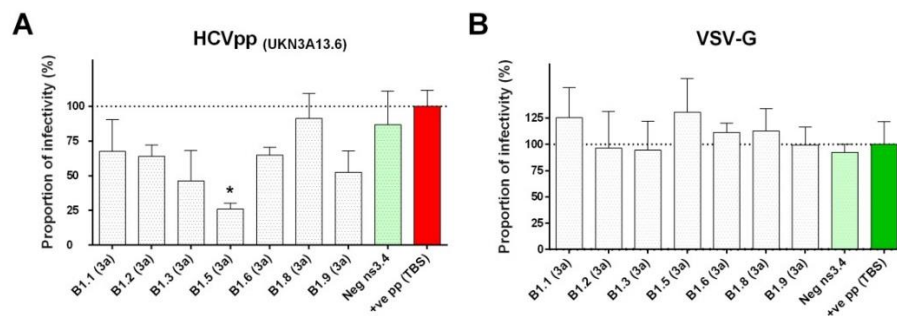


Figure 3-7 Neutralisation of HCV entry (UKN3A13.6) using phage-displayed peptides enriched following a detergent-free selection with bacterial elution strategy

(A) The inhibitory effect of phage clones selected against HCV E2 envelope glycoprotein from genotype 3A (Table 3-4) were tested against its equivalent HCV pseudoparticles. (B) The same phage clones were tested against vesicular stomatitis virus as a control. No neutralisation observed with the control VSV-G pseudotyped particles. Results are expressed as percentage of infectivity compared to untreated pseudoparticles. Comparisons were made using one-way ANOVA (* $p < 0.05$). Error bars represent $\pm$ SEM ($n=3$).

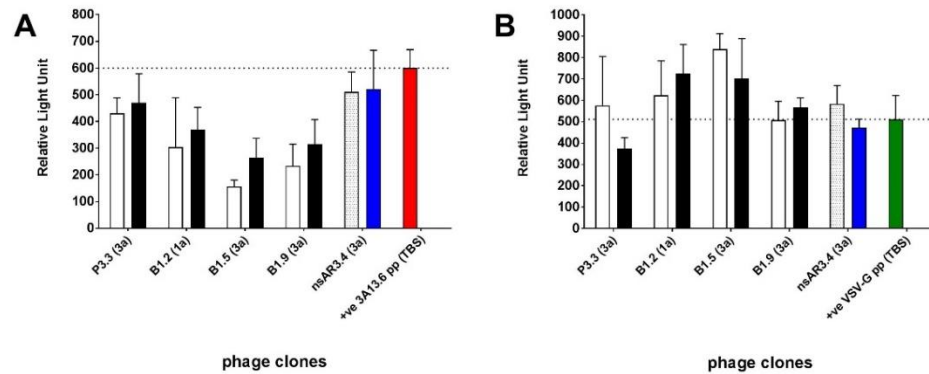


Figure 3-8 Repeated neutralisation assay at high (stock) phage concentration and 10-fold less concentration

(A) In line with the previous results, the promising phage clones were selected for repeated neutralisation assay of HCVpp (UKN3A13.6) at two different titres of phage particles: 10^9 (empty bars) and 10^{10} (black bars). (B) No neutralisation observed with the control VSV-G pseudotyped particles. The control phage (blue bars) did not show an inhibitory effect on both pseudotyped viral particles. Results are expressed as percentage of infectivity compared to untreated pseudoparticles. Error bars represent $\pm$ SEM ($n=3$).

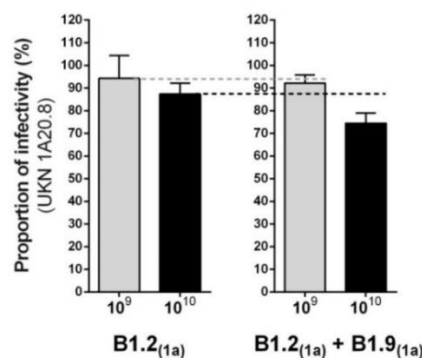


Figure 3-9 Additive neutralisation effect of B1.2 and B1.9 phage clones compared to B1.2 inhibitory effect alone

In line with the previous neutralisation tests, this assay was repeated at two concentrations (10^9 and 10^{10}) using newly prepared cell lines and HCV pseudoparticles (UKN1A20.8). To combine both clones, a half concentration of each phage was added: 5×10^8 in the first set (grey bar) and 5×10^9 (black bar) in the second set. Error bars represent $\pm$ SEM ($n=3$).

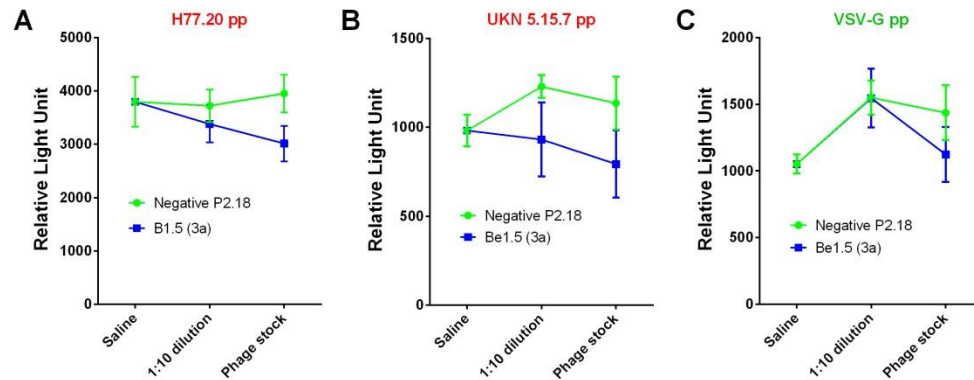


Figure 3-10 Neutralisation assay using HCV pseudoparticles of H77.20 and UKN5.15.7 genotypes treated with B1.5 phage clone

B1.5 lead was tested against two HCV isolates: H77.20 (A) and UKN5.15.7 (B) as well as VSV-G pseudotyped particles (C). Each 90µl of HCVpp was treated with 10µl stock (which represents 10^{10} phage particles) and 1:10 dilution of the stock (10^9). P2.18 clone was used as a control. Results are expressed as relative light units. Error bars represent $\pm$ SEM (n=4).

3.5.5 Neutralisation of cell-cultured HCV (HCVcc) by selected lead

Having identified favourable peptides that cause neutralisation of HCVpp, chimeric JFH-1based HCVcc (with the Core-NS2 genes from genotype 1a) was treated with the promising phage leads in cell culture system as previously described (Wakita et al., 2005). Intriguingly, B1.9 peptide was a promising lead for genotype 1. The other peptide (B1.5) might be a better lead for genotype 3 (section 3.5.4). This conforms with the genotype representing the HCV envelope glycoproteins used against a phage library. The peptide sequence selected against the viral envelope of genotype 1a showed a clear inhibition of HCVcc entry by nearly 50% in comparison with untreated virus particles (Figure 3-11).

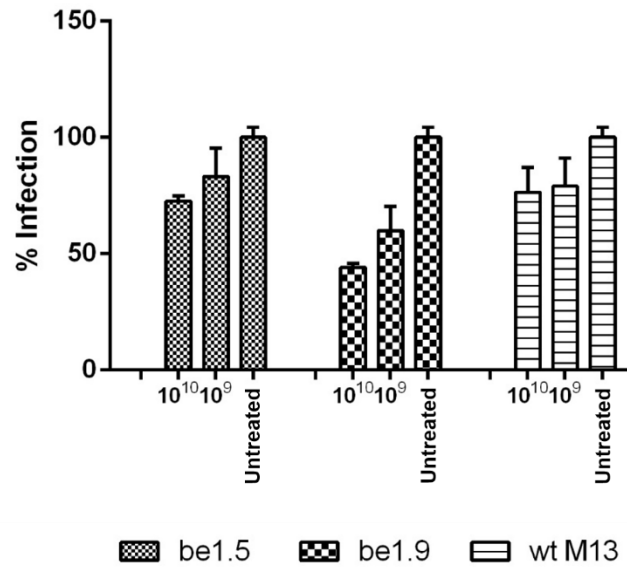


Figure 3-11 HCVcc neutralisation using lead phage-displayed peptides

Hepatitis c virus infectious particles (HCVcc) were treated with each phage clone at two concentrations (10^9 and 10^{10}) in cell-cultured derived system using the whole viral RNA (HCVcc) of genotype 1A. The assay was performed in three replicates for each treatment, and the results are expressed as percentage of infectivity compared to untreated HCVcc particles. Wt: wild type.

3.5.6 Peptides selected using biopanning with HCV E2 as bait have weak binding affinity

In contrast to previous studies where strong binding affinity interactions were selected following library screening with antibodies (Tarr et al., 2006, Tarr et al., 2012b), HCV-selected phage peptides were weakly cross-reactive to the envelope glycoprotein. In three repeats, B1.5_(3a) phage-displayed peptide sequence (TMRHMPDAYNKE) showed statistically significant binding tested with E2 from genotype UKN3A13.6 (Figure 3-12), with no significance found

with P2.18 control phage-displayed peptide (LLADTTHHRPGT). The challenge is to find a sequence that can cross-react and neutralise various genotypes. This is still far from easy. The peptide lead B1.9_(1a) selected against an envelope of genotype 1A20.8 has the potential to cross-react E2 from genotype 4.11.1 (Figure 3-15). Having tested the binding affinity of individual phage clones, the phage pool was also reacted in the presence of HCV E2 alongside an individual promising clone (Figure 3-14). Interestingly, the additive binding of 11 phage clones from pool B1_(1a) (Table 3-4) to HCV E2 suggests that each peptide sequence might bind to different domain on the envelope glycoprotein, but the affinity is still low.

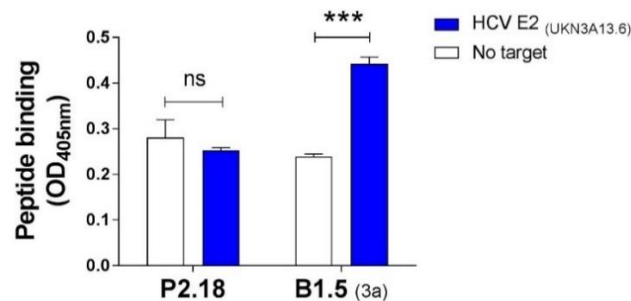


Figure 3-12 B1.5 phage-displayed peptide binding to HCV E2 of genotype UKN3A13.6

B1.5 phage clone was tested for binding to HCV E2 glycoprotein at high concentration ($10\mu\text{g.mL}^{-1}$). P2.18 was the control phage-displayed peptide. Comparison was made using paired t-test ($***p < 0.001$). Error bars represent $\pm\text{SD}$ of the mean ($n=3$). NS: not significant.

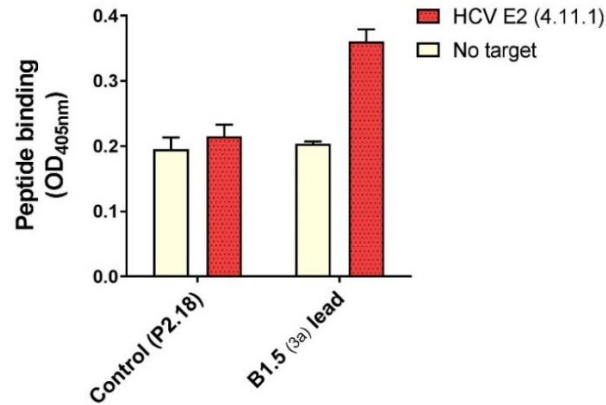


Figure 3-13 Repeated binding assay with B1.5_(3a) phage clone against HCV E2 of genotype 4.11.1

ELISA plate was coated with E2 envelope glycoprotein at $2\mu\text{g.mL}^{-1}$. P2.18 was the control phage-displayed peptide. Error bars represent $\pm\text{SD}$ of the mean (n=2).

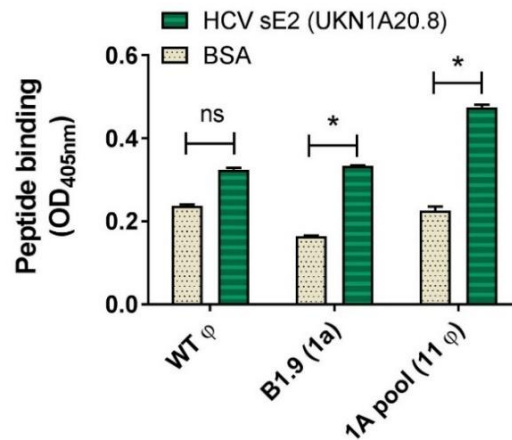


Figure 3-14 Binding affinity of B1.9 clone and phage pool to E2 from genotype UKN1A20.8

ELISA plate was coated with E2 envelope glycoprotein at $2\mu\text{g.mL}^{-1}$. A concentration of 10^{11} phage particles of B1.9 clone were added to the coated protein. All eleven phage clones that represent the eluted pool against HCV envelope protein of genotype 1a (Table 3-4) were tested in phage binding assay. A concentration of 9.09×10^9 of each phage clone was added in a tube before the binding reaction to reach a total concentration of 10^{11} . P2.18 was the control phage-displayed peptide. Comparison was made using paired t-test (* $p < 0.05$). Error bars represent $\pm\text{SD}$ of the mean (n=2). NS: not significant. Wt: wild type.

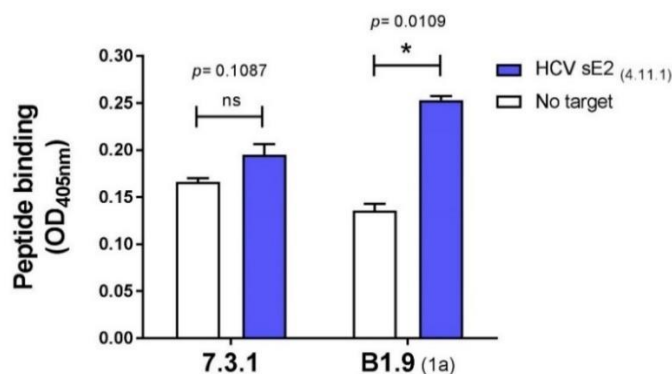


Figure 3-15 B1.9 phage-displayed peptide cross-reaction with HCV E2 from genotype 4.11.1

B1.9 promising clone that was selected from a genotype 1a target glycoprotein was tested against genotype 4.11.1 envelope glycoprotein at $1\mu\text{g.mL}^{-1}$. Phage clone 7.3.1 was the control phage-displayed peptide. Comparison was made using paired t-test. Error bars represent $\pm$ SD of the mean (n=2). NS: not significant.

3.5.7 Direct comparison of different batches of commercially-prepared phage-displayed peptide libraries

At the end of this project, another phage library, with different amino acid distribution than that used to generate the majority of peptides in this study, was screened against HCV E2 genotype 1 (UKN1A20.8) using two rounds of a bacterial elution method. With another representation of libraries being considered, another group of sequences presented new leads for future investigation (Table 3-8). Sequences were included in the multiple peptide alignment with HCV receptors and some candidates were tested in phage ELISA (Figure 3-16, Figure 3-17 & Figure 3-18). An interesting alignment was

observed with EB2.9 peptide with high alignment scores with CD81 and SR-BI (Figure 3-21).

Table 3-8 Phage-displayed peptide sequences isolated by biopanning against HCV E2 (UKN1A20.8) using another representation of phage library after two rounds of bacterial elution method

The phage library used (NEB, #E8111) showed different observed frequencies of amino acid distribution carried out by the commercial industry using deep sequencing compared to the previous constructed libraries (NEB, #E8110).

