
UNIVERSITI PUTRA MALAYSIA

# SELECTION OF HIGH AFFINITY PEPTIDES AGAINST HEPATITIS B CORE ANTIGEN FROM A PHAGE DISPLAYED CYCLIC PEPTIDE LIBRARY

HO KOK LIAN

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# SELECTION OF HIGH AFFINITY PEPTIDES AGAINST HEPATITIS B CORE ANTIGEN FROM A PHAGE DISPLAYED CYCLIC PEPTIDE LIBRARY

By

HO KOK LIAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

March 2002

UPM

*This thesis is dedicated to my family, beloved one and friends……*

Abstract of thesis submitted to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science.

**SELECTION OF HIGH AFFINITY PEPTIDES AGAINST HEPATITIS B CORE ANTIGEN FROM A PHAGE-DISPLAYED CYCLIC PEPTIDE LIBRARY**

**By**

**HO KOK LIAN**

**March 2002**

**Chairman: Dr. Tan Wen Siang, Ph.D.**

**Faculty: Science and Environmental Studies**

Hepatitis B virus is the prototype member of the family *Hepadnaviridae* which causes acute and chronic liver diseases worldwide. The viral nucleocapsid containing a partially double stranded DNA is surrounded by an envelope comprises three distinct but related surface proteins (HBsAg), termed as small (S), medium (M) and large (L)-HBsAg. The essential subunit of the nucleocapsid is a polypeptide comprising 183 amino acids known as core protein (HBcAg). HBcAg produced in *Escherichia coli* is capable of self-assembly into core-like particles and can be purified easily with ammonium sulphate precipitation and sucrose gradient centrifugation. Core particles make of full-length HBcAg were used as substrate in biopanning with a cysteine constrained phage-displayed heptapeptide library. The most frequently identified phage clones displayed the cyclic peptides C-WSFFSNI-C and C-WPFWGPW-C. The relative dissociation constant ($K_d^{rel}$) values for the interaction between the phages and HBcAg were determined by an equilibrium binding assay in solution. The $K_d^{rel}$ values for

phage bearing peptides C-WSFFSNI-C and C-WPFWGPW-C for full-length and truncated HBcAg are less than 10 and 30 nM, respectively, which are 17- and 7-fold stronger than that of phage bearing the linear peptide LLGRMK. The selected phages were able to compete with monoclonal antibody C1-5 for a binding site on the surface of core particles, suggesting that the docking site of these phages may partially overlap with the epitope of mAb C1-5, which was mapped at amino acid positions 78 to 83 at the tips of the core particles. The heavy chain of mAb C1-5 is hydrophobic and was proposed to be the contact region for HBcAg. Interestingly, the isolated peptides C-WSFFSNI-C and C-WPFWGPW-C are mainly composed of hydrophobic amino acids and may bind to the same region as mAb C1-5. A synthetic linear peptide bearing the sequence WSFFSNI inhibited the binding of L-HBsAg to core particles *in vitro* with an inhibition concentration ($IC_{50}$) approximately 9.8 μM. The additional of cysteine residues to both the N- and C-termini of the peptide greatly reduced the solubility of this cyclic peptide, and as a result the $IC_{50}$ is approximately 20-fold higher than that of WSFFSNI. A suitable recombinant carrier therefore is needed in order to reduce the hydrophobicity of the peptides and subsequently acts as a delivery system for targeting the peptide to virally infected cells.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia untuk memenuhi keperluan bagi mendapat Ijazah Master Sains.

**PEMILIHAN PEPTIDA-PEPTIDA BERAFFINITI TINGGI TERHADAP ANTIGEN TERAS HEPATITIS B DARIPADA PERPUSTAKAAN PEPTIDA PAMERAN FAJ YANG TERBATAS SECARA DISULFIDA**

**Oleh**

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**Mac 2002**

**Pengerusi: Dr. Tan Wen Siang, Ph.D.**

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Virus Hepatitis B adalah ahli kepada keluarga *Hepadnaviridae* yang menyebabkan masalah kesihatan sedunia serta merupakan punca utama penyakit hati kronik dan akut. Nukleokapsid HBV mengandungi DNA bebenang dua separa yang diselaputi oleh sarung yang terdiri daripada tiga jenis protein permukaan (HBsAg) iaitu HBsAg kecil (S), sederhana (M) dan besar (L). Subunit nukleokapsid ini merupakan satu polipeptida yang terdiri daripada 183 asid amino yang dikenali sebagai protein teras (HBcAg). Unit-unit HBcAg yang dihasilkan dalam *Escherichia coli* bergabung untuk membentuk partikel teras yang boleh ditulenkan dengan pemendakan ammonium sulfat dan pengemparan kecerunan sukrosa. Partikel teras yang diperbuat daripada HBcAg berpanjangan asal telah digunakan sebagai substrat dalam "*biopanning*" dengan menggunakan perpustakaan peptida pameran faj yang terbatas secara disulfida. Faj-faj yang membawa peptida C-WSFFSNI-C dan C-WPFWGPW-C merupakan faj-faj yang paling banyak dipilih. Pemalar penceraian relatif ($K_d^{rel}$) antara faj-faj dan HBcAg

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telah ditentukan dalam asai keseimbangan pengikatan dalam cecair. Nilai-nilai $K_d^{rel}$ bagi faj yang membawa peptida C-WSFFSNI-C dan C-WPFWGPW-C dengan HBcAg berpanjangan asal dan bundung adalah kurang daripada 10 dan 30 nM masing-masing, iaitu, 17 dan 7-kali lebih kuat daripada faj yang membawa peptida lurus LLGRMK. Faj-faj yang terpilih juga berupaya bersaing dengan mAb C1-5 untuk tapak pengikatan pada permukaan partikel teras. Penemuan ini mencadangkan bahawa tapak pengikatan faj-faj tersebut adalah bertindih secara separa dengan epitop mAb C1-5 yang telah dipetakan dalam kedudukan asid amino 78 hingga 83 pada penghujung duri partikel teras. Selain daripada itu, rantai berat C1-5 adalah kaya dengan asid amino yang hidrofobik dan sifat ini telah disarankan sebagai bahagian yang bergabung dengan HBcAg. Peptida-peptida yang terpilih juga terdiri daripada asid amino yang hidrofobik, maka, peptida-peptida ini mungkin ikat pada bahagian yang sama dengan rantai berat mAb C1-5. Peptida sintetik lurus WSFFSNI berupaya menyekat pengikatan di antara L-HBsAg dan partikel teras secara *in vitro* dengan kepekatan penyekatan ($IC_{50}$) lebih kurang 9.8 μM. Penambahan asid amino sisteina yang bersifat hidrofobik pada kedua-dua penghujung C dan N peptida WSFFSNI telah menurunkan keterlarutannya, dan seterusnya mengakibatkan $IC_{50}$ peptida gelang ini 20-kali lebih tinggi daripada peptida WSFFSNI. Sesuatu pembawa diperlukan untuk mengurangkan hidrofobik peptida-peptida tersebut dan seterusnya bertindak sebagai sistem penghantaran peptida-peptida ini ke sel-sel yang dijangkiti oleh virus.