Phage	Peptide sequences and redundancy
EB2.1	MSVNSGQHPTSM
EB2.3	HDSLTRQNRPHP
EB2.4	AAHYSLSMAQAAQ
EB2.5	HPATSNTKYWIN (3X)
EB2.6	ATYRTAGWVVEK
EB2.7	HPATSNTKYWIN (3X)
EB2.8	LAHPTSIQKSGK (2X)
EB2.9	NDKPLTQTVVFT
EB2.10	LAHPTSIQKSGK (2X)
EB2.12	SDINAVSKVANG
EB2.13	YNEPILSPRFTT
EB2.14	HPATSNTKYWIN (3X)

3.5.8 Repeated binding assay with selected hits

With a diverse library as complex as 1.3 billion phage clones, precious sequences are not easy to isolate, and requires careful selection methods for successful identification. Selected phage candidates were tested by binding assay in different repeats to evaluate the reproducibility. Initial results suggest that some of these hits have the potential of binding specifically, albeit at low affinity (Figure 3-16). Independent repeated experiments confirmed the

significant binding of the selected peptides, indicated that they are potential leads for future investigations (Figure 3-17 & Figure 3-18).

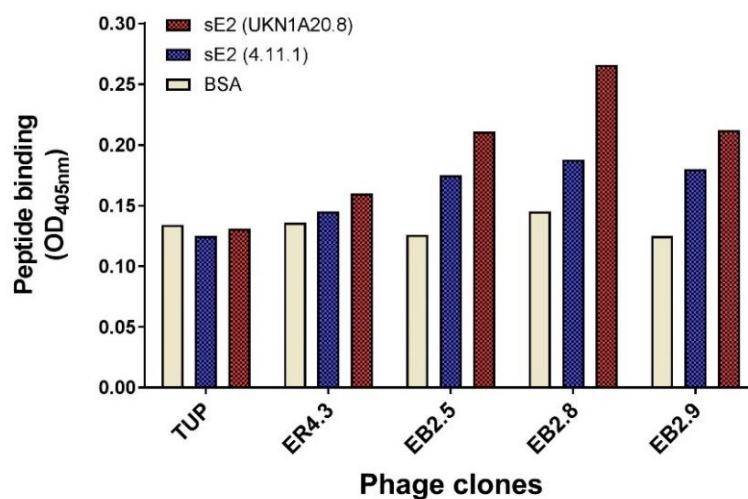


Figure 3-16 Preliminary ELISA result with the new hits tested against two genotypes of HCV envelope sE2

Two HCV sE2 genotypes (UKN1A20.8 and 4.11.1) were included to test four phage candidates with a control (n=1).

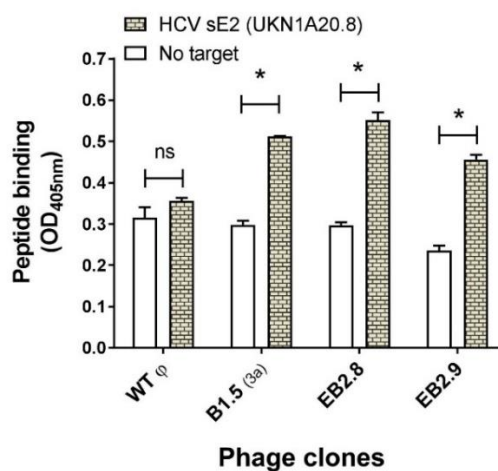


Figure 3-17 Binding assay using the new potential leads against E2 from genotype (UKN1A20.8)

EB2.8 and EB2.9 were selected in line with the previous assay. B1.5 lead was included in this binding assay. Comparisons were made using paired t-test (* $p < 0.05$). Error bars represent $\pm$ SD of the mean ($n=2$).

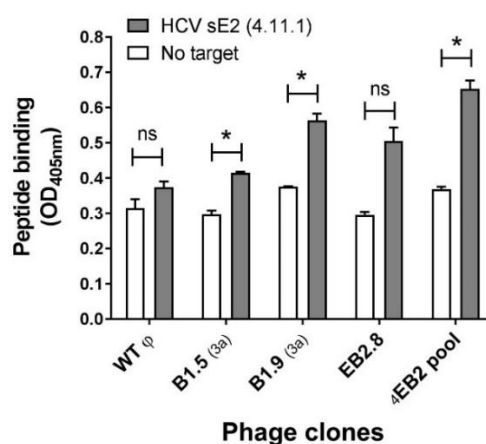


Figure 3-18 Binding assay using the new potential leads against E2 from genotype 4.11.1

B1.9_(3a) sequence (EMNLMGAHNLTSS) and B1.9_(1a) sequence (DLTFTVNPLSKA) were tested against 2 μ g.mL of HCV E2 glycoprotein from genotype 4.11.1. Comparisons were made using paired t-test (* $p < 0.05$). Error bars represent $\pm$ SD of the mean ($n=2$).

3.5.9 Effect of time on the optical density in ELISA

In line with the previous Chapter, assay parameters can have a dramatic impact on the final ELISA binding result. Because the selected phage-displayed peptides were weak target-binders, careful investigation of these ligands was required to differentiate the signal from the background. Figure 3-19 shows the binding signal of one peptide sequence measured at two time points following substrate addition. The data suggests that B1.2 sequence is a promising lead, which is a aa163-174CD81-mimicking sequence. The test was done at $10\mu\text{g.mL}^{-1}$ coated protein. Such a high target concentration favours binding of those ligands with weak affinity. Characterising several phage clones requires high concentrations of the protein of interest. The limited concentration of the precious HCV envelope glycoprotein in this study is an obvious drawback. Owing to this limitation, low target concentration was coated for most binding assays.

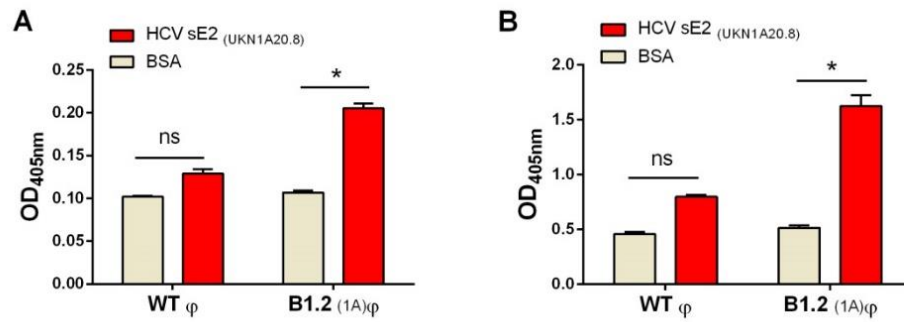


Figure 3-19 Phage binding to its target and essential time for colour development

B1.2_(1a) phage clone was reacted to the same target in which it was biopanned (HCV E2 of the UKN1A20.8 isolate). Optical densities were measured after ten minutes (A) and after two hours (B). This was tested at 10 μg.mL⁻¹ coated target. Comparisons were made using paired t-test (**p* < 0.05). Error bars represent ±SD of the mean (n=2).

3.5.10 Multiple peptide alignment with HCV receptors

All selected peptides against different HCV E2 genotypes under various assay conditions were subjected to multiple peptide alignment with HCV receptors. Interesting similarity were seen with many regions of specific HCV receptors, suggesting that they are mimics of the natural ligand of E2. Conservation of amino acid residues were found in a non-gapped multiple peptide alignment with CD81, SR-BI and occludin (Figure 3-20, Figure 3-21, Figure 3-22 & Figure 3-23). However, and because any short peptide can show similarity to a protein sequence, this does not provide substantive evidence that all these peptides are real mimics. Future investigations of the ability of these peptides to inhibit receptor binding will be required to support this initial analysis with various experiments.

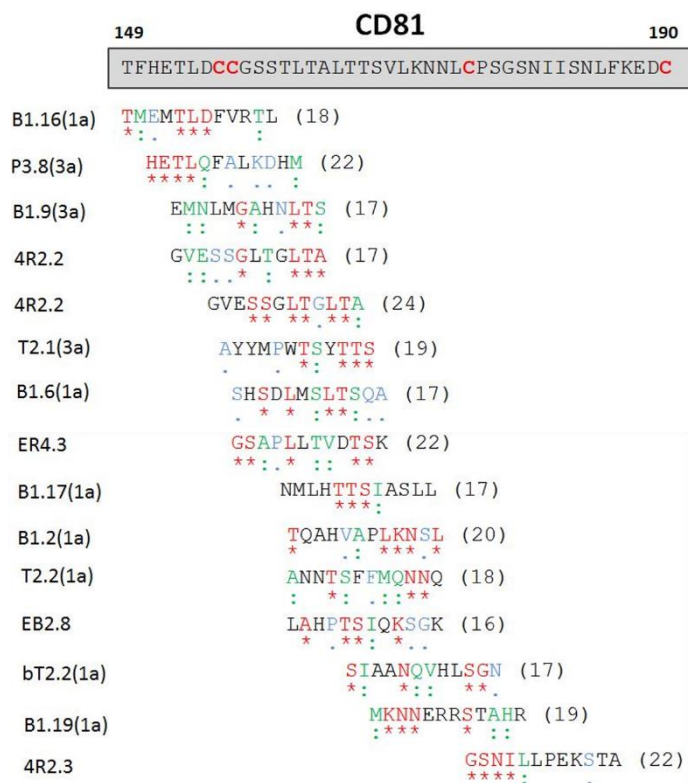


Figure 3-20 Alignment of phage-displayed peptide sequences with CD81 receptor

Fifteen peptides were aligned with the sequences of the large extracellular loop of human CD81 receptor. Numbers indicate BLOSUM62 scoring system.

* Fully conserved residues

: Conservation of strong groups

. Conservation of weak groups



Figure 3-21 Alignment of EB2.9 and B1.16_(1a) peptide sequences with CD81 and SR-BI receptor

EB2.9 and B1.16_(1a) peptide sequences were aligned with SR-BI and CD81 receptors in a non-gapped alignment with high BLOSUM62 scores.

- * Fully conserved residues
- : Conservation of strong groups
- . Conservation of weak groups



Figure 3-22 Alignment of phage-displayed peptide sequences with human SR-BI receptor

Five different domains in the extracellular loop of SR-BI (aa 149—161; 229—250; 252—279; 356—378 and 431—447) were aligned with many sequences isolated by various methods using phage-displayed peptide libraries against HCV E2 envelope glycoprotein. Numbers indicate BLOSUM62 scoring system.

* Fully conserved residues

: Conservation of strong groups

. Conservation of weak groups

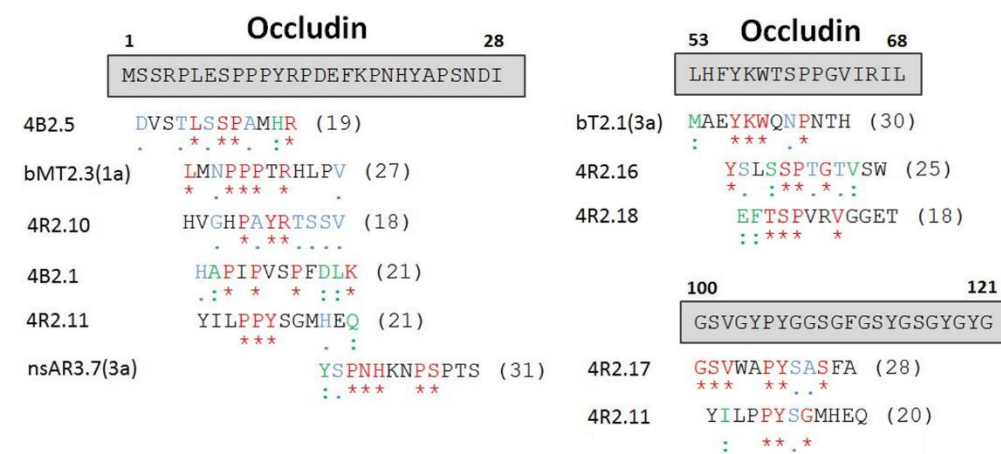


Figure 3-23 Alignment of phage-displayed peptide sequences with human occludin receptor

Three domains of occludin (aa 1—28 and 53—68 of the cytoplasmic domain; and 100—121 of the extracellular domain) were aligned with 10 phage-displayed peptide sequences at high alignment scores. Numbers indicate BLOSUM62 scoring system.

* Fully conserved residues

: Conservation of strong groups

. Conservation of weak groups

3.5.11 Cytoplasmic and helical domains and peptide alignment

Extracellular domains of viral receptors are primarily involved in the interaction with the viral glycoproteins. As such, selected peptides would be expected to mimic the extracellular regions more commonly than the intracellular of transmembrane domains. Interestingly, HCV-E2-selected peptides were aligned with the cytoplasmic and helical topological domains of all HCV receptors (Table 3-9). Figure 3-24 & Figure 3-25 show some of these alignments. For example, R4.8 peptide sequence (TWSPPSARTLIF) presented the highest alignment score of 35 with the cytoplasmic domain of SR-BI (499 AWSPPPAVLRL 510).

Table 3-9 Number of peptides aligned with the topological domains of HCV receptors

Positions	High score (≥ 18)*	Low score (< 18)*	Total
CD81			
(aa1-12 cytoplasmic / aa13-33 helical)	11	11	22
(aa13-33 helical / aa34-63 extracellular)	6	27	33
(aa113-201 extracellular / aa202-224 helical)	10	12	22
SR-BI			
(aa1-11 cytoplasmic / aa12-32 helical)	10	9	19
(aa12-32 helical / aa33-443 extracellular)	3	11	14
(aa33-443 extracellular / aa444-464 helical)	13	28	41
(aa444-464 helical / aa465-552 cytoplasmic)	5	14	19
(aa490-500 cytoplasmic)	9	8	17
(aa520-535 cytoplasmic)	12	17	29
Claudin			
(aa1-7 cytoplasmic / aa8-28 helical & aa8-28 helical / aa29-81 extracellular)	5	56	61
(aa29-81 extracellular / aa82-102 helical)	5	16	21
(aa82-102 helical / aa103-115 cytoplasmic / aa116-136 extracellular)	4	7	11
(aa137-163 extracellular / aa164-184 helical)	8	5	13
(aa164-184 helical / aa185-211 cytoplasmic)	25	22	47
Occludin			
(aa1-20 cytoplasmic)	14	21	35
(aa1-66 cytoplasmic / aa67-89 helical)	13	4	17
(aa90-135 extracellular / aa136-160 helical)	2	4	6
(aa136-160 helical / aa161-170 cytoplasmic)	2	10	12
(aa171-195 helical / aa196-243 extracellular)	2	5	7
(aa244-265 helical / aa266-522 cytoplasmic)	9	1	10
(aa300-336 cytoplasmic)	3	0	3
(aa400-420 cytoplasmic)	10	8	18
(aa441 cytoplasmic)	9	4	13
(aa470-480 cytoplasmic)	4	3	7
(aa495-500 cytoplasmic)	1	3	4

*Scores based on BLOSUM62 system.

SRBI_490 EB2.9	SDRTLWPTAAWS NDKPLTIQIVVFT .*:. * * . . .	Occludin_50 B1_8_3a	DEILHFYKWTSP DNLMYYNKITRL *:.
SRBI_493 EB2.8	TLWPTAAWSPPP LAHPTSIQKSGK *:. . .	Occludin_51 B1_2_3a	EILHFYKWTSP NILHGLEPPLLY :*** : .
SRBI_493 EB2.6	TLWPTAAWSPPP ATYRTAGWVVEK : : * * *	Occludin_53 T2_1_3a	LHFYKWTSPPGV AYYMPWTSYTTS : : * * *
SRBI_493 4B2_21	TLWPTAAWSPPP HPKPTTEHWRALA * * * . .	Occludin_53 BT2_1_3a	LHFYKWTSPPGV MAEYKQNPENTH : * * * *
SRBI_496 nsAR3_11_3A	PTAAWSPPPAAV QTSPSSPPSLTV *:. * * * . : *	Occludin_56 B1_16_3a	YKWTSPPGVIRI HKMESYPGPFPL : * * * * : :
SRBI_497 B.R2_8	TAAWSPPPAAVL SIAGSPTNVRWI : * * * . . :	Occludin_56 4R2_16	YKWTSPPGVIRI YSLSSPTGTVSW * . : * * * . :
SRBI_497 4B2_17	TAAWSPPPAAVL GSVWAPYSASFA : * * * . * : .	Occludin_57 4R2_18	KWTSPPGVIRIL EFTSPVRVGGET : : * * * *
SRBI_499 R4.8	AWSPPPAAVLRL TWSPPSARTLIF : * * * * . * :	Occludin_58 B1_6_3a	WTSPPGVIRILS YLSDDQGVAVTM : * * * * :
SRBI_499 4B2_2	AWSPPPAAVLRL DPGMPATATNR * * * * . .		
SRBI_501 R4.5	SPPPAAVLRLCR SLVPAQHRLTD * * * * : * *		

Figure 3-24 Alignment of phage-displayed peptide sequences with SR-BI and occludin at their cytoplasmic domains

Eighteen peptide sequences were aligned with the cytoplasmic domain of SR-BI (aa 490—501) and occludin (aa 50—58).

Claudin_181 4R2_3	LLCCSCPRKITS GSNILLPEKSTA * * * * :	Claudin_190 4R2_9	TTSYPTPRPYPK TVSIPTHDSTDR * * * * . :
Claudin_182 4R2_16	LCCSCPRKITSY YSLSSPTGTVSW * * * * * :	Claudin_191 T2_2_3a	TSYPTPRPYPKP GSFSIARAVPNP *:. . * * * :
Claudin_183 B1_191a	CCSCPRKITSYP MKNNERRSTAHR * * * * :	Claudin_194 B_R2_3	FTPRPYPKFAPS GTFTNYQQPADA * * * * * :
Claudin_185 T2_1_3a	SCPRKITSYPTP AYYMPWTSYTTS : * * * * .	Claudin_195 B_MT2_1_1a	TPRPYKFPAPSS EPMKLPPPDTRI * * * * .
Claudin_185 MT2_1_3a	SCPRKITSYPTP MSGRTNPFPAH * * * * * :	Claudin_197 EB2_8	RPYPKFPAPSSGK LAHPTSIQKSGK : * * * . * * *
Claudin_186 4B2_6	CPRKITSYPTPR AVVFKTSSDGPR * * * * *	Claudin_197 4B2_2	RPYPKFPAPSSGK DPGMPATATNR * * * * * :
Claudin_188 B1_163a	RKITSYPTPRPY HKMESYPGPFPL : * * * * *	Claudin_199 4R2_21	YPKFPAPSSG--- HPKPTTEHWRALA : * * * :
Claudin_188 4R2_1	RKITSYPTPRPY VSPAQHSNTRPG * * * * * *	Claudin_201 EB2_5	KPAPSSGKDYV- HPATSNKTYWIN : * * * . * * :
Claudin_189 EB2_3	KTTSYPTPRPY HDSLTRQNRHP : : * * *		

Figure 3-25 Alignment of phage-displayed peptide sequences with claudin at its cytoplasmic and helical domains

3.5.12 Random mutagenesis and cloning results

Having selected weakly inhibitory peptides, lead optimisation can bring better bioactive peptides by introducing random mutations into the leads. A series of experiments were performed (sections 3.4.7 through 3.4.12). Random-mutagenesis PCR was used to generate DNA products that were purified for cloning experiments (Figure 3-26). High primer concentration is usually required in random mutagenesis experiment. This justifies the presence of primer dimer in the absence of the template. This will not be a concern as most primers bind the template. Then, M13KE plasmid was measured by Nanodrop spectrophotometry (Figure 3-27). A ratio of ~1.8 is generally acceptable purity for DNA measurement. The estimated ratio of adenine (4.50) is higher than guanine (1.15), cytosine (1.51) and thymine (1.47). Due to the higher ratio of adenine in M13KE plasmid than guanine and cytosine, the observed ratio is slightly higher (2.03), which is still acceptable for this justification.

Following random mutagenesis, M13KE plasmid was digested with the same restriction enzymes used with the randomised PCR-products. Ligated plasmids were purified, spectrophotometrically measured (Figure 3-28) and electroporated in competent cells as summarised in section 3.4.11. Unfortunately, no blue plaques were formed following *E. coli* electroporation. This could be due to electroporation poor efficiency or other parameters during electroporation.

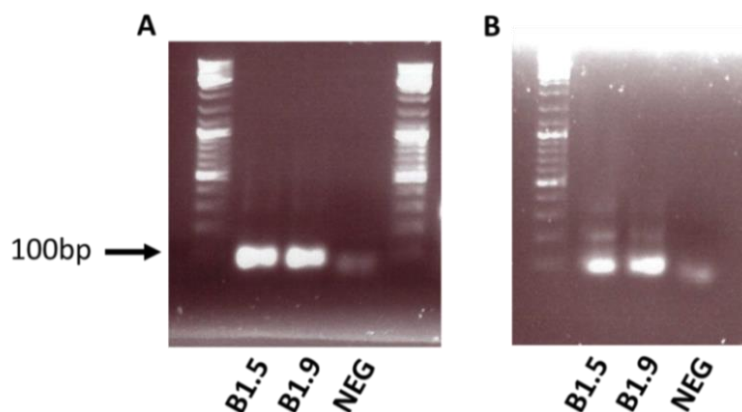


Figure 3-26 Gel electrophoresis of the randomised PCR product

Random mutagenesis was performed using phage particles (A) and PCR-products (B) of the two lead clones as template for epPCR.

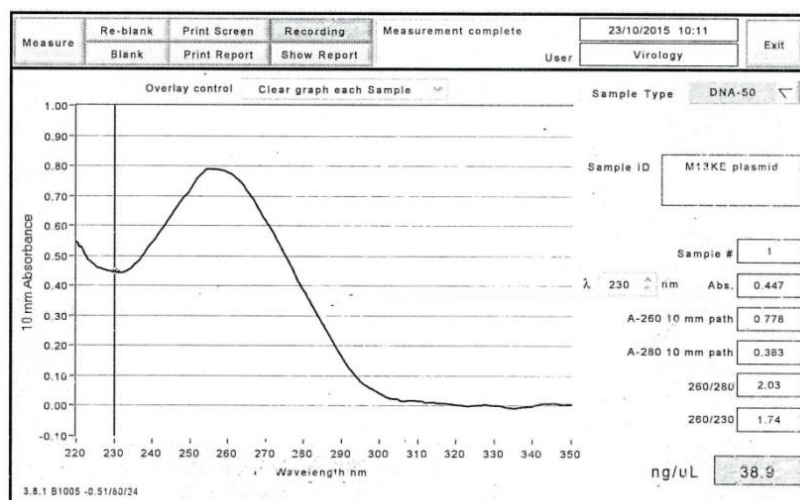


Figure 3-27 NanoDrop measurement of M13KE plasmid

The purity of M13KE plasmid DNA was assessed by measuring the ratio of absorbance at 260nm/280nm.

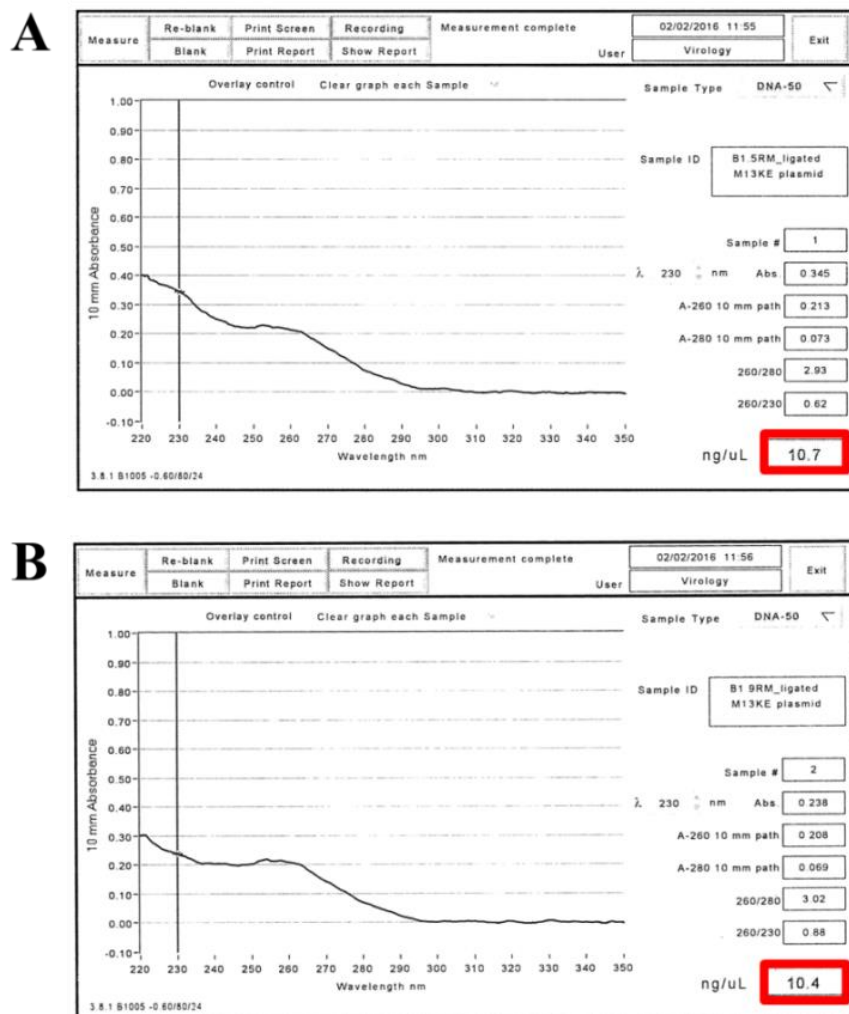


Figure 3-28 Concentration of ligated M13KE plasmids with two leads

Concentrations of M13KE plasmids ligated with B1.5 (A) and B1.9 (B) lead sequences after being randomly mutated were measured by NanoDrop spectrophotometry.

3.6 Discussion

There is a growing concern about the scale of global HCV distribution (Gower et al., 2014). This Chapter aimed at finding inhibitory peptides that block HCV entry. According to Chapter 2, phage library screening was performed in varied ways to increase the possibility of better leads being discovered. Together, all peptides have shown some interesting alignments with HCV receptors and few were able to neutralise HCVpp to some extent. However, the vast majority did not present significant neutralisation, suggesting that the viral envelope glycoprotein presents an intricate surface with many available binding regions. With these results, HCV E2 is a classic example of a recalcitrant protein. Hence HCV is remarkably diverse, discovering pangenotypic entry inhibitors is highly unlikely. To address this, three common genotypes were included (section 3.4.1).

In this Chapter, 30 peptides were tested in neutralisation assays. The presence of weak non-specific as well as specific binders in the phage pool is also likely. Sequences containing WXW motif with high redundancy of histidine are usually target unrelated peptides (Lu et al., 2014). Another study revealed a notorious peptide (SVSVGMKPSRP) as an E2-binder (Hong et al., 2010). Such peptides can be selected due to reduced quality of target bait or inefficient selection method (Table 3-3).

Strikingly, HCV infectivity was enhanced when treated with bM2.6 and bM2.7, and probably bT2.5 phage peptides (Figure 3-5). The three peptides might induce a conformational change upon binding the envelope glycoprotein that favours infectivity. A well-known example is the dramatic conformational change of HIV gp41 upon binding to chemokine receptors CXCR4 or CCR5 (Dimitrov et al., 2005). Peptides presenting an opposite biological activity are particularly desirable. Therefore, no further investigations were made for these three peptides.

B1.9_(1a) is a possible lead for genotype 1 and B1.5_(3a) has the potential to inhibit genotype 3 (Figure 3-6 & Figure 3-7). Evidence based on enrichment (Table 3-7), alignment and ELISA suggest that both lead sequences can bind the envelope of genotype 4. In HCVcc system, nearly 50% neutralisation of infectious particles from genotype 1 was observed with B1.9_(1a) phage treatment (Figure 3-11). But due to their weak affinity, lead optimisation is essential before peptide synthesis and before any further biological assays. This may explain why competitive inhibition assays failed to determine the binding domain using anti-HCV antibodies that are known to bind specifically to a defined region on HCV E2. Background binding is another factor that limits the interpretation of such a competitive assay with weak binders.

With regard to alignment, we have observed SR-BI-mimicking peptides. Having used Δ HVR1-bait protein, the finding suggests that multiple binding sites for SR-BI may exist on the E2 protein. Previous investigations have demonstrated

that hypervariable region 1 (HVR1) of the E2 envelope glycoprotein interacts with SR-BI. It has previously been demonstrated that passage of a Δ HVR1 virus results in adaptive mutations that rescue infectivity and required for the functional interaction with SR-BI (Scarselli et al., 2002, Bartosch et al., 2005). Alignment seems more significant with CD81 than SR-BI, because the truncated E2 construct includes the E2-CD81 binding region aa 474—494 and aa 522—551 (Barth et al., 2006). The tetraspanin CD81 is a key receptor involved in the multistep process of HCV entry mediated by the large extracellular loop. It also forms complexes with claudin-1 to mediate viral entry. CD81 is involved in various cellular responses. Therefore, targeting the receptor to inhibit HCV entry can initiate adverse effects (Feneant et al., 2014).

While HVR1 is involved in HCV life cycle, HCV H77_{N476D/S733F} entry was restored in deleted HVR1. Unlike H77, no SR-BI dependency observed with J6 Δ HVR1 (Prentoe et al., 2014). It is possible that there is an undiscovered domain in between HCV E2₄₁₀₋₆₄₄ responsible for SR-BI binding. Despite that SR-BI is a crucial receptor for HCV E2 binding, specific amino acid determinants essential for HCV recognition are not known. However, 10 single-substitution SR-BI mutants and three swapping mutants (aa 70—87; 114—124 and 187—195), were expressed and tested for HCV sE2 binding. Significant reduction of HCV sE2 binding was observed with the swapping mutant (aa 70—87) and the single point mutant (E210A) (Catanese et al., 2010). The study conducted limited number of mutants.

A group has shown the first tacit evidence that the cytoplasmic and helical domains are implicated in HCV binding (Si et al., 2012). They used a library of 18-mer overlapping peptides that cover HCV receptors (CD81, SR-BI, claudin and occludin). Their best inhibitory peptide (designated CL58) covers the cytoplasmic and helical domains (aa 1—18 of claudin).

Based on the results, peptide candidates should be optimised to make a more potent bioactive ligand. An example of such a development is the quantitative structural activity relationship (QSAR) study aided with computer-based drug design (Jenssen et al., 2008). With time running out, developing these lead peptides as well as optimising electroporation parameters can be continued by the next researcher in the years to come.

4 Epitope Mapping Using a Phage Display Library as a Technical Platform

4.1 Introduction and Aim

A quintessential goal in molecular biology is to understand protein-protein interaction. Effective methods in studying functional amino acid residues that contribute to binding were alanine scanning and competitive binding (Baerga-Ortiz et al., 2002, Boersma et al., 2008). However, comprehensive analysis using this scanning system is laborious. Phage display of a peptide library expedites epitope mapping process considerably.