## ACKNOWLEDGEMENTS

It is my pleasure to express my deepest appreciation to my main supervisor Dr. Tan Wen Siang, thanks for his teaching, guidance and support in supervising this study. I really learn a lot from him.

Not forgetting to Prof. Datin Dr. Khatijah Yusoff and Assoc. Prof. Dr. Seow Heng Fong, thanks for their guidance, encouragement, and invaluable advice throughout this project.

My appreciation is also extended to the Virology Laboratory's members, Dr. Majid Eshagi, Tang Yik Kiong, Loke Chui Fung, Subha Bahsu, Priadarisni Ramanujam, Kho Chiew Ling, Lau Wei Hong, Wong Sing King, Amir Rabu, Kok Wai Ling, Ong Swee Tin, Siti Salwah and Sharifah Tuan Ibrahim. Many thanks for their helpful commends and stimulating discussion. My gratitude also goes to Electron Microscopy Unit, Universiti Putra Malaysia. Special thanks go to Mr. Ho for his assistance in electron microscopy observation.

To the person who willing to share my "happiness and sadness" throughout this study, Siew Yeong, I would like to thank for her love, support and understanding throughout these years.

Last but not least, to my parents and my brothers, I wish to dedicate my heartiest gratitude and thanks for their support and never-ending love.

This study was supported by IRPA Grant No. 06-02-04-0054.

I certify that an Examination Committee met on 7th March 2002 to conduct the final examination of Ho Kok Lian on his Master of Science entitled "Selection of High Affinity Peptides against Hepatitis B Core Antigen from A Phage-Displayed Cyclic Peptide Library" in accordance with Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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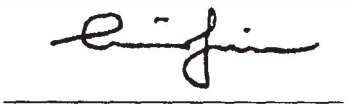

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## DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

HO KOK LIAN

Date: 8th March 2002

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ε | encapsidation signal |
| α | alpha |
| β | beta |
| °C | degree centrigrade |
| µg | microgram ($10^{-6}$ g) |
| µl | microlitre ($10^{-6}$ l) |
| µM | micromolar ($10^{-6}$ M) |
| ρmole | picomole |
| A | adenine |
| Å | Ångstrom |
| Amp | ampicillin |
| ATP | adenosine triphosphate |
| bp | basepair |
| BSA | bovine serum albumin |
| C | cytosine/ core |
| ccc | covalently closed circular |
| Ci | curies |
| CITE | Cap-independent translation enhancer |
| cpm | count per minute |
| C-terminus | carboxy terminus |
| CTLs | CD8+ cytotoxic T lymphocytes |
| dATP | 2'-deoxy-adenosine-5'-triphosphate |
| dCTP | 2'-deoxy-cytidine-5'-triphosphate |
| ddATP | 2',3'-dideoxy-adenosine-5'-triphosphate |
| ddCTP | 2',3'-dideoxy-cytidine-5'-triphosphate |
| ddGTP | 2',3'-dideoxy-guanosine-5'-triphosphate |
| ddNTP | dideoxy-nucleoside triphosphate |
| ddTTP | 2',3'-dideoxy-thymidine-5'-triphosphate |

| | |
|---|---|
| dGTP | 2'-deoxy-guanosine-5'-triphosphate |
| DHBV | duck hepatitis B virus |
| DNA | deoxy-ribonucleic acid |
| dNTP | deoxynucleoside triphosphate |
| DR | direct repeat |
| dsDNA | double-stranded DNA |
| DTT | 1,4-dithiothreitol |
| dTTP | 2'-deoxy-thymidine-5'-triphosphate |
| ELISA | enzyme-linked immunoabsorbent assay |
| ER | endoplasmic reticulum |
| g | gram |
| GSHV | ground squirrel hepatitis virus |
| h | hour |
| HBcAg | hepatitis B core protein |
| HBsAg | hepatitis B surface protein |
| HBV | hepatitis B virus |
| HBxAg | hepatitis B x protein |
| HCC | hepatocellular carcinoma |
| HIV | human immunodeficiency virus |
| HSV | herpes simplex virus |
| IPTG | isopropyl-β-d-thiogalactopyranoside |
| kb | kilobase |
| $K_d$ | dissociation constant |
| kDa | kilodalton |
| $K_d^{rel}$ | relative dissociation constant |
| l | litre |
| LB | Luria broth |
| L-HBsAg | large surface antigen |
| LTR | long terminal repeat |
| M | molar |
| mAb | monoclonal antibody |

| | |
|---|---|
| mg | milligram ($10^{-3}$ g) |
| M-HBsAg | medium surface antigen |
| MHC | major histocompatibility complex |
| min | minute |
| ml | millilitre ($10^{-3}$ l) |
| mm | millimetre ($10^{-3}$ m) |
| mRNA | messenger ribonucleic acid |
| NDV | Newcastle disease virus |
| NET-gel | sodium-Tris-EDTA-gelatin buffer |
| nM | nanomolar ($10^{-9}$ M) |
| NP-40 | Nonidet p40 |
| N-terminus | amino terminus |
| OD | optical density |
| ORF | open reading frame |
| P | polymerase protein |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PEG | polyethylene glycol |
| pfu | plaque forming unit |
| pH | *Puissance hydrogene* |
| *pol* | polymerase protein |
| PreS1 | N-terminal region of L-HBsAg comprising 108 or 119 amino acids |
| PreS2 | region of M and L-HBsAg comprising 55 amino acids |
| PVDF | polyvinylidene difluoride |
| RF | replicative form |
| RNA | ribonucleic acid |
| RNAsin | RNA inhibitors |
| rpm | revolutions per minute |
| s | second |

| | |
|---|---|
| SDS | sodium dodecyl sulphate |
| S-HBsAg | small surface antigen |
| ssDNA | single stranded DNA |
| STE | sodium-tris-EDTA buffer |
| SV40 | simian virus 40 |
| T | thymine/ triangulation number |
| TBE | tris-buffered EDTA solution |
| TBS | tris-buffered saline |
| TE | tris-EDTA buffer |
| TEMED | tetramethyl ethylenediamine |
| TP | terminal protein |
| tRNA | transfer RNA |
| U | unit |
| UV | ultraviolet |
| v | volt |
| v/v | volume/volume |
| vol | volume |
| w/v | weight/volume |
| WHV | woodchuck hepatitis virus |
| x *g* | centrifugal force |
| X-gal | 5-bromo-4-chloro-3-indol-β-D-galactopyranoside |
| Y | fraction bound |