Epitope mapping of antibodies possessing linear or contiguous epitopes was very successful in previous studies. However, antibodies with discontinuous, conformation-sensitive epitopes have proven much more difficult to map with linear peptides. Epitope mapping of anti-HCV antibodies was successful by enrichment of random phage-displayed peptide libraries. This included subtype-specific antibodies such as AP213 that targets the hypervariable region (HVR) of HCV E2 (Vieyres et al., 2011), mAb 2/69a (Tarr et al., 2012b), AP33 and 3/11 (Tarr et al., 2006) and mapping of B-cell epitopes of E2 protein (Bugli et al., 2001).

In this Chapter, we explored the potential for phage-displayed peptides to map a wide range of antibody formats, using antibodies directed to linear and conformation-sensitive epitopes, and to antibodies in different expression formats including Fab2 and full IgG. Different antibodies were used to map their epitopes. With nearly 1.3 billion variants, the 12-mer phage library may contain hot spot residues necessary for favourable interaction with immunoglobulins,

including anti non-primate hepacivirus (NPHV) antibodies, without a full representation of 10^{15} sequences.

4.2 Materials and Methodologies

4.2.1 Target proteins used for epitope mapping

Details on biopanning and binding analysis are outlined in Chapter 2. The following target proteins were expressed and purified by other groups.

4.2.1.1 Anti-NPHV antibodies

A recombinant soluble ectodomain of the major NPHV glycoprotein E2 was expressed in insect cells as previously described (Krey et al., 2010). Three monoclonal antibodies recognising the NPHV E2 protein were raised in mice by a collaborator (Thomas Krey; unpublished data). These antibodies recognised their antigens in a western blot assay, suggesting that the epitopes recognised by these antibodies are linear. To further characterise these three antibodies, phage-displayed peptides were selected by iterative rounds of biopanning as described in the previous Chapters.

4.2.1.2 Anti-Herpes Simplex Virus antibodies

The immunogen used to generate HSV antibodies (Fab regions from three different antibodies) was pUL32 (also called pp150 or phosphoprotein 150) of cytomegalovirus (CMV). It was unknown if these Fabs were specific for a linear or conformation-sensitive epitopes.

4.2.1.3 HC33.4 monoclonal antibody

HC33.4 human monoclonal antibody (HMAb) is capable of binding to a soluble form of HCV E2. Mimotopes that cross-react this HMAb have not been discovered. Fab region of HC33.4 was provided by Dr Thomas Krey for affinity selection.

4.2.1.4 L-ficolin

Ficolin is an innate molecule that neutralises HCV and other microbes. It has a broad range of specificity, and targets acetylated compounds on microorganisms and activates lectin pathway of the complement system (Lynch et al., 2004, Krarup et al., 2008). Purified recombinant L-ficolin (E4) was prepared (Hamed et al., 2014) and used as a target for phage library screening.

4.3 Results

4.3.1 Peptide sequences using anti-NPHV antibodies

To determine the epitopes of monoclonal antibodies F13.18, G19.3, L18.1 targeting NPHV sE2, two or three rounds of affinity enrichment of a 12-mer random peptide phage-library was performed. The sequence of affinity enriched peptides was determined according to the two elution methods described earlier (section 2.2.2). Peptide sequences were aligned to the NPHV polyprotein amino acid sequence (Figure 4-1). This selection process isolated specific peptides matching two discrete regions of the E2 protein. mAbs F13.18 and L18.1 both

selected peptides that had sequence similarity to amino acids 433—437 of the NPHV polyprotein (SPYNP). In contrast, mAb G19.3 selected peptides matching amino acids in the region 522—527 (PPTYRW) (Table 4-2).

Selected peptides were assayed for interaction with the mAbs by phage binding assay as previously described (Hakami et al., 2015). Further verification of interaction with selected peptides by ELISA revealed a cross-reactivity of mAbs F13.18 and L18.1 with peptides selected by both of these antibodies, while the peptide selected by mAb G19.3 reacted only with the selecting mAb, confirming the specificity of these interactions (Figure 4-2). This revealed that at least two immunogenic regions exist in the NPHV E2 protein.

Table 4-1 Enrichment calculation of NPHV-selected phage pools

Round	Target	PFU/Euate	Titre of amplified pool	Enrichment (Output/input ratio)
R1	F13.18 Ab	Not titrated*	$1.81 \times 10^{10}/100\mu\text{l}$	-
	L18.1 Ab	Not titrated*	$1.07 \times 10^{11}/100\mu\text{l}$	-
	G19.3 Ab	Not titrated*	$1.66 \times 10^{10}/100\mu\text{l}$	-
R2	F13.18 Ab	3.207×10^4	$1.00 \times 10^{12}/100\mu\text{l}$	-
	L18.1 Ab	1.725×10^6	$1.44 \times 10^{12}/100\mu\text{l}$	-
	G19.3 Ab	6.261×10^3	$1.00 \times 10^{10}/100\mu\text{l}$	-
R3	F13.18 Ab	6.005×10^7	Not amplified**	339X
	L18.1 Ab	6.197×10^7	Not amplified**	38X
	G19.3 Ab	1.060×10^5	Not amplified**	28X

* $1\mu\text{l}$ might contain $\sim 10^2 - 10^3$ phage. Wanted clones from round one were not titrated to avoid excessive clonal loss.

** Clones were randomly selected from the third round eluate (not the amplified pool) to avoid amplification-associated bias which may give rise to misleading sequence redundancy.

Table 4-2 NPHV-antibodies-selected phage clones after two rounds of bacterial elution strategy and three rounds with the chemical elution method

Conserved residues by physicochemical properties are indicated by colours. Fully conserved amino acids are indicated in red letters. Green colour indicates strong conservation. Weak conservation is shown as blue.

Target	Φ Clones (Standard)	Sequence	Φ Clones (Bacterial)	Sequence
F13.18	R3.2	SSPYNPTKVEAP	Be2.1	MTMSAAPQGTSV
	R3.3	GNTSVVHLEPY	Be2.2	SGGVLAGKGGST
	R3.6	GNTSVVHLEPY	Be2.4	SPYNPYKPSAIR
	R3.7	GNTSVVHLEPY	Be2.5	DMSYTSKPAALS
	R3.8	SSPYNPTKVEAP	Be2.6	MQSAYQHNPRQA
			Be2.7	SPYNPYKPSAIR
L18.1	R3.1	GTVSPYNPWKLQ	Be2.1	GSSSIWHVYSSD
	R3.2	VNPNIYVTKPYWT	Be2.2	SGANPFYLSKSN
	R3.5	WSPYNPHKMIDY	Be2.3	FVNPHYVSKLRE
	R3.6	GTVSPYNPWKLQ	Be2.4	FVNPHYVSKLRE
	R3.7	IGTNGGPYNPSK	Be2.6	SGANPFYLSKSN
	R3.8	IGTNGGPYNPSK	Be2.7	WSPYNPHKMIDY
G19.3	R3.1	ELANRYPPTYRW	Be2.1	DNMTTGDYSPTTE
	R3.2	SMYTWGMTAESG	Be2.2	TSTASTPPSYNW
	R3.3	ELANRYPPTYRW	Be2.3	TSTASTPPSYNW
	R3.5	IPSYHWRGTNTH	Be2.4	TSTASTPPSYNW
	R3.6	SMYTWGMTAESG	Be2.5	TSTASTPPSYNW
	R3.7	SMYTWGMTAESG	Be2.6	TSTASTPPSYNW
	R3.8	ELANRYPPTYRW	Be2.7	TLYSWGQAPYTT
	R3.9	ELANRYPPTYRW	Be2.8	KLWAPSQPSYLW
	R3.10	ELANRYPPTYRW	Be2.9	TSTASTPPSYNW

	423	444		
	NPHV	GQRLNCTYGSPYNPICKGPLD		BLOSUM62 score
F_R3.7&8		IGTNGGPPYNPSK	2X	36
		* * . * * * *		
F_R3.2&8		SSPYNPTKVEAP	2X	31
		. * * * * *		
F_be2.4&7		SPYNPYKPSITIR	2X	31
		* * * * * . . .		
L_R3.1&6		GTVSPYNPWKLQ	2X	31
		* * * * *		
L_R3.5		WSPYNPHKMIDY		31
		* * * * *		
L_R3.2		VNPNYVTKPYPWT		
		* *		
F_be2.6		MQSAYQHNPRQA		
		* . * . . .		
F_be2.5		DMSYTSKPASLS		
		. : * * . . .		
F_R3.3&6&7		GNTSVWHLREPY	2X	
		* : . * *		
L_be2.2		SGANPFYLSKSN		
		. : . * :		
L_be2.3		FVNPHYVSKLRE		
		. : . * :		

Figure 4-1 Alignment of anti-NPHV-selected phage clones with NPHV envelope

Viral envelope protein belongs to the hepacivirus horse isolate DH1/HUN/2013.

* Fully conserved residues

: Conservation of strong groups

. Conservation of weak groups

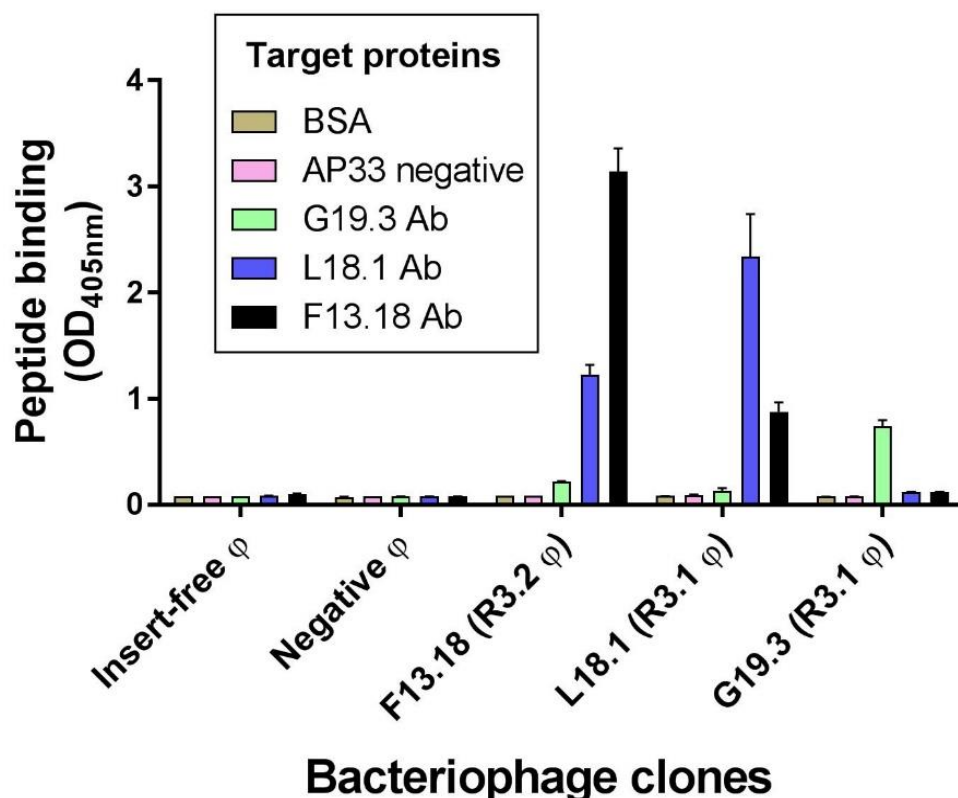


Figure 4-2 Binding of representative individual affinity-selected phage clones to different target proteins

One phage clone selected from each antibody target was tested for binding against all three antibodies (F13.18, L18.1 and G19.3 antibodies). BSA and mAb AP33 are included as negative control targets. A phage possessing no insert, and one possessing an insert selected against an anti-HIV mAb were used as negative control phage. Because of the unique SPYNP consensus, along with limited antibody concentration provided, only one phage representative from each antibody target was tested. Error bars represent $\pm$ SD of the mean (n=2).

4.3.2 Peptide sequences using HC33.4 antibody Fab region

As done with anti-NPHV antibodies, HC33.4 Fab region was the target bait in a phage library sorting. With enrichment being monitored, an abundant phage output was conspicuous with HC33.4, exceeding 3 million, in comparison with 1:7 Fab as a control (Table 4-3). Sequencing analysis reveals redundant

sequences and consensus amino acids worthy of further investigation (Table 4-4 & Figure 4-3). All selected peptides (HC3.2 through HC3.28) were highly reactive to the original HC33.4 target except HC3.5 that showed less similarity with the other peptide candidates. And most striking of all, HC3.7 peptide adopts two conformations by its exclusive interaction with AP33 and HC33.4. AP33.12 and 3/11.30 control peptides were specific to their original target antibodies. Compared to previous assays, this was the most specific and sensitive measurement of phage clones against their original target, read after few minutes of substrate addition. Some peptides have the ability to align the viral protein (aa 406—426) at various residues in a non-gapped alignment. However, these peptides represent typical mimotopes for HC33.4 Fab region compared to the wild type sequence (QLINTNGSWHIN). Despite the low alignment score of HC3.7 mimotope, it is the most interesting sequence by being highly cross-reactive to HC33.4 mAb and AP33 but not 3/11 (Figure 4-4).

Table 4-3 Enrichment calculation between the second and third round

Target	Round	Output (per eluate)	Enrichment
HC33.4 Fab	R2	6.516×10^4	-
	R3	2.172×10^{11}	$3,333,333 \times^*$
1:7 Fab	R2	3.833×10^4	-
	R3	5.365×10^4	No enrichment

*Highest enrichment ever observed.

Table 4-4 HC33.4-selected phage clones

Fully conserved amino acids are indicated in red blocks. Green blocks indicate strong conservation. Weak conservation is shown as blue.

Φ Clones	Peptide sequences
HC3.2	GPKDSLAL T SEW
HC3.3 and HC3.4	KAP M GW S P S SEW
HC3.5	TAYTSN T KNVLW
HC3.7 and HC3.29	KPSDL T LYRAHW
HC3.8 and HC3.11	KPH L V T I P LSEW
HC3.9	KPQAL T AIPQWQ
HC3.28	GKPT I TAS P GEW
HC3.12	SKPQLQYS I SEW
HC3.13	SVASNWY F TNEW
HC3.25	KPV L H S TEGDWT
HC3.27	KAP M GW S P S SEW

	406	426	
HCV E2	GAKQNIQLINTNGSWHINSTA		Score
HC3.2	GPKDSLAL [*] TSEW		16
HC3.3		KAPMGWSP [*] SEW	12
HC3.7	KPSDL [*] TLYRAHW		11
HC3.8	KPHLVT [*] IPLSEW		11
HC3.9	KPQALTAIPQWQ		12
HC3.12	SKPQLQYSISEW		13
HC3.13		SVASNWYFTNEW	15
HC3.25	KPVLHSTEGDWT		24
HC3.28	GKPTITASPGEW		17

Figure 4-3 Alignment of HC33.4-selected phage clones with HCV E2

HC33.4 peptide sequences showed conservation with residues in the wild type sequence of HCV envelope glycoprotein E2 (QLINTNGSWHIN) such as lysine (412) and tryptophan (420).

* Fully conserved residues

: Conservation of strong groups

. Conservation of weak groups

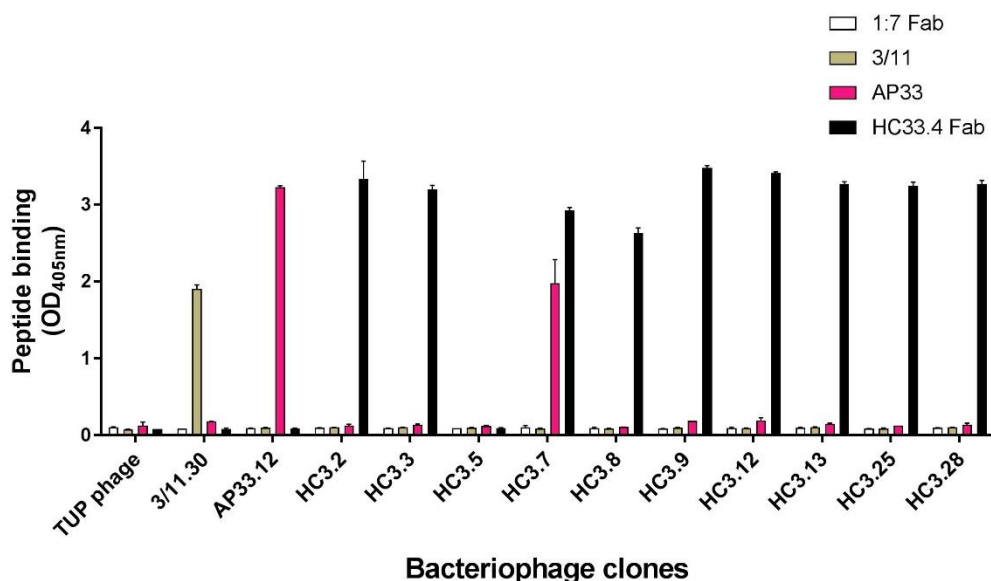


Figure 4-4 Binding results of HC33.4-selected phage clones

All HC33.4 peptides were tested using HC33.4 Fab, AP33, 3/11 and 1:7 Fab region. AP33.12 and 3/11.30 were used as control phage clones which are specific to AP33 and 3/11 antibodies respectively. Error bars represent $\pm$ SD of the mean (n=2). TUP: Target un-related peptide.

4.3.3 Peptide sequences using HSV-1 antibody Fab regions

Having discovered favourable peptides and robust data with anti-NPHV and HC33.4 antibodies, 3 herpes antibodies were subjected to peptide selection using the same protocol. Despite conducting a fourth round, no enrichment observed between rounds. Sequencing analysis of selected phage members from the third and the fourth round did not reveal a clear consensus (Table 4-5). No strong and specific binding was obvious for HSV Fab #4 and #5. However, 1.3.5 peptide binding showed a statistical significance ($p < 0.0434$) and showed amino acid conservation with 3 domains in the CMV pUL32 protein that are consistent with 1.3.5 peptide alignment. Therefore, 1.3.5 phage-displayed peptide (VSPPSLKVEHRH) might be a specific binder. Its alignment with the

immunogen (CMV pUL32) used to raise the target antibodies and the binding results suggest a possible peptide binder (Figure 4-5).

Table 4-5 Peptide sequences selected against three HSV Fab regions

Fully conserved amino acids are indicated in red letters. Green colour indicates strong conservation. Weak conservation is shown as blue.

Target	Φ Clones	Sequence
#1 HSV Fab	1.3.2	EMQLDGRGPWGI
	1.3.5	VSPPSLKVEHRH
	1.4.9‡	SGVYKVAYDWQH
	1.4.11	SGVYKVAYDWQH
	1.4.14	SGVYKVAYDWQH
	1.4.16	SGVYKVAYDWQH
	1.3.4	HTPAATLHPVFL
	1.3.7	LLAMPDTMLPRS
	1.3.8	NHLSTPVWSITG
	1.4.13	HPLTWNLRSSPA
	1.4.15	HPLTWNLRSSPA
#4 HSV Fab	4.3.2	HSPGPLAIKLAG
	4.3.4	MTREPSPSNLGH
	4.3.8	SPVLSKVELPPT
	4.3.7	HLHHGERSKNAW
	4.4.9‡	VFAYHLRIPSGD
	4.4.10	VFAYHLRIPSGD
	4.4.11	VFAYHLRIPSGD
	4.4.16	VFAYHLRIPSGD
	4.4.12	QFDYMRPANDTH
	4.4.13	QFDYMRPANDTH
#5 HSV Fab	5.3.1	TQAAFNMNVSRL
	5.3.8	HLNMMDLLRKDD
	5.3.5	FPRLSLPLDGSS
	5.3.6	FPRLSLPLDGSS
	5.3.7	FPRLSLPLDGSS
	5.4.11‡	FPRLSLPLDGSS
	5.3.3	SVGLSNHFGLHV
	5.4.12	GFPITATWPVRG
	5.4.13	MQKTTTPMMPLSN

‡: Due to the absence of enrichment, round 4 was performed and compared with round 3 sequences. Phage clones were named as: [target].[round of enrichment].[clone number].

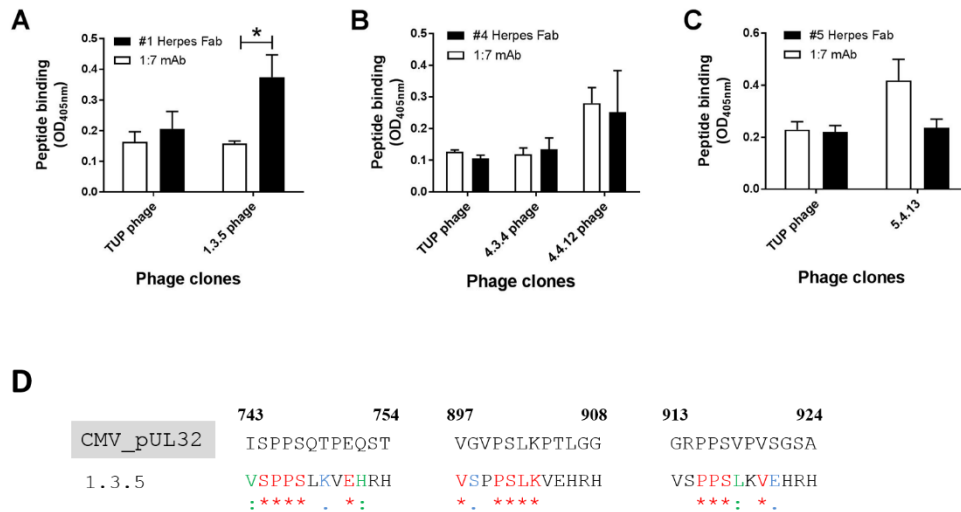


Figure 4-5 Selected phage representatives for HSV Fab binding

One phage-displayed peptide candidate from HSV Fab #1 (A), two phage clones from Fab #4 (B) and one clone from Fab #5 (C) were selected for binding test using enzyme immunoassay. Peptide candidate 1.3.5 (A) showed amino acid conservation with three domains in CMV pUL32 protein used as immunogen to generate the three HSV Fab regions (D). Comparison in figure A was made using paired t-test (* $p < 0.05$). Error bars represent $\pm$ SD of the mean ($n=2$).

4.3.4 Peptide sequences using L-ficolin

Biopanning was performed using purified L-ficolin as bait. No clear consensus or specific peptide sequences were selected against ficolin based on alignment and phage binding assay. This shows the difficulty of biopanning large proteins along with the presence of peptides that can interact with the target in a non-specific manner. No strong phage binders were selected. However, one peptide (Fic.B2.6) demonstrated a homology with the HCV E2 protein (aa 686). It is plausible that Fic.B2.6 peptide (STGTIDLTHRRF) is a probable ficolin binder

based on independent binding results and alignment with HCV envelope glycoprotein from which this innate protein interacts (Table 4-6 & Figure 4-6).

Table 4-6 Ficolin-selected phage clones

Fully conserved amino acids are indicated in red letters. Green colour indicates strong conservation. Weak conservation is shown as blue. Grey blocks represent dicysteine-containing motifs.

Target	Φ Clones (Standard)	Sequence	Φ Clones (Bacterial)	Sequence
Ficolin E4	R4.1	TTS ^{blue} HNGRL ^{green} PHAP ^{red}	Be2.3	STPG ^{red} CC ^{blue} AH ^{green} DHFR
	R4.4	YNFR ^{blue} PPTV ^{green} PGQP ^{red}	Be2.4	GMT ^{red} GVAT ^{blue} IRPLD
	R4.2	VSFTNTHIERLD	Be2.6	ST ^{red} GT ^{blue} ID ^{green} LTHRRF
	R4.6	GALLPSMNK ^{grey} GHW	Be2.1	DGAPTLKRPYPR
			Be2.5	GVVLPPVGLSQR

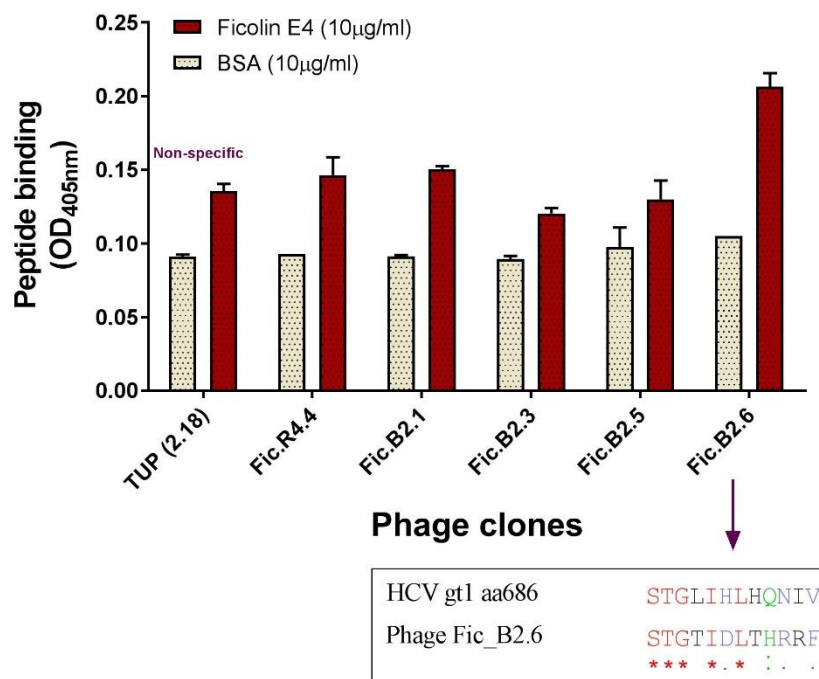


Figure 4-6 Ficolin-selected phage binding assay

Five phage clones were selected from Table 4-6 and tested against ficolin and BSA as a control target. Conservation between Fic.B2.6 clone and HCV E2 glycoprotein sequence at position 686 is shown. Error bars represent $\pm$ SD of the mean (n=2). TUP: Target un-related peptide.

4.4 Discussion and Conclusion

Epitope mapping using phage-displayed peptide libraries is exceedingly useful tool for rapidly determining the specific molecular interactions of many epitopes, with the selected peptides acting as antigen mimics. Epitope mapping helps in developing vaccines and therapeutic discoveries; as well as an essential process in research and diagnostics. In this Chapter, several mimotopes that mimic viral antigens were identified. However, there are many different factors that must be taken into consideration that can result in selection failure (section 6.1).

There are no previous reports of antibodies directed to the non-primate hepacivirus (NPHV) surface glycoproteins. This represents the first demonstration of the antigen region of NPHV E2 glycoprotein. Mimotopes selected against anti-NPHV antibodies and HC33.4 Fab region provided the most successful peptide enrichment results and discovered the most convincing epitopes. They were confirmed by multiple peptide alignment with their natural ligands, enrichment during selection and by binding data. For instance, peptide alignments with NPHV envelope have shown high scores using BLOSUM62 matrix tool (Figure 4-1), and also confirmed by phage binding assay with specific and high affinity interaction (Figure 4-2). Noticeably, it was discovered that the SPYNP motif is shared by two antibodies: F13.18 and L18.1. Epitope mapping of monoclonal anti-NPHV E2 mAbs by a phage-displayed peptide library proved both specific and efficient if conditions permit. The work in its entirety was aimed at finding evidence regarding non-primate hepacivirus transmission from horses to humans and whether NPHV antibodies are able to detect HCV antigens. Being the closest relative to HCV, NPHV might broaden our understanding of HCV evolution, pathogenesis and immune response (Scheel et al., 2015).

As to HC33.4 target, it is notable to briefly review HC33.1 as one of the HC33 subset of anti-HCV antibodies. Asn417Ser mutation in the HCV E2₄₁₂₋₄₂₃ epitope can be potently neutralised by HC33.1 and overcomes the glycan shield. However, cell culture infectious virions (HCVcc) possessing L413I mutation are

resistant to HC33.1 at high concentration (Keck et al., 2014). HC33.1 has already been crystallised with a 13-mer soluble peptide (RQLINTNGSWHIN) that represents HCV E2₄₁₂₋₄₂₃ epitope (Li et al., 2015). Interestingly, Figure 4-3 shows that position Asn415 can be replaced with serine or threonine, underpinning the study of Li et al.

Phage library screening with HC33.4 was different from other selection experiments in terms of the considerable phage output eluted after the third round, exceeding 10^{11} in comparison with 10^4 as usually selected in earlier rounds with most target proteins. This is, by far, the greatest enrichment factor ever reported for phage-displayed peptide library biopanning against any target protein. We interpret this high enrichment as selection of very high affinity interactions. When we performed sequence analysis and ELISA binding assays, this interpretation was confirmed. Most selected phage members were highly reactive to HC33.4. Similarity between peptide candidates and HCV E2 was clear. Such a high alignment score supported by strong binding is a categorical evidence of the specificity of interaction. AP33 and 3/11 mAbs bind the same region of the HCV E2 protein, but in discrete conformations (Tarr et al., 2015).

The wild type HCV E2₄₁₂₋₄₂₃ epitope (QLINTNGSWHIN) adopts at least three different conformations to bind AP33, 3/11 and HC33.4. Strikingly, HC3.7 phage clone was highly reactive to HC33.4 HMAb as well as AP33, while all other selected members did not exhibit this binding characteristic. Despite that HC3.7 and HC3.8 are alike in their sequence, the latter did not bind AP33 as

HC3.7 does. Previous AP33-reactive phage (AP33.12) and 3/11-reactive phage (3/11.30) were amplified and tested with HC33.4. Control phage clones (Tarr et al., 2006) were reproducible but there was no cross-reaction with antibodies (Figure 4-4).

The major determinants of AP33 mouse monoclonal antibody recognition to HCV E2 are L413, N415, G418 and W420 (Tarr et al., 2006, Potter et al., 2012). Based on peptide alignment (Figure 4-3) and binding assay (Figure 4-4), HC3.7 unique peptide confirms that L413 and W420 are key residues for AP33 recognition. The remaining two determinants can be replaced by N415L and G418A. Having found a conserved tryptophan, a recent investigation underpins the significant role of W420 as a CD81-binding residue (Cowton et al., 2016). The data show that AP33 and HC33.4 mAbs have strict preference to tryptophan at position 420.

To add more on HCV envelope glycoprotein, molecular dynamics analysis suggested that E2₄₁₂₋₄₂₃ is a flexible region (Barone et al., 2016). In two domains of HCV E2, aa 425—443 and 529—535 contact residues are shared by HC-11 and CBH-2 antibodies. HC-1 showed a restricted binding to the region aa 529—535. From this study, epitope mapping revealed the significance of HCV E2 region aa 529 to 535 in CD81 binding (Keck et al., 2011).

Previously selected AP33.12 peptide with the sequence HLANHQGKW, which can adopt only one conformation of the naturally occurring peptide 412—423 with AP33 antibody, matched HC3.7 sequence DLTLYRAHW with many conserved residues. Asparagine in AP33.12 is the only amino acid change with no conservation in its HC3.7 counterpart. The lysine single residue change in HC3.7 could provide an explanation of its flexibility by forming two different conformations.