## AMINO ACID ABBREVIATIONS

| | **One letter code** | **Three letter code** |
|---|---|---|
| Alanine | A | Ala |
| Arginine | R | Arg |
| Asparagine | N | Asn |

| | | |
|---|---|---|
| Aspartic acid | D | Asp |
| Cysteine | C | Cys |
| Glutamic acid | E | Glu |
| Glutamine | Q | Gln |
| Glycine | G | Gly |
| Histidine | H | His |
| Isoleucine | I | Ile |
| Leucine | L | Leu |
| Lysine | K | Lys |
| Methionine | M | Met |
| Phenylalanine | F | Phe |
| Proline | P | Pro |
| Serine | S | Ser |
| Threonine | T | Thr |
| Tryptophan | W | Trp |
| Tyrosine | Y | Tyr |
| Valine | V | Val |

# CHAPTER I

## INTRODUCTION

Hepatitis B virus (HBV) is an enveloped DNA virus of the family *Hepadnaviridae*, which causes a variety of acute and chronic liver diseases such as cirrhosis and hepatocellular carcinoma in human and other higher primates such as chimpanzees (Ganem and Varmus, 1987). The virus is transmitted through direct contact with serum of an infected patient or body fluid, such as saliva, semen and vaginal fluid. In endemic areas, perinatally transmission from an HBV-infected mother to her offspring is more common (Mahoney, 1999). Generally, HBV infection becomes clinically apparent in less than 50% of all infected individuals after an incubation period of 35-150 days and a complete remission occurs in 90-95% of the cases within 3 to 4 months (Caselmann, 1996). The people infected with HBV either recover from the infection or they may remain chronically infected. According to World Health Organisation (WHO), there are about 400 million carriers worldwide and approximately 2 million carriers die annually (Caselmann, 1996), despite the existence of an effective HBV vaccine.

Currently, there is no safe and effective therapeutic agent treatment available for hepatitis B infection. Several inhibitors have been used in the therapy of chronic liver disease such as arabinoside A, acycloguanosine, β- and α-interferon

(Bassendine *et al.*, 1981; Weller *et al.*, 1983; Thomas and Scully, 1985). At present, α-interferon appears to hold the best hope to clear the virus. However, the overall respond rate is only approximately 50 % (Thomas and Scully, 1985). Lamivudine (also known as 3TC) is the currently licensed chemotherapeutic, which proved to be actively suppressed hepatitis B infection (Hilleman, 2001). Until recently, there is no safe and effective antiviral compound against the viral assembly and infectivity. To overcome these problems, small molecules such as peptide inhibitors, which bind to subunit interfaces that interfere the virus morphogenesis have been extensively studied.

Filamentous bacteriophage displaying millions of random peptide sequences on the minor coat proteins has been used to define ligand-binding sites that are difficult to identify by conventional methods (Scott and Smith, 1990). Peptide sequences that react with ligands such as monoclonal antibodies (D'Mello *et al.*, 1997), carbohydrate (Harris *et al.*, 1997), virus receptor (Ramanujam *et al.*, 2002) and animal organ (Pasqualini and Ruoslahti, 1996) were successfully isolated. Peptide sequences that bind to the core antigen of HBV were successfully isolated from a random linear hexapeptide library displayed on gpIII proteins of filamentous phage (Dyson and Murray, 1995). The relative dissociation constants for the linear hexapeptide and the core particles are in micromolar range. The peptides block the association between the core particles and the long surface antigen (L-HBsAg) *in vitro* and also inhibit the virus assembly in cell culture system (Dyson and Murray, 1995; Böttcher *et al.*, 1998). The linear peptide

sequences were not found as a continuous sequence within the L-HBsAg polypeptide, suggesting that some of the amino acids are brought from different positions of the polypeptide to form a discontinuous binding region or mimotope. Furthermore, Tan *et al.*, (1999) showed that the interaction of L-HBsAg and core particles is rather complex and involves at least two binding sites. It is therefore of interest to select for tighter ligands that bind to these sites with a disulfide constrained phage-displayed peptide library.

Isolation of cyclic peptides that associate with the core particles from a conformational phage display peptide library would be advantageous in providing high affinity binding clones to the core particles. As a result, synthetic peptides based upon the selected sequences would inhibit the association of HBcAg and L-HBsAg and thus block the assembly of HBV. Therefore, the objectives of this study were:

1. To select filamentous phage bearing cyclic peptide sequences that interact with HBcAg by biopanning;
2. To determine the relative dissociation constants ($K_d^{rel}$) between the selected phages and core particles;
3. To study the binding site of the phages on core particles and;
4. To evaluate the inhibitory effects of the synthetic peptides derived from the selected sequences upon the association of core particles and L-HBsAg.

# CHAPTER II

# LITERATURE REVIEW

## 2.1 Hepatitis B Virus (HBV)

### 2.1.1 Hepatitis B Virus Classification

HBV is an etiologic agent of human liver diseases, which poses the major public health problem, causing acute, chronic and fulminant hepatitis (Tiollais *et al.*, 1981). HBV is the prototype of the family *Hepadnaviridae* and subdivided into genus *Orthohepadnavirus*.

### 2.1.2 Epidemiology and Transmission

HBV is transmitted by exposure to blood or body fluid from HBV-infected individuals, and also sexually contact with HBV-infected patients (Mahoney, 1999). In the high endemicity areas such as Southeast Asia and China, perinatal transmission from infected mother to her offspring and intra-familial spread are most common. However, in the moderate and low endemicity areas such as United States, Canada, Western Europe, Middle East and Japan, most infections occur among the high-risk group for HBV infection such as intravenous drug users, sexual contact, haemodialysis patients and occupational exposure to HBV-

contaminated products (Mahoney, 1999). Additionally, HBV markers (HBsAg or HBV DNA) have been detected in tears, saliva, sweat, breast milk, faeces, semen and urine (Blum *et al.*, 1998).