Earlier studies on HCV have investigated the interaction of various forms of the virus glycoprotein to CD81 (Owsianka et al., 2001). This suggests that mimetics of HCV E2 can be identified from phage peptide libraries using linear antibodies. Having identified strong HC33.4-peptide binders that mimic HCV envelope, synthesised peptides can be used to detect similar anti-HCV antibodies in previously infected patients. Another possibility is to immunise animal models with these peptides that mimic HC33.4 and comparing antibodies with those raised against HC3.7 peptide that adopts two different forms.

In collaboration with another group as to herpes antibodies mapping, peptide microarray of pUL32 protein was used to probe its 260 overlapping peptides for binding to the same Fab regions used as target bait in phage biopanning. Microarray did not show conclusive results. These inconclusive findings from both experiments suggest that the preliminary data of this target bait was probably not correct, or the antibodies might bind to a different CMV protein. The only peptide that might be specific is 1.3.5 peptide (VSPPSLKVEHRH)

targeted against HSV Fab #1, albeit with poor signal, that showed a non-gapped alignment with the original immunogen (Figure 4-5).

According to these results, and considering the optimised selection technique in Chapter 2, phage libraries of random peptides prove useful in lead peptides discovery and epitope mapping if conditions were optimum. In this Chapter, two Fab regions were used. One with success as observed with HC33.4 Fab and the other with neither clear consensus nor strong binding interaction as with HSV Fab regions. Three whole antibody molecules that neutralise NPHV were used to investigate their antigenic determinants. Despite using whole antibody molecules, the results showed that peptides were specific to the Fab region based on binding and alignment results. Chapter 5 details the characteristics of Fc-binding ligands. Some antibodies are easy to map than others, and may be more tractable than large carbohydrate-binding proteins.

In relation to biopanning against L-ficolin, Fic.B2.6-phage-borne sequence (STGTIDLTHRRF) might be a specific peptide. Non-specific binding with other phage clones was observed (Figure 4-6). This drawback is discounted by peptide alignment with HCV E2₆₈₆₋₆₉₇ and statistical analysis. Ficolin specifically binds targets bearing acetyl groups and glycosylated sites (Krarup et al., 2005, Hamed et al., 2014). This might justify the inability to select high affinity peptides to this elusive target. The problems of biopanning against large proteins such as L-ficolin is that it is highly flexible, and may not adopt a stable structure that peptides can bind to. Phage peptide libraries are not glycosylated

to thoroughly study glycosylation effect on binding. However, Fic.B2.6 is still promising as a non-glycosylated ligand for future investigations including peptide synthesis. It has been shown that ficolin enhances ebola infection. Ebola glycoprotein is highly glycosylated and interacts with lectin receptors (Favier et al., 2016). A likely medical application is to use Fic.B2.6 peptide or its advanced form, if properly developed, as an antagonist of ficolin. All in all, proteins that naturally bind to acetylated carbohydrates may be difficult as targets for affinity-selection of phage-displayed peptides. They might lack the regions necessary for molecular interactions with short peptides, and some proteins require special ligands to interact. This can complicate the process of finding ligand-binding pockets of a given target conformation.

5 Discovery of Immunoglobulins- Binding Peptide

5.1 Introduction

The technique of phage-displayed peptide libraries has long been used as a promising application for the development of miniprotein scaffolds and identification of novel binding domains. Understanding the binding characteristics of protein-peptide interactions and antibodies-specific peptides is fundamental for many biotechnical applications, and can predict novel interactions (Olsson et al., 2012). Such identifications can provide striking new observations and patentable peptide entities. For instance, phage libraries of cyclic peptides revealed two interesting Fc-peptide binders sharing the two cysteines spacing (DeLano et al., 2000). In addition, a discovery of specific peptide that binds the anti-HIV b12 antibody was fulfilled using phage display technology (Zwick et al., 2001).

Biopanning against a specific target protein can be complicated by the selection of unwanted peptides. This is influenced by the type of target protein, the purity of a protein preparation and the biopanning methodology. It is widely described that affinity selection of peptides representing linear epitopes are easier to mimic with peptide libraries. However, antibodies recognising conformation-sensitive epitopes have been much harder to map using phage-displayed peptides. While many antibodies have been successfully mapped with this approach, off-target enrichment of peptides interacting with regions of an antibody outside the paratope of the antibody is possible.

In this Chapter, we describe the selection of Fc binding peptides that were selected by biopanning. We affinity selected a peptide library against the monoclonal antibody 1:7, which is known to recognise a conformation-sensitive epitope in the E2 protein of HCV.

Identification of Fc-specific ligands as well as manipulation of Fc domain for therapeutic purposes has become an interesting field of research. The binding to Fc-receptor is important for anti-viral function of anti-HIV antibodies. In an outstanding example, the ability of a broadly neutralising anti-HIV antibody to neutralise virus challenge was achieved by engineering out the Fc receptor binding domain but not complement binding (Hessell et al., 2007). On the functional level, preliminary data showed that peptides bind the Fc fragment of immunoglobulin G may downregulate FcR-initiated inflammatory responses (Wang et al., 2014). Having observed an antibody-binding peptide, blocking Fc receptors may restrain diseases where pathology is influenced by Fc-mediated effects (Aleyd et al., 2015).

Successful identification of peptides that specifically bind IgM and no other antibody classes was possible using a phage library (Glee et al., 1999). Specific peptides against antibodies might be considered as artefacts when using similar antibody targets for epitope mapping. On the other hand, they can be used for other useful applications if properly validated. Therefore, peptides selected from phage libraries can also be useful ligands for various applications including antibody purification. Peptides have been used as affinity ligands to isolate

immunoglobulins G by binding to their Fc region (Yang et al., 2010). The use of antibodies as affinity ligands was hampered by the necessity of extensive purification prior affinity chromatography and variation in their quality, and this may delay their approval (Huang and Carbonell, 1995).

Phage display of random peptide libraries revealed a novel ligand with specific antibody binding properties. For instance, the peptide sequence HWRGWV was successfully used as a peptide resin to retain pFc fragment of human IgG antibodies (Yang et al., 2010). In addition, peptide libraries were effectively used to select specific ligands for use in affinity chromatography separations. The peptide sequence YNFEVL was used as a ligand to purify S-protein mixed with bovine serum albumin (BSA) via affinity chromatography (Huang and Carbonell, 1995).

Purification of proteins using affinity chromatography depends on specific interactions between the protein of interest and the ligand. Peptide affinity tags have also been used for purification of proteins. For instance, polyhistidine affinity-tagged proteins can be purified using immobilised metal-affinity chromatography (IMAC) (Bornhorst and Falke, 2000). In this study, a novel peptide sequence that interacts with the Fc region of human antibodies was validated by competitive inhibition, peptide synthesis and binding assays. This suggests its possible use in biotechnical and therapeutic applications.

5.2 Materials and Methodologies

5.2.1 Affinity selection using a conformational antibody

A 12-mer phage peptide library was screened for four iterative rounds of affinity selection (biopanning) against 1:7 human anti-HCV antibody, which is a conformation-sensitive immunoglobulin (Allander et al., 2000, Johansson et al., 2007). Enrichment was monitored using plaque assay (Table 5-1).

5.2.2 Phage binding immunoassay

Phage binding assay was performed considering our optimised method (Hakami et al., 2015), with the addition of mixing phage with the blocking buffer. Selected phage (7.3.1) was tested for binding affinity using its original target (1:7 mAb) and other human antibodies (Edwards et al., 2012). Single chain variable fragment (ScFv) and Fab regions from these antibodies were also utilised.

5.2.3 Investigating the quality of peptide library and selected phage members

Two non-binder (Nb) peptides from the first round of selection were recovered, titrated for plaque assay, PCR-amplified and sequenced to investigate the library diversity. The two peptides (Nb1.1 ‘ELSFERKELRLD’ and Nb1.2 ‘NALPLQWTASDT’) do not show any homology, and this indicates that the library is diverse enough to identify promising ligands. This was supported by screening the same library against other target baits.

5.2.4 Competition assay

Having identified a ligand that cross-reacts with several antibodies, we hypothesised that 7.3.1 peptide binds the Fc portion of the IgG. To test this, competition assay was performed using proteins known to bind Fc fragment of human antibodies such as the unconjugated anti-human IgG (Fc-specific) antibody (Sigma, I2136) as a competitor to 7.3.1 phage candidate.

5.2.5 Peptide synthesis and binding studies

The peptide sequence HHFLCENPDFCT (with its GGGS naturally occurring linker and biocytin) was synthesised by ThermoFisher Scientific with >96% purity [NH₂]HHFLCENPDFCTGGGS-Lys(Biotin)[COOH]. Direct coating of the target antibody followed by peptide detection with streptavidin-alkaline phosphatase (RD System, #AR001) showed some background noise. Alternatively, 2 µg.mL⁻¹ NeutrAvidin (Pierce) was coated on a 96-well plate for sandwich enzyme-linked immunosorbent assay (ELISA). After blocking the plate with 1% fish gelatin, 2 µM peptide dissolved in 0.1% dimethyl sulfoxide (DMSO) was added to react with a panel of human and mouse antibodies, followed by detection with secondary labelled antibodies. Unlike phage binding assay (Figure 2-17), fish gelatin (Sigma, G7041) showed less background noise compared to BSA in direct biotinylated peptide assay.

5.2.6 BCA assay

Bicinchoninic acid (BCA) assay for colorimetric detection was used to measure peptide concentration (Pierce, 23227) and using a flat-bottom polystyrene plate (Costar, 9017). This assay offers a more sensitive protein measurement than NanoDrop spectrophotometry. By using a standard curve from different BSA concentrations, peptide concentrations were determined from the best-fit line (Figure 5-1).

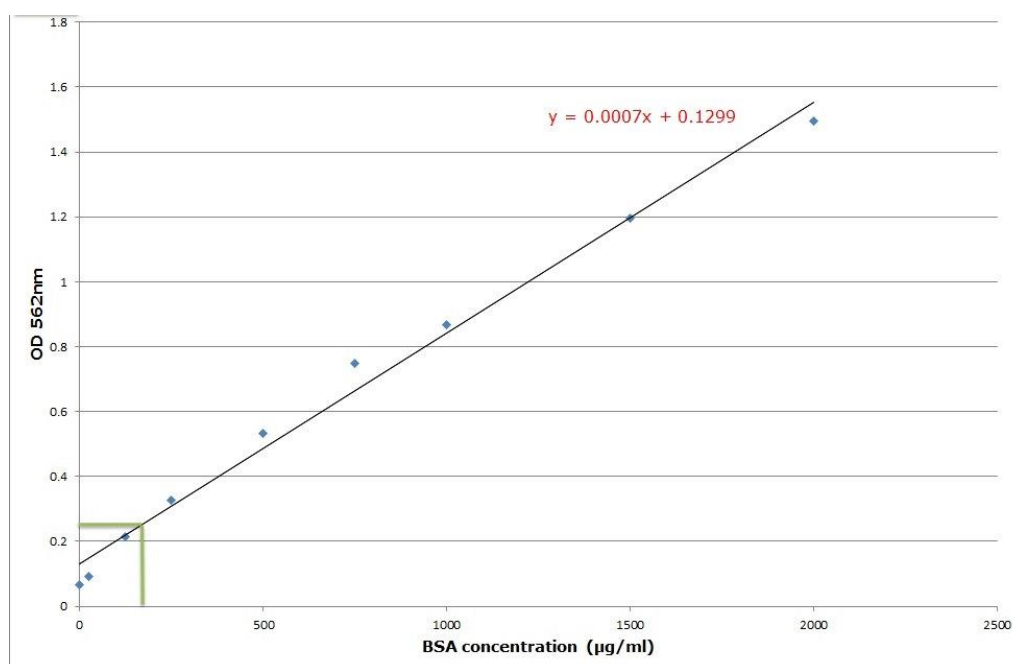


Figure 5-1 A standard curve from BCA assay

Five different concentrations of albumin standards were reacted with BCA reagent for the colorimetric detection of the unknown 7.3.1 peptide concentration. The optical density was measured at 562nm. The absorbance of 7.3.1 peptide was plotted with the best-fit line to determine its concentration. The peptide was diluted ten-times, therefore the concentration was multiplied by the dilution factor to determine the stock concentration of 7.3.1 peptide.

5.3 Results

5.3.1 Enrichment and quality of library

Biopanning was performed using mAb 1:7 as a target. In general, most peptide selection experiments generate different peptide sequences at different frequencies with some consensus sequence. Here, we found an interesting unique peptide sequence and 156 times enrichment after 4 rounds of biopanning (Table 5-1). The fact that only a single peptide was selected with an enriched

phage pool provides a clue of its specificity to the target protein. There was no mutation in gene II of phage 7.3.1 (section 2.2.9). This excluded the possibility that the selected phage clones have a propagation advantage.

Table 5-1 Phage enrichment and peptide frequency

Round	Φ Input	Φ Output	Enrichment	Peptide redundancy
1	1×10^{11}	Not titrated	-	Not sequenced
2	1×10^{11}	1.456×10^4	-	Not sequenced
3	1×10^{11}	1.495×10^4	No enrichment	HHFLCENPDFCT 8/8 (100%)
4	1×10^{11}	2.338×10^6	156 X	HHFLCENPDFCT 2/2 (100%)

5.3.2 Phage reactivity with other immunoglobulins

The highly redundant peptide was preliminary tested against the background substrate and a control antibody along with its intended 1:7 mAb target. Another phage displaying a di-cysteine peptide was used as a control. The high signal observed with the control antibody and the very low background noise urged us to investigate this further (Figure 5-2, A). This ligand might behave as an antibody-binding peptide.

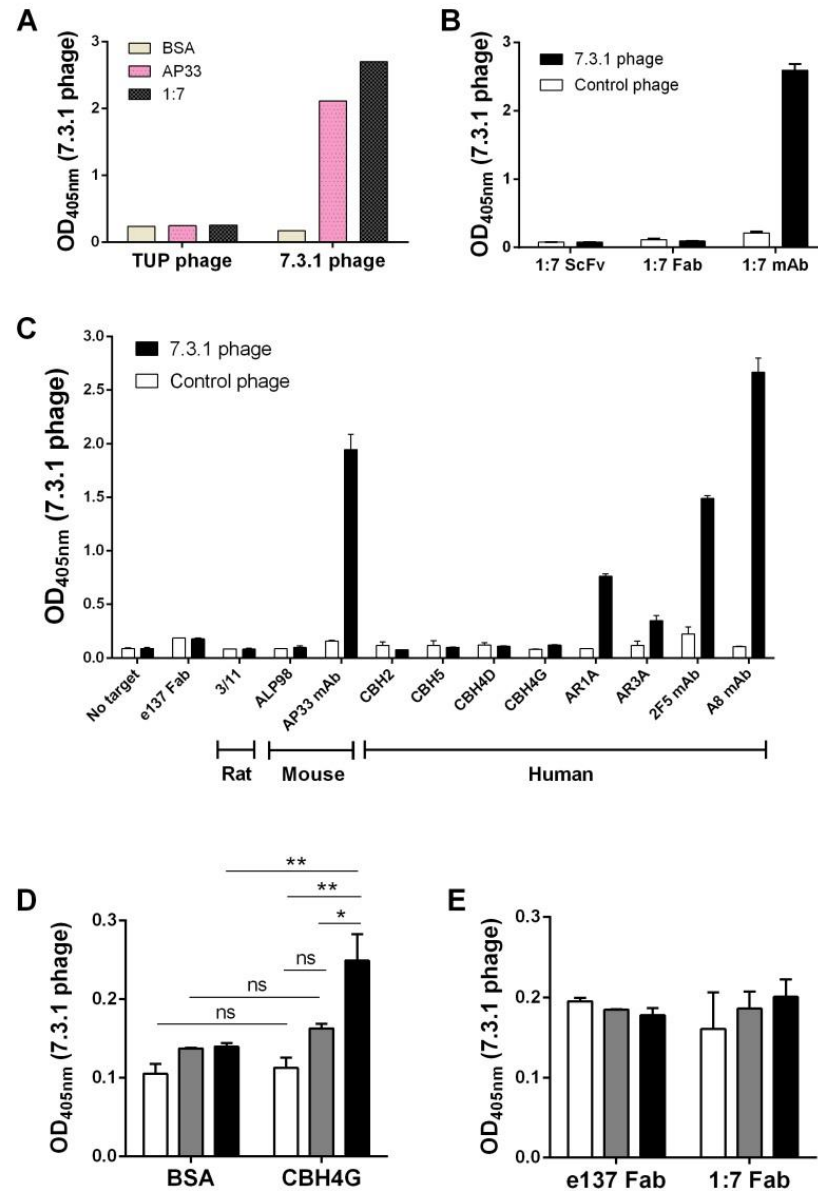


Figure 5-2 Binding of 7.3.1 phage to human and other antibodies

(A) The mAb 1:7-selected phage-displayed peptide 7.3.1 was observed to interact both with 1:7 and a control mouse monoclonal antibody (n=1). (B) The test was repeated using expressed Fab and single chain variable fragment (ScFv) of 1:7 (n=2). (C) The specificity of 7.3.1 phage was tested against some antibodies including a human anti-HIV antibody (mAb 2F5) and other anti-HCV monoclonal antibodies such as A8, AR1A and AR3A (n=2). In figures D and E, two phage controls were used: A di-cysteine phage-displayed peptide (grey) and a target unrelated peptide with no cysteine (blank). All error bars represent the mean $\pm$ SD of duplicate samples. Figure D was analysed using two-way ANOVA (* p < 0.05; ** p < 0.01).