### 2.1.3 Prevention and Treatment

Hepatitis B vaccine was first derived from the plasma of chronic carriers in the replicative phase of the disease (Schödel, 1998). The non-infectious particles consists of HBsAg were used as the first licenced vaccine in the United States in 1981 (Ellis and Kniskern, 1991). However, despite of its efficacy and safety profile, acceptance of the vaccine was less than anticipated. Furthermore, suitable donors became limiting for the production of sufficient quantities of vaccines for worldwide demands. Therefore, recombinant-derived hepatitis B vaccines were developed (Valenzuela *et al.*, 1982; McAleer *et al.*, 1984; Murray *et al.*, 1984). The recombinant yeast-derived HBsAg particles proved to be antigenically and immunologically identical to plasma-derived vaccines (McAleer *et al.*, 1984).

Therapy of chronic HBV infections require an immunostimulator, which is able to eliminate the hepatocytes containing the episomal and integrated viral DNA (Foster and Thomas, 1998). Most of the immunostimulators such as interleukin-2 and γ-interferon are too toxic to routine clinical use but α-interferon clearly shows clinical control of the infections (Foster and Thomas, 1998). It acts as an enhancer in expression of major histocompatibility (MHC) class I by infected hepatocytes.

HBV specific peptides are then presented in sufficient amount by MHC class I to allow $CD8^+$ cytotoxic T lymphocytes (CTLs) to lyse the infected cells (Dimmock and Primrose, 1994). α-interferon is able to clear the virus but the overall respond rate is approximately 50% (Thomas and Scully, 1985). At the present time, lamivudine is an effective antiviral drug (Hilleman, 2001). However, the problem of drug resistance development supports the need for new antiviral compounds.

## 2.2 Biology of Hepatitis B Virus

### 2.2.1 HBV Morphology

Three morphological distinct forms of particle are found in the serum of an infected individual: (i) spheres and (ii) filamentous particles with 22 nm diameter and (iii) 42 nm double-shelled infectious particles or known as Dane particles (Dane *et al.*, 1970). The infectious agents are Dane particles (Figure 2.1), which consists of a 7 nm lipoprotein bilayer (envelope protein) derived from the endoplasmic reticulum (ER) membrane of the host cell. Embedded inside the envelope are three distinct but related surface proteins of varying sizes known as S (small), M (middle) and L (large) hepatitis B surface antigen (HBsAg) (Ganem and Varmus, 1987; Nassal and Schaller, 1993). These surface antigens surround the core particle of HBV, which in turn encapsidates a partially double stranded circular DNA of 3.2 kb, a protein kinase, an RNA-dependant polymerase and a genome-bound protein, which linked covalently to the 5'-end of the minus strand

of HBV DNA (Caselmann, 1996). The 22 nm spherical and filamentous structures are about 1000 to 10,000-fold in excess than the Dane particles in the serum of an infected patient and comprise exclusively the viral surface proteins but do not contain the viral DNA (Caselmann, 1996).

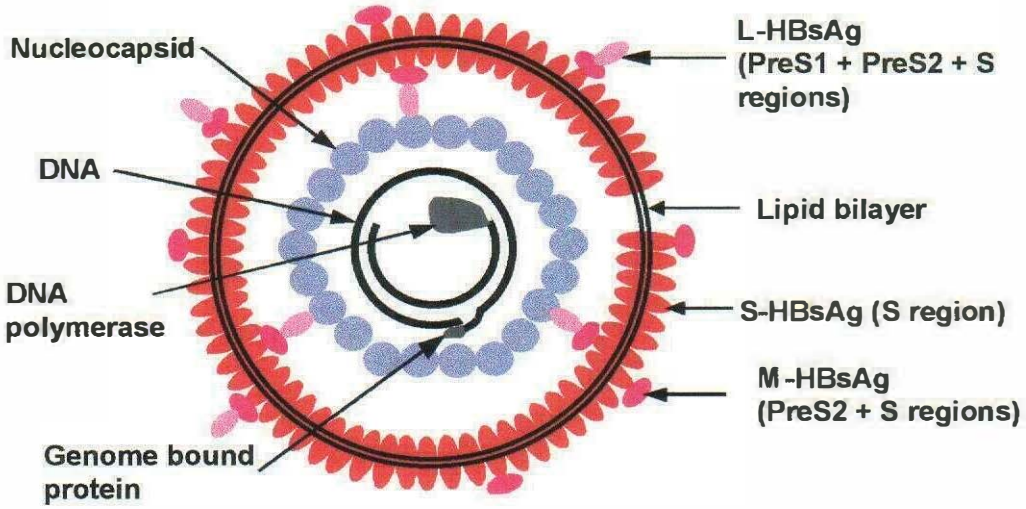



**Figure 2.1: A schematic representative of virion structure of HBV**
The representative of the L, M, and S forms of HBsAg has no quantitative or positional significance. (adapted from Caselmann, 1996).

### 2.2.2 Genomic Organisation

The complex organisation of HBV is encoded by a 3.2 kb genome and the studies of the complete nucleotide sequence demonstrated a compact coding organisation (Figure 2.2). Circulation of the DNA is achieved by base pairing of the free 5' end of the plus strand with the nicked 5' end of the minus strand in a region flanked by two 11 bp direct repeats: DR1 and DR2 (Caselmann, 1996). The large (minus) strand of partially dsDNA carries four major overlapping open reading frames

(ORFs): PreS1/S2 (surface protein), PreC/C/e (core/e protein), P (DNA polymerase), and X (Ganem and Varmus, 1987).