Phage 7.3.1 bound several human and some mouse antibodies such as AP33, but not 3/11, an antibody isolated from a rat. AP33 and 3/11 antibodies bind the same linear region on HCV glycoprotein E2 (Tarr et al., 2006). Having observed a positive interaction with irrelevant immunoglobulins, these preliminary data suggest that Fab regions do not interact with 7.3.1 peptide. In other words, Fc region might be implicated in this interaction. To underpin the finding, 1:7 Fab region was used to screen the same peptide library in order to confirm specificity of 7.3.1 peptide selectivity to 1:7 Fc region.

5.3.3 Competition between 7.3.1 phage and Fc-specific antibody

To characterise the specificity of interaction, 7.3.1 phage was used to compete the binding of the unconjugated anti-human IgG (Fc-specific) antibody. Phage candidate was competed with the Fc-specific IgG in a dose dependent manner using 1:7, A8 and 2F5 human antibodies as coating materials. A control competitor was anti-complement C4 antibody (Figure 5-3, B). The result showed that the more competitor is added, the less 7.3.1 phage binds to human antibodies. By adding less concentration of phage candidate, a dose-dependent curve was clearly observed.

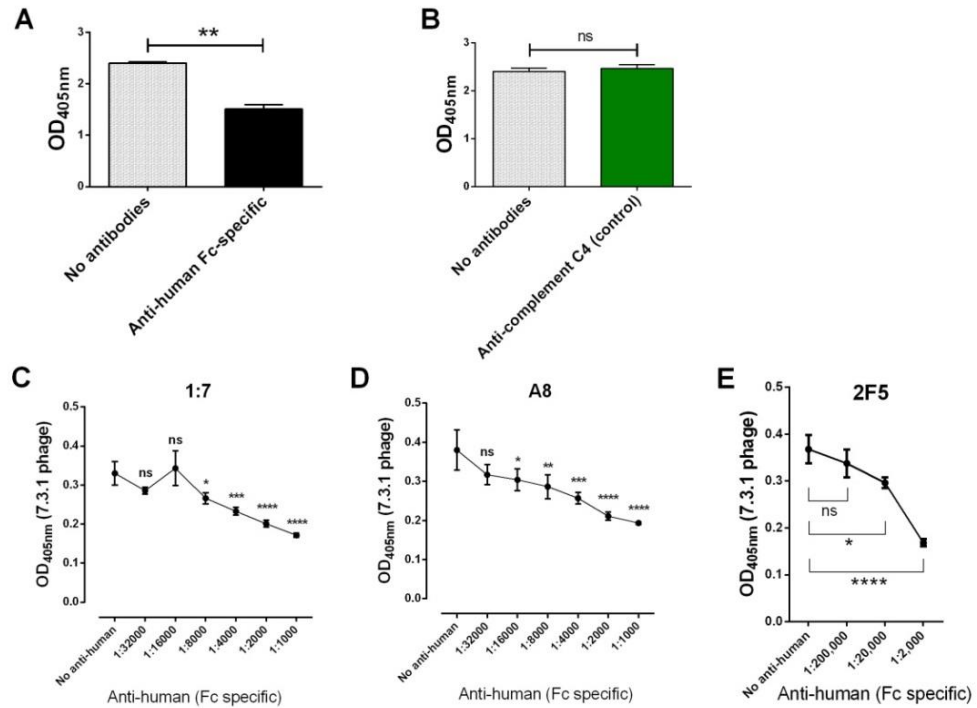


Figure 5-3 Competitive binding between 7.3.1 phage-displayed peptide and anti-human Fc-specific antibody

At high phage concentration (10^{11}), inhibition of binding was tested using 1:2000 dilution of anti-Fc specific human antibody as a competitor (A) and anti-complement C4 antibody as a control. Figures A and B were analysed using paired t-test. At low phage concentration (10^{10}), the competitive binding between 7.3.1 phage and anti-human Fc-specific antibody was measured using 1:7 (C), A8 (D) and 2F5 (E) antibodies as coated targets, and the results were analysed using one-way ANOVA with the Bonferroni method (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). Error bars represent $\pm$ SD of the mean ($n=2$).

5.3.4 Reproducibility with the biotinylated peptide

Affinity of immunoglobulins to the phage candidate was validated. Antibodies were added to coated biotinylated 7.3.1 peptide and detected by secondary labelled-antibodies. Interestingly, and as hypothesised, CHB4G mAb specifically interacted with 7.3.1 ligand, but was negative using phage binding

assay due to low concentration of displayed peptide on the phage surface that only supports a high affinity interaction (Figure 5-2 & Figure 5-4).

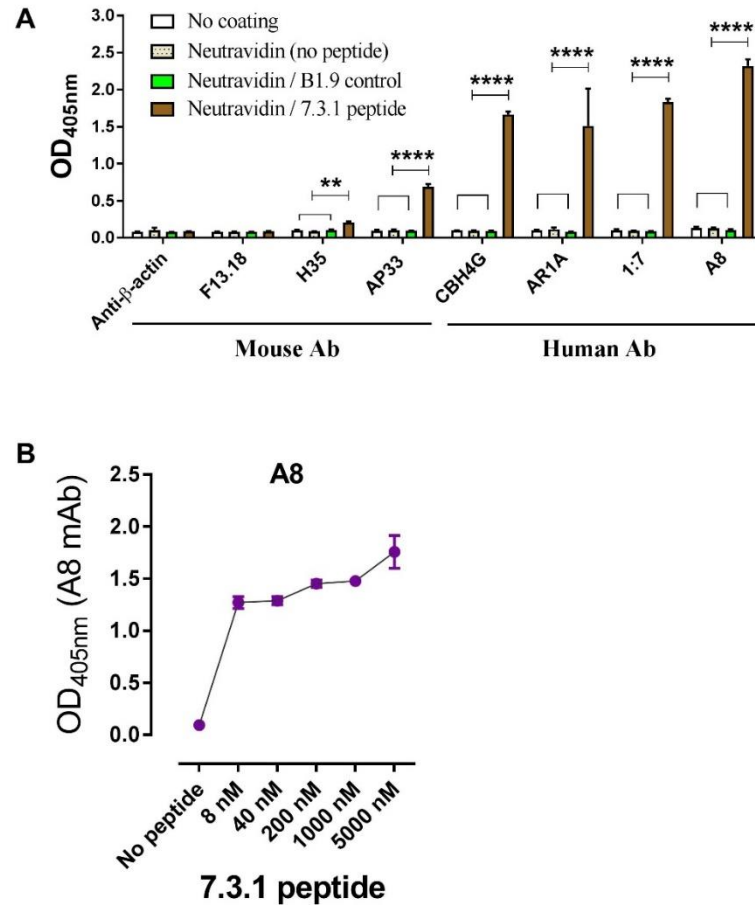


Figure 5-4 Human and some mouse antibodies interaction with 7.3.1 biotinylated peptide

(A) Four human antibodies (A8, 1:7, AR1A and CBH4G) and four mouse antibodies (AP33, H35, F13.18 and anti-β-actin) were reacted with a coated 7.3.1 biotinylated peptide. Error bars represent the mean $\pm$ SD (n=3). The results were analysed using paired t-test (** $p < 0.01$; **** $p < 0.0001$). (B) A8 monoclonal IgG antibody was reacted with a range of concentrations of 7.3.1 biotinylated peptide at five-fold serial dilutions. Error bars represent the mean $\pm$ SD (n=2).

5.3.5 Peptides selected against 1:7 Fab

The same peptide library used for mAb 1:7 was affinity selected against the 1:7 purified Fab region. This time, the peptide HHFLCENPDFCT was not selected, supporting the hypothesis that the Fc portion is involved in the interaction. No enrichment was observed, and selected phage were mostly without peptide insert (6 out of 7 selected M13KE clones). The only peptide sequence recovered was HKDLYPSTFENT. It was rather a negative binder to both 1:7 whole molecule and Fab region. As often is the case, this selection was due to weak non-specific interaction of phage particles. This is inevitable in any phage affinity selection.

5.4 Discussion and Conclusion

A conformational antibody (1:7 monoclonal antibody 'mAb') was one of the test proteins in one of the preceding selections as a difficult antibody which is conformation-sensitive. The initial result was interesting enough to synthesise a biotinylated peptide. Confirmed by competitive binding and peptide assay, one noticeable and novel peptide was validated with unique binding characteristics, and this remained to be investigated. Remarkably, this peptide (designated 7.3.1) has not been selected despite using other whole antibody molecules. It is possible that this ligand would be of great benefits in different applications, given its high cross-reactivity to various antibodies.

Using antibodies as target bait, screening phage display libraries could identify peptides at any region due to its unbiased nature. Here, we demonstrated that a phage-displayed peptide library can identify useful ligands. Following peptide selection against the whole 1:7 antibody molecule, a mere one peptide was selected with unique binding characteristics. Having identified a peptide that cross-reacts 1:7 mAb, 1:7 Fab was used to screen the same peptide library as a control. No specific peptide was selected using 1:7 Fab, suggesting that 7.3.1 ligand is an interesting sequence to investigate.

Two consensus residues at the N-terminus of 7.3.1 (HHFLCENPDFCT) with another similar ligand (HWRGWV) that also binds IgG constant domain (383 SNGQPEN 389) were observed. The second and third position of 7.3.1 ligand (HF) matches the first two amino acids of the aforementioned 6-mer ligand (HW). These ligands differ from those plastic-binders that have already been investigated (Huang et al., 2010, Hakami et al., 2015), and might suggest that the consensus indicates a special ligand with interesting characteristics. Two cysteines flanked by five amino acids in our 12-mer ligand might form a disulfide bond. There is no evidence the two cysteines within 7.3.1 peptide is essential to its unique characteristic as an antibodies-binding ligand, but it might maintain a rigid structure of the peptide.

While these experiments failed to identify a peptide that can interact with the paratope of the 1:7 mAb/Fab, peptides that interact with the IgG Fc binding domain are interesting and can be useful for other research applications. An interesting study increased the half-life of a model protein by fusing it with a 13-mer IgG Fc region binding peptide; (sequence: DCAWHLGELVWCT). This allows the engineered protein to bind serum IgG *in vivo* and enhances its half-life in the circulation by reducing recombinant protein clearance (DeLano et al., 2000, Sockolosky et al., 2014). Interestingly, the last three amino acids of the mentioned 13-mer ligand at the C-terminus (WCT) are homologous to the penultimate positions of 7.3.1 ligand (FCT). Having this aligned, the third (F) and the seventh (N) amino acids of 7.3.1 have the same physicochemical properties with the fourth (W) and the eighth (E) residues of the 13-mer peptide, and they all present the same binding property. Both peptides share odd numbers of cysteine spacing.

The 42kDa *Staphylococcus aureus* protein A has already been investigated and used as an IgG-Fc-fragment binder (Atkins et al., 2008), but expensive and should be highly purified from *S. aureus* before use. Therefore, other purification methods and optimisations are desirable (Lao et al., 2013). The fact that 7.3.1 peptide binds 1:7 whole antibody molecule and other human IgGs, but not the Fab or ScFv, indicates that the peptide targets the Fc region, and can be an alternative ligand to protein A.

The role of antibody Fc fragment in controlling viral infections has been characterised, but little is known about anti-HCV antibodies Fc region in controlling HCV infection (Ball et al., 2014). Tissue damage and inflammation can be enhanced by antibody-mediated Fc receptor activation. Interfering with this mechanism may prevent autoimmune diseases and other maladies such as ulcerative colitis and rheumatoid arthritis (Aleyd et al., 2015). Such unique peptides might prevent deleterious antibody-mediated diseases. On the other hand, ligands that bind the Fc portion of antibodies may hinder phagocytosis (Foster, 2005).

In earlier studies, specific ligands from peptide libraries have been suggested for use in affinity purification of biological molecules. The peptide sequence YNFEVL was successfully selected as a specific ligand to S-protein with potential use in purification techniques (Huang and Carbonell, 1995). In another example, histidine residues show the strongest interaction with immobilised metal ions, hence used in immobilised metal-affinity chromatography (IMAC). However, non-specific binding of untagged protein is a drawback of polyhistidine affinity tags that may contaminate the final product (Bornhorst and Falke, 2000).

When screening phage libraries using human antibodies, careful investigation must be considered. An early study has identified IgM-specific peptides

inadvertently using phage libraries. The specificity of interaction has not been identified. They have suggested a conserved domain implicated in that interaction (Glee et al., 1999).

Novel peptides have several advantages over immunoglobulins when used as antivirals. Antibodies large multi-domain proteins provoke immune response that limits its therapeutic use. Compared to small peptides, they require complex and cost-intensive manufacturing process for synthesis (Zoller et al., 2011). We confirm that 7.3.1 peptide was serendipitously selected against 1:7 mAb. Further detailed characterisations revealed the possibility of this peptide as a candidate for human, and perhaps some mouse, antibodies binding for purification purposes on affinity matrices. The failure to interact with all mouse immunoglobulins could be attributed to allotypic variation in the constant regions (Vidarsson et al., 2014).

Phage display offered a cheap method for the discovery of novel protein-specific probes, i.e. mimtags or mimotopes, that can tag epitopes in detection assays. In a successful discovery, mimtags were able to interact with interleukin-4 receptor (IL-4) and IL-13 (Ahmed et al., 2014). In summary, this Chapter provides compelling evidence of a robust mimtag with ability to interact most human and some mouse antibodies which was synthesised, biotinylated and used for *in vitro* enzyme immunoassays. The unique binding characteristic makes this peptide a possible candidate for some applications including human antibody purification and blocking the Fc-receptor-mediated maladies.

5.4.1 Future work

Additional experiments are still required to confirm binding to other antibody classes and other Fc species including sheep, goat, horse and donkey. It is unlikely that this ligand will bind goat antibodies. Competitor immunoglobulins used in the inhibition assay (section 5.2.4) were produced in a goat. In addition to that, selective labelling to immunoglobulins and testing its possible use in affinity purification of human Ig are also crucial. To increase potency of binding, a cyclic multimeric version of 7.3.1 ligand should be generated.