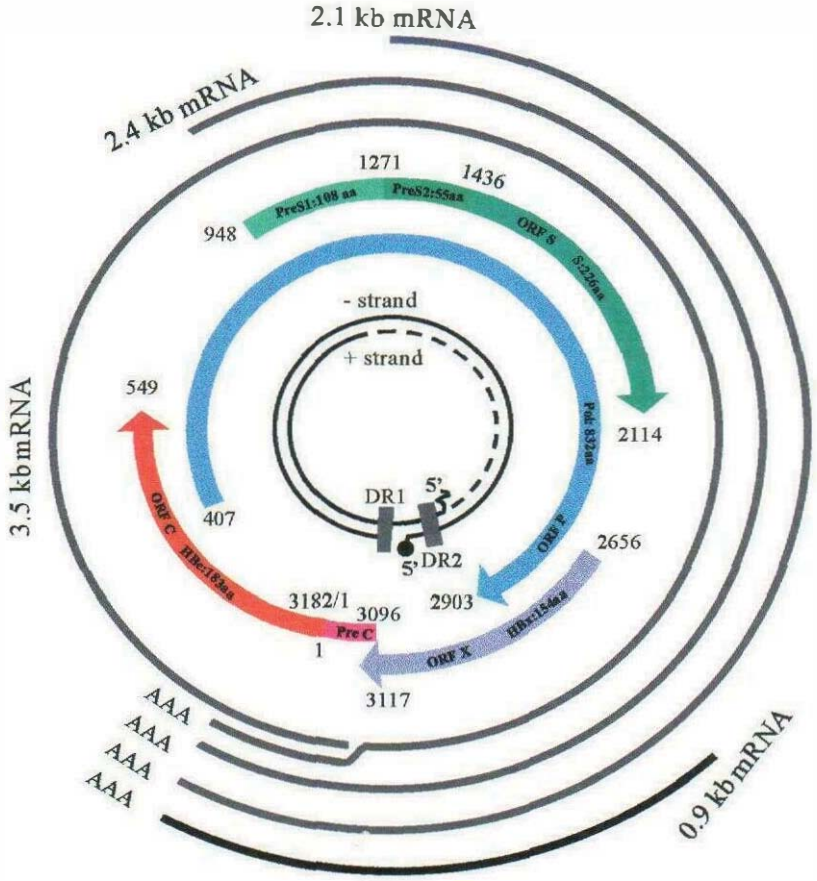



**Figure 2.2: Genome organisation of HBV**
The outer bold lines represent different classes of RNA transcripts and the inner circles represent the dsDNA genome as present in the virion. The four major open reading frames are labelled and differentiated by colours (adapted from Nassal and Schaller, 1993).

The surface antigen ORF, ORF S, comprises 678 nucleotides and it is the largest coding region for the synthesis of S-HBsAg. Upstream of the ORF S is another in-frame ORF, ORF PreS, with two conserved ATG codons. These codons subdivide the ORF PreS into PreS1 and PreS2 coding regions, which direct the synthesis of L- and M-HBsAg, respectively (Galibert *et al.*, 1979).

The core antigen ORF, ORF C, is preceded by an upstream short in-phase ORF, ORF PreC, which has the capacity to encode a 24 kDa polypeptide (Standring *et al.*, 1988). Production of e-antigen (HBeAg) is resulted from the proteolytic processes of both N- and C-termini of the primary translation product from the complete ORF C whereas the HBcAg is synthesised from a second in-frame ATG codon, which located 87 bases downstream of the ORF PreC (Pasek *et al.*, 1979). The ORF P is believed to encode the viral DNA polymerase (~94 kDa). The X gene ORF, ORF X, encodes the 17 kDa X protein (Moriarty *et al.*, 1985).

## 2.3 Replication of Hepatitis B Virus

The major problems in the studies of virus infections are due to the narrow host range of HBV and the absence of susceptible cell lines for virus propagation (Nassal and Schaller, 1993). However, promising results have been achieved in the DHBV model by using primary duck hepatocytes in the infection studies (Tuttleman *et al.*, 1986; Urban *et al.*, 1999). Several glycoproteins were identified as candidates for the DHBV receptor such as 170-189 kDa (gp180/p170) carboxylpeptidase (Kuroki *et al.*, 1995; Tong *et al.*, 1995) and 120 kDa preS-binding protein (Li *et al.*, 1996). Recently, the virus attachment site was found located at the C-domain of carboxypeptidase D, which is a soluble form of DHBV receptor (Urban *et al.*, 1999; 2000). The liver-specific binding sites have been identified *in vitro* within the preS regions (Neurath *et al.*, 1986; Pontisso *et al.*, 1989; Gerlich *et al.*, 1993).

The life cycle of HBV is schematically shown in Figure 2.3. The first step of viral replication is the conversion of virion DNA to cccDNA in the nucleus of infected hepatocytes (Miller and Robinson, 1984). cccDNA serves as a template for transcription by host RNA polymerase to produce several classes of transcripts. The 3.5 kb genomic RNA contains the complete viral genome serves as template for the synthesis of reverse transcriptase, HBcAg and reverse transcription of minus strand DNA (Huang and Summers, 1991). The pregenomic RNA is about 10% larger than cccDNA because it is terminally redundant (Seeger and Mason, 1998).

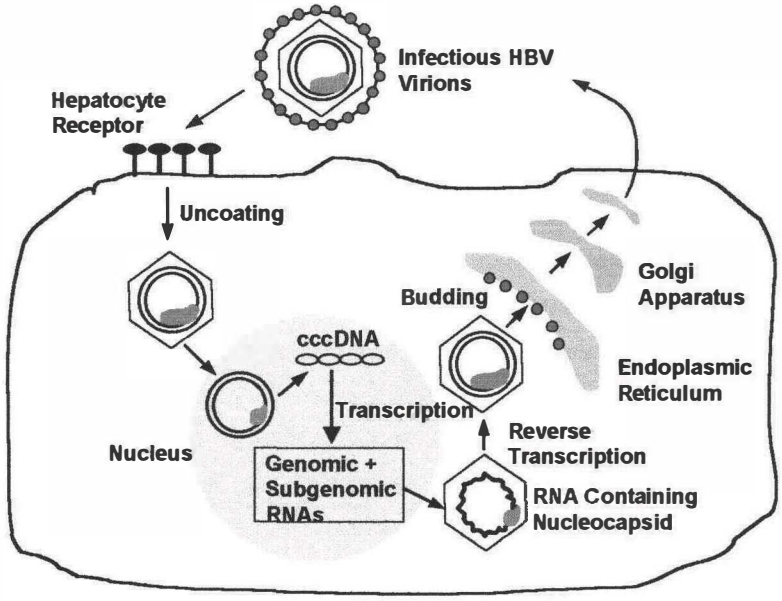



**Figure 2.3: The basic replication cycle of HBV**
Following the attachment and penetration of the host cell, the virion DNA is transported into the nucleus and converted to cccDNA, which serves as template for transcription. RNA transcripts are then entered cytoplasm, where translations occur. After the assembly of RNA containing nucleocapsid and reverse transcription of RNA, the premature virions are budded into ER lipid bilayer and eventually released from the infected host cell (adapted from Tan, 1997).

Once sufficient quantities of HBcAg, P protein and RNA pregenome have accumulated, these components are coassembled into premature core particles as

shown in Figure 2.4 (Nassal and Schaller, 1993). Following the assembly of capsid, the pregenomic RNA and reverse transcriptase are encapsidated in these structures (Figure 2.4).

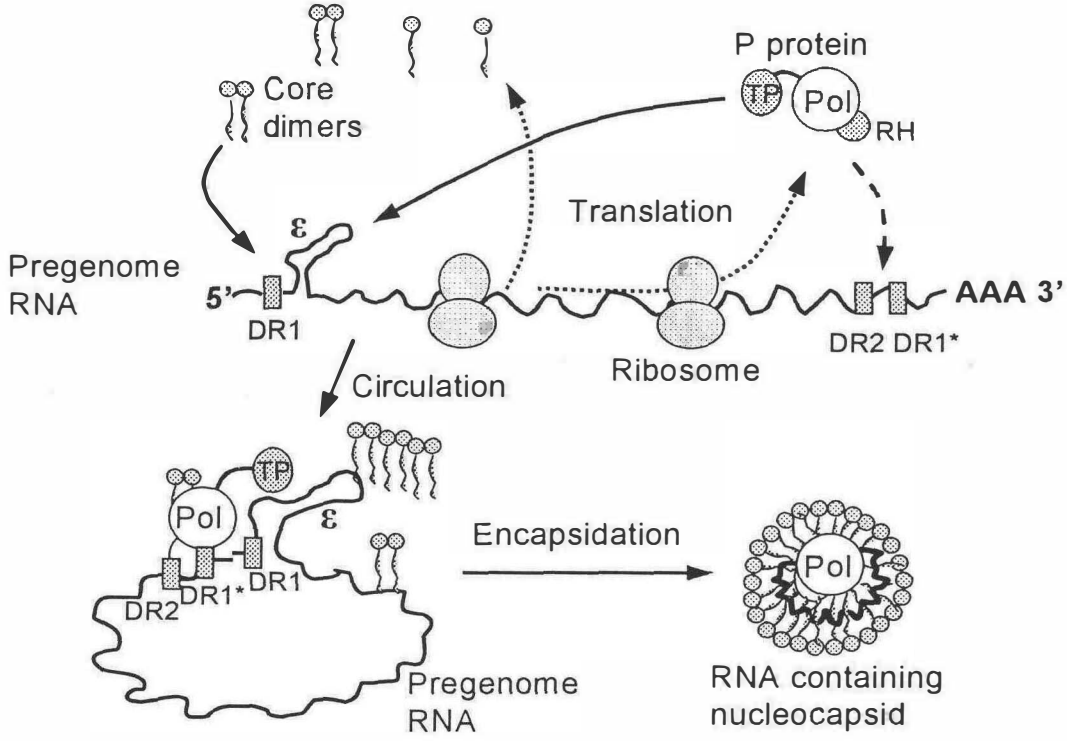



**Figure 2.4: Nucleocapsid assembly**
Core and P proteins are translated from the pregenome RNA. Core dimers are attached to the ribosome-deprived RNA via non-specific binding domain and complete the shell formation. P interacts with the ε on its own mRNA and triggers the addition of core dimers. P protein interacts with additional site between DR2 and DR1* at the 3' end of pregenome which induces circularisation and prepare the RNA for replication (adapted from Nassal and Schaller, 1993).

The RNA packaging and priming of reverse transcriptase depend on the presence of ε, which located close to the 5' end of the pregenome RNA. ε contains DR1 and an initiation codon for synthesis of HBcAg. This region has been proposed to fold into moderately stable hairpin structure (Junker-Niepmann *et al.*, 1990). Mutagenesis study revealed that specific nucleotide sequences in the loop and in regions of the upper stem are critical for RNA encapsidation (Knaus and Nassal,

1993). The assembled particles are either exported as enveloped virions or returned to the nucleus to increase the copy number of cccDNA.

## 2.4 Viral Proteins

HBsAg was discovered before the virus itself was identified and these proteins are produced persistently into the blood of infected individuals (Heermann and Gerlich, 1991). The first efforts to purify the HBsAg from carrier blood led to the isolation of pleiomorphic round particles (~ 20 nm in diameter) in the absence of viral DNA. Dane and Colleagues (1970) discovered double-shell particles in the serum of a chronic carrier and the core particles were discovered when the outer shell of the Dane particles removed with non-ionic detergent (Almeida *et al.*, 1971). Dane particles were hypothesised as infectious particles when the endogenous DNA polymerase activity and nucleic acid were detected within it (Kaplan *et al.*, 1973; Robinson and Greenman, 1974).

### 2.4.1 Surface Antigen (HBsAg)

HBsAg is encoded by three alternatives initiation and a common stop codon in the ORF S (Ganem and Varmus, 1987). The S-HBsAg is translated from the third initiation codon (Figure 2.5). This 226-amino acid polypeptide of about 24 kDa (p24) contains the major antigen determinants 'a', as well as epitopes, which

define the virion subtypes (Stirk *et al.*, 1992). Glycosylation at Asn-146 produces the glycosylated form of S-HBsAg (gp27) (Peterson *et al.*, 1977).

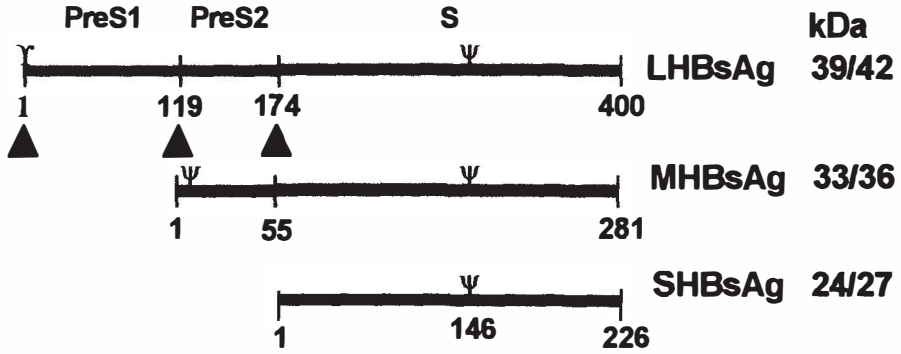



**Figure 2.5: HBsAg proteins**
Numbers refer to amino acids of primary translation products. (ϒ) represents myristic acid, which replaced Met-1 of L-HBsAg. (ψ) represents the glycosalation sites at Asn-4 of M-HBsAg and Asn-146 of S-domains. S, M, and L-HBsAg are translated from a common ORF by using 3 in-frame initiation codons (▲) at the 5' end of the PreS and S genes (adapted from Heermann and Gerlich, 1991).