6 Final Conclusion

Phage-displayed peptide libraries are promising tools for discovery of novel peptide sequences. This is one of the most effective molecular techniques for epitope mapping and ligand discovery. Despite being invented more than 25 years ago, this cutting-edge technology is still rapidly expanding. Advances in phage display techniques have improved our understanding of molecular interactions. Peptides identified from affinity selections can show interesting reaction kinetics. To find a rare variant within a diverse library, an optimised protocol is essential, using various experimental conditions. This optimisation can broaden our understanding of affinity screening and phage binding characteristics. Antibodies and large proteins were used as target bait (Table 6-1). In Chapter 2, I proposed revised approaches to biopanning and phage ELISA protocols. The Chapter aimed at optimising the phage selection techniques to be performed in a much more precise fashion to allow selection of promising peptides and to discriminate specific signal from the noise. Even with these optimisations, they are not a sure-fire way of getting the desirable ligands. In Chapter 3, I attempted to examine these optimisation steps to discover inhibitory peptides that neutralise HCV entry. Few candidates have been suggested, but in this research we identified 2 lead peptides that appeared to have inhibitory properties, and which have sequence similarity to the receptors known to interact with the HCV E2 glycoprotein. In Chapter 4, an attempt to map the antigenic determinants of some target proteins was conducted. The high affinity peptides outcompeted the non-specific binders. An interesting finding with one unique peptide sequence from chapter 4 led us to study this interaction in greater detail (as described in Chapter 5). While biopanning is a lengthy technique, it

offers an efficient and relatively inexpensive approach for investigating receptor-binding sites by mapping their epitopes.

Table 6-1 Target proteins used in this study

Target name	Category
HCV E2	Envelope glycoprotein
L-ficolin	Large oligomeric protein
HC33.4 anti-HCV	Fab region
Anti-HSV-1	Fab region
Anti-non-primate hepacivirus	Whole antibody molecule
1:7 anti-HCV	Whole antibody molecule

6.1 Factors Determining the Effectiveness of Peptide Selection

6.1.1 Amino acid compositional bias

Following the first electroporation of a diverse M13KE library, natural selection due to *E. coli* and amino acid change counteract the experiential selection performed by the operator (Zanconato et al., 2011). To minimise this biological constraint, a maximum of three rounds of recursive cycles of peptide selection should be implemented. Section 2.2.2 summarises an optimised selection method which helps identifying consensus in one or two rounds of selection.

6.1.2 Type and nature of protein bait

A fundamental factor for the success of selection is the quality and type of the target bait. According to the results presented in this thesis, the protein of interest can be categorised into ‘easy’ or ‘difficult’ targets. For instance, it is much more difficult to generate useful data from phage-displayed peptide libraries when using a conformational antibody as bait, and paratope-specific mimotopes were not generated for these antibodies. However, peptides against different sites might be selected (Chapter 5). Antibodies recognising linear epitopes are the most readily mapped targets for phage library screening. To identify inhibitors, a well-folded microbial antigen is required. Large oligomeric proteins such as ficolins were recalcitrant. The same difficulty was noticed with HCV envelope glycoprotein.

6.1.3 Propagation-related bias

Peptides with α -helical structure on phage pIII slow down amplification. On the other hand, fast propagation was linked with β -turn peptides (Derda et al., 2011). Furthermore, mutation in the Shine-Dalgarno (SD) sequence of phage gIIp results in a fast propagation rate. This has also been observed in phage clones lacking displayed peptides. Several laboratories have selected HAIYPRH-displaying phage using different target proteins. Mutation in the SD region was a coincidence with this peptide, suggesting that this sequence has nothing to do with the target as well as with the fast propagation characteristic (Brammer et al., 2008). Phage mutants with enhanced propagation rate were discovered

recently that include G6813A mutation for clones displaying HAIYPRH, AKIDART, HTPQVHHPELTH and G6793A mutation in phage displaying no peptide. Other mutations included C6810T, A6809C, A6802T, C6799T and T6798C (Nguyen et al., 2014).

It is worth mentioning that phage production was affected most by media composition and temperature, while other factors also played a role such as bacteria concentration. This is particularly significant when using M13 phage for large-scale applications (Warner et al., 2014).

6.1.4 Peptide length, library diversity and hot spot residues

As a general rule, the more diverse the peptide library, the higher the chance for finding peptides to the protein of interest. When using a 12-mer library, there are 4×10^{15} (20^{12}) possible peptide sequences, but other factors affect this huge representativeness of peptide diversity. *E. coli* transformation efficiency allows the uptake of M13KE plasmids that produce 10^9 dodecapeptide library. Despite that, such exhaustive peptide variants could generate desirable ligands if hot spot residues are presented in a sequence that favours the target protein. The 7-mer library on the other hand presents all possible amino acid sequences; (20^7) or 1.28×10^9 .

In Chapter 4, one of the selected peptides against L18.1 antibody is IGTNGGPYNPSK (Table 4-2). The antibody requires the motif TXGXPYNP

for binding which spans a length of 8 amino acids. This specific motif is impossible to select from a 7-mer library. In such an example, the dodecapeptide library is favoured over the fully diverse heptapeptide library. A good example to further this point is the motif SPYNP in Chapter 4. The target antibodies require this overt consensus of residues for a highly robust interaction. Absence of necessary and specific order of amino acids within the peptide library would fail the selection process or produce feeble cross-reactivity at best.

Peptides work at different lengths. Short peptides have the potential of binding receptors to induce cellular functions. For instance, oxytocin hormone in fin whales consists of 9 amino acids (Acher et al., 1964), yet produces a significant biological function. This suggests that available phage libraries provide a suitable peptide length for the study of protein-protein interaction.

Another point worth considering is the amino acid frequency within a phage library. Following library construction, the redundancy of each residue in the entire peptide repertoire cannot be predicted. Knowing how frequent each amino acid is in a library would greatly help characterising enriched sequences and exclude non-specific peptides based on the frequency observed. A study used next-generation pyrosequencing methodology and Sanger-sequencing of unselected phage library and revealed that glycine, serine, arginine and leucine were the most redundant, whereas cysteine was the least frequent amino acid (Dias-Neto et al., 2009). Tryptophan was not highly presented. From Chapter 4, tryptophan was observed in many reactive peptides, suggesting that its structure,

which is the largest and the rarest amino acid with two aromatic rings, is essential for binding interactions, although it is encoded by one codon (TGG). Different naive libraries would have different observed frequencies of amino acids. Deep sequencing of a commercial library (NEB, #E8110) showed similar redundancy observed by Dias-Neto et al., 2009 study. Serine, leucine and proline were the most redundant, whereas lysine, tryptophan and cysteine were the rarest residues observed.

6.1.5 Temperature and target concentration

It is worthwhile to test the effect of temperature (25—37°C) during peptide selection. This should depend on the type of target protein used. In general, room temperature provides a favourable environment for many target proteins as many antibodies were tested in this study at room temperature with committed peptides being selected. In regards to target concentration, high concentrations are recommended in the first round. Lower concentrations in later rounds can bring high affinity binders and avoid avidity effect.

6.1.6 Washing stringency

This is particularly critical at the library screening in earlier rounds. Notwithstanding that the recommended washing stringency at the first round is 10 times, the very few copies of precious peptides might get washed at such a washing stringency. We recommend reducing that in the first round. To get a better view, Figure 2-15 in Chapter 2 shows the number of phage particles washed off in each washing cycle.

6.1.7 Elution method

Various elution methods were suggested to liberate phage clones after the washing step. These include chemical elution and the use of a specific eluent. We suggested another method (section 2.2.2) to be included for more efficient elution of all reactive and target-bound phage.

6.2 Study Limitations

Given the above, it can be generally observed that a ‘successful’ final result from a phage library screening is not guaranteed. Phage display is a time consuming technique, involving a biological step of phage propagation in a bacterial host. The growth advantage of each clone displaying unique peptide sequence might vary greatly in each iterative round. This might result in inconsistency of phage titres within the pool. Peptide complexity in phage display is limited to 10^9 sequences due to reduced transformation efficiency. Ribosome display on the other hand has exhaustive diversity and permits a selection experiment of nearly a trillion sequences (Chen et al., 2010). When using a phage library for the sake of discovering and developing antimicrobial peptides, organisms can develop mechanisms of resistance (Yeaman and Yount, 2003).

Before screening, target proteins need to be prepared at high quantity. Apart from antibodies, some target proteins have to be in high quality as the case with viral proteins. By targeting HCV E2, peptides may not inhibit all genotypes,

unlike genotype-independent entry inhibitors that targets host receptors such as the SR-BI antagonist ITX 5061 (Zhu et al., 2012). But these inhibitors might show adverse events as receptors have other biological functions.

Lead optimisation as well as development is labour-intensive and time consuming. This process is subject to failure at any step throughout the optimisation period. It requires a whole array of experiments and expertise to turn a bioactive peptide into a marketed drug. It covers a series of numerous methods such as peptide production, optimisation for pharmacological and physiological properties, engineering these properties to generate drug-like traits of the peptides and replacing peptide bonds with alternatives. Finally, I hope that this thesis benefits researchers in the foreseeable future.

7 Appendix

7.1 Display of Lead Peptides as Monovalent Maltose-Binding Protein Scaffolds

The low peptide concentration on phage pIII protein does not allow detailed investigations of the lead peptide such as use in surface plasmon resonance (SPR) and in inhibition studies for the determination of IC_{50} . Peptide synthesis is essential for these types of analyses. The downside remains in the high cost of chemical peptide synthesis. Display of peptides as monovalent maltose-binding proteins makes a cost-effective approach. An alternative way to chemical peptide synthesis is the pMAL-pIII plasmid vector which replaces its leader sequence of maltose binding protein with M13 phage leader sequence (Figure 7-1) that contains Acc65I/EagI restriction sites. This allows subcloning of phage peptides for more binding investigations.

Primers were designed based on pMAL-pIII plasmid which include malE reverse primer 5'-ACCGTTATAGCCTTTATCGC-3' (nucleotides 1628—1647 of the pMAL-pIII plasmid) (Zwick et al., 1998) used with gIIIs primer (section 2.2.9). Other primers were designed (Koressaar and Remm, 2007, Untergasser et al., 2012) to confirm malE region of the vector. They are pMAL FWD primer 5'-GGTCGTCAGACTGTCGATGAAGCC-3' (nucleotide 2647) and pMAL REV primer 5'-TGTCCTACTCAGGAGAGCGTTCAC-3' (nucleotide 3244). The lead B1.5 peptide was cloned into pMAL-pIII vector (Figure 7-2). Due to time limitation, the project was suspended for the next researcher. The high efficiency express competent *E. coli* (NEB, #C2523) is

recommended for expression of pMAL-pIII plasmid and purified by affinity chromatography on amylose resin (NEB, #E8021).

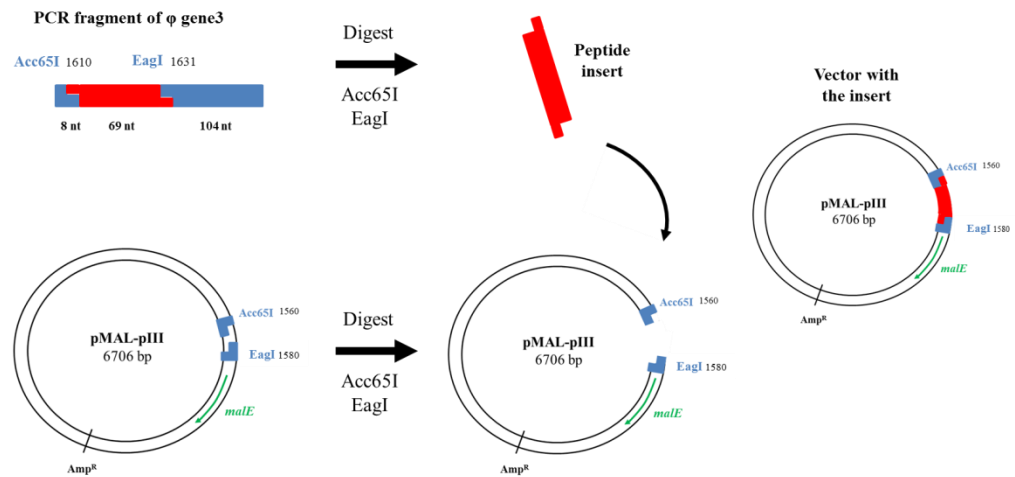


Figure 7-1 Overview of pMAL-pIII plasmid and restriction sites

The pMAL-pIII vector and M13KE recombinant phage clones share *Acc65I* and *EagI* restriction sites. Purified phage nucleotide insert (red) can be cloned into pMAL-pIII plasmid using DNA ligase enzyme. The small size of maltose binding protein (42kD) compared to phage (~16.3MD) allows expression of high peptide concentration.

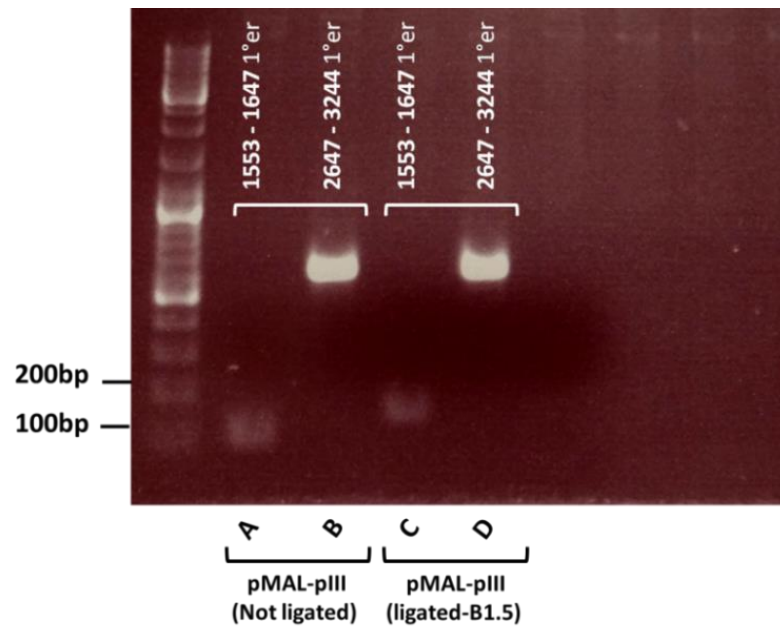


Figure 7-2 Gel electrophoresis of pMAL-pIII vector following digestion

After digesting an uncloned pMAL-pIII vector, the cut insert was purified and run on a gel (A). Leader sequence of phage B1.5 lead was digested and cloned (C). As a control, the malE region was PCR-amplified using 2647 FWD primer and 3244 REV primer. Both plasmids (cloned and uncloned) show a band of 598bp (B and D).

7.2 Unpaired Cysteine in Phage Libraries

There was a strong negative selection against single cysteine residues in displayed peptides. Unpaired cysteine is almost completely absent in phage display systems (Kay et al., 1993). Other researchers have tried to introduce (NNK)₆-TGC-(NNK)₆ that encodes single cysteine within the peptide.¹¹ Strikingly, DNA sequencing revealed that a stop codon appeared before the TGC codon (Fukunaga and Taki, 2012). It has been hypothesised that lone cysteine residues are detrimental to phage propagation and can form an intermolecular disulfide cross-bridges with another cysteine in different protein (Kay, 1996). Interestingly, an acid-resistant phage displaying single cysteine (sequence: SHPSECEHQTRM) was selected in our laboratory non-specifically. The clone propagation was not affected by the peptide sequence.

¹¹ K = G or T, whereas N is any nucleotide with equimolar ratio.

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