Polypeptides translated from the middle initiation codon are M-HBsAg with an additional 55 residues at the N-terminus of S-HBsAg, known as PreS2. The glycosylation at position Asn-4 is invariably in PreS2. Glycosylation of the 'a' determinant in the S region of M-HBsAg produces two forms of glycosylated proteins, gp33 and gp36 (Stibbe and Gerlich, 1983). While the translation of the entire length of ORF S produces the L-HBsAg with an N-terminal extension of 108 or 119 amino acids (depends on subtype) known as PreS1 region. However, the PreS2 of L-HBsAg is not glycan linked and it presents in non-glycosylated (p39) and glycosylated (gp42) forms depending upon glycosylation at the 'a' determinant (Heermann *et al.*, 1984).

Both the N- and C-termini of S-HBsAg are exposed on the ER lumen and up to four transmembrane domains have been proposed (Ostapchuk *et al.*, 1994).

However, only the first two N-terminal α-helix domains have been shown to participate in membrane interaction (Gavilanes *et al.*, 1982; Eble *et al.*, 1986; Eble *et al.*, 1987). These domains correspond to residues 11-28 and 80-98, respectively, are separated by a hydrophilic region exposed on the cytoplasmic of ER membrane (Figure 2.6). A second hydrophilic loop (residues 99-168) was proposed to be disposed on the ER lumen due to the fact that it carries the major antigenic determinant of HBsAg and acquires carbohydrate modification (Eble *et al.*, 1987; Stirk *et al.*, 1992). The S-HBsAg is unique among HBsAg because when expressed in uninfected cell, it does not require nucloecapsid interactions to be released from the cell as it capable to aggregate in the ER bilayer prior to budding into the lumen as a subviral particles (Liu *et al.*, 1982; Simon *et al.*, 1988; Huovila *et al.*, 1992). The transmembrane topology for the M-HBsAg is believed to be very similar to S-HBsAg and the translocation is regulated by a signal sequence located at the N-terminus of S-domain (Eble *et al.*, 1990).

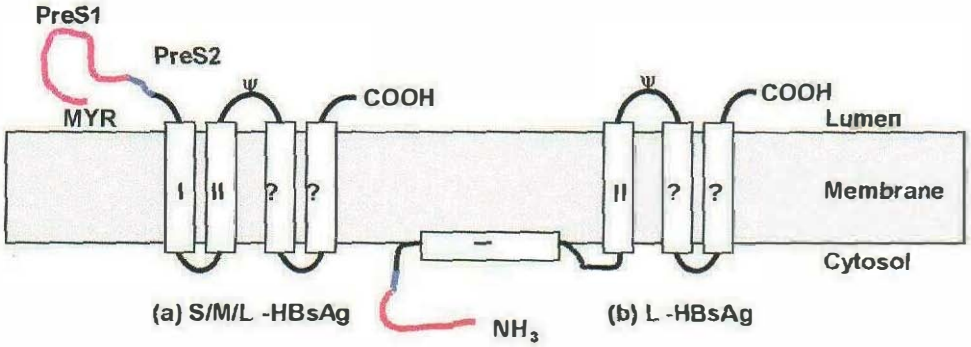



**Figure 2.6: Proposed topologies for S-, M-, and L-HBsAg**
The luminal and cytoplasmic sides of ER are indicated and the Asn-glycosylation sites are marked with (ψ). (a) Topology of S-,M- or L-HBsAg with two well defined transmembrane domains (I and II). The other two transmembrane domains marked with "**?**" was proposed based on modelling and not empirically defined (Guerrero *et al.*, 1988). The PreS regions of the M and L-HBsAg were proposed largely based on analyses of subviral and viral particles (modified from Ostapchuk *et al.*, 1994). (b) Another transmembrane topology of the L-HBsAg. The cytoplasmic disposition of the preS regions enforces an altered structure on the first hydrophobic domain of the S region (adapted from Prange and Streeck, 1995).

The transmembrane topology of L-HBsAg is remained controversial. During virus morphogenesis, the L-HBsAg mediates the attachment of PreS regions to hepatocyte and also participates in nucleocapsid envelopment. In order to accomplish this dual function, the PreS regions of the L-HBsAg must be both luminally and cytoplasmically disposed. The non-glycosylation of its PreS2 region suggests that an alteration in topology is occurred. Both the PreS1 and PreS2 regions of L protein are proposed to be cytoplasmically disposed and post-translationally translocated across the lipid bilayer following the association with nucleocapsid or reorganisation of the HBsAg, lipid bilayer or both (Ostapchuk *et al.*, 1994). Nevertheless, Prange and Streeck (1995) demonstrated that alteration of L-HBsAg occurred post-translationally and yielded two different topologies, which dispose PreS regions luminally and cytoplasmically (Figure 2.6). Moreover, the S regions of M and L- HBsAg are not uniformly oriented in the membrane of microsomes as assayed in a cell-free system. The first hydrophobic domain [Figure 2.6 (b); domain I] might be embedded in the plane of the lipid bilayer rather than spanning the membrane (Prange and Streeck, 1995). Therefore, this is inconsistent with the model illustrated in Figure 2.6 (a). The disposition of PreS regions in cytosolic side of ER is essential for nucleocapsid envelopment and mutagenesis studies showed that deletion up to 102 residues at the N-terminus of preS1 sequence still allowed virion morphogenesis (Bruss and Thomssen, 1994).

Dyson and Murray (1995) showed that several linear peptides selected from the phage display library were able to inhibit the binding of L-HBsAg onto the nucleocapsid in a cell-free system. The peptide LLGRMKG were not found as continuous sequence but matched partially amino acid sequences within the L-HBsAg and believed to be mimicking the internal regions of HBsAg resulting from protein folding. The proposed docking sites for HBsAg on nucleocapsid are located at amino acid 19-24 (LDPAFR) and 63-65 (LLG) of PreS1 region and amino acid 21-26 (LLTRIL) of S region (Figure 2.7).

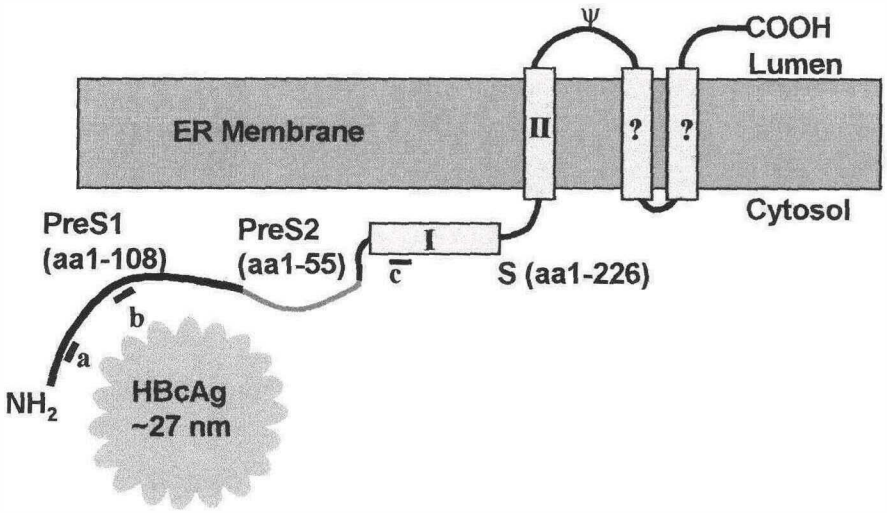



**Figure 2.7: Domain of L-HBsAg associated with the HBcAg**
The diagram shows the proposed docking sites of the PreS and S domains on HBcAg. a, b and c represent the amino acid sequences LDPAFR (19-24), LLG (63-65) of the PreS domain and LLTRIL (21-26) of the S domain, respectively (modified from Dyson and Murray, 1995).

Mutational analysis showed that two distinct segments located between amino acids 24-191 and 191-322 of L-HBsAg contributed synergistically to high affinity binding to HBcAg in a cell-free system (Tan *et al.*, 1999). Furthermore, interactions between the corresponding deletion mutants were blocked by small

peptides that bind to the tip of the HBcAg (Tan *et al.*, 1999). Amino acid substitution of Arg-92 with Ala greatly reduced the binding affinities, thus, Arg-92 might involve in the interaction (Tan *et al.*, 1999).

### 2.4.2 Core Protein (HBcAg)

The viral capsid is surrounded by an outer envelope derived from the host cell membrane and the essential subunit of the nucleocapsid is a single polypeptide chain of 183 residues (~22 kDa). When the gene for the HBcAg is expressed in *E. coli*, the expressed HBcAg self-assembles into shells, which are identical to the core particles isolated from virally infected liver (Pasek *et al.*, 1979; Cohen and Richmond, 1982). HBcAg has an Arg-rich C-terminus that required for nucleic acid packaging (Petit and Pillot, 1985; Gallina *et al.*, 1989). Mutants without Arg-rich C-terminus still able to form spherical shell but the RNA packaging is abolished (Birnbaum and Nassal, 1990; Zheng *et al.*, 1992).

As investigated by Crowther *et al.* (1994), two classes of spherical particles with 180 and 240 subunits arranged into T=3 and T=4, respectively, have been investigated by using electron cryomicroscopy and three-dimensional image processing (Figure 2.8). Deletions of the protamine-like structure at C-terminus systematically decreased the proportion of T=4 capsid and the truncation at residue 149 leads to production of predominantly T=4 capsids with no morphological changes (Zlotnick *et al.*, 1996). Böttcher *et al.* (1997) has

determined the structure of T=4 capsid containing 240 copies of truncated HBcAg expressed in *E. coli* at 7.4 Å resolution by electron cryomicroscopy. The overall diameters measured to the tips of the spikes are 36 and 32 nm for the T=4 and T=3 particles, respectively. Both particles are penetrated by holes and the largest holes are around 2 nm, whereas the smaller holes are estimated to be 1.3 nm. The larger particles comprising 80 larger and 30 smaller holes; and there are 60 larger and 20 smaller holes for the small particle (Crowther *et al.*, 1994).

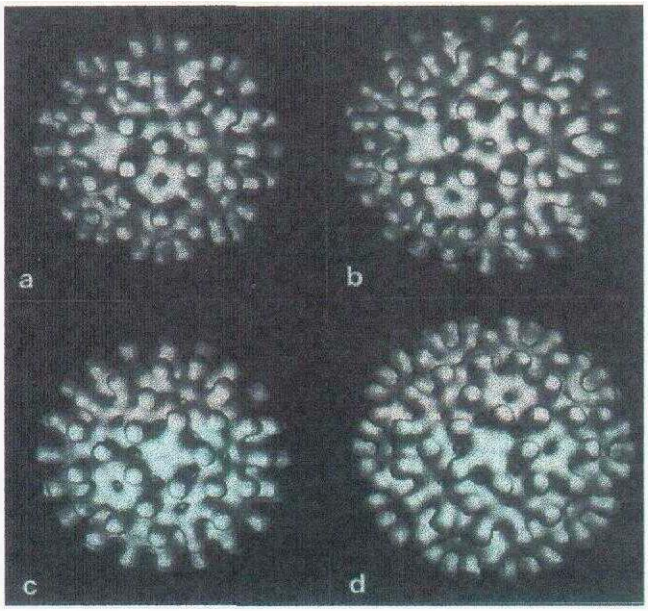



**Figure 2.8: Three dimentional maps of hepatitis B viral core particles**
Computed 3D maps of small empty particles (a and c), and large empty particles (b and d). (a) and (b) show the view along a 2-fold axis of symmetry, (c) and (d) show the view along 5 fold axis of symmetry. 90 and 120 protruding spikes are shown in small and larger particles, respectively (Source: Crowther *et al.*, 1994).

Two monomers of HBcAg associate to give a compact dimer (Figure 2.9), so that each dimer spike consists of a four-helix bundle (Böttcher *et al.*, 1997; Conway *et al.*, 1997). The dimer is linked together in a back-to-back manner and there is a disulfide bridge between the residues Cys-61 of two monomers, however, all Cys

residues (at positions 48, 61, 107 and 185) are not essential for dimer formation (Nassal *et al.*, 1992; Zhao and Standring, 1992).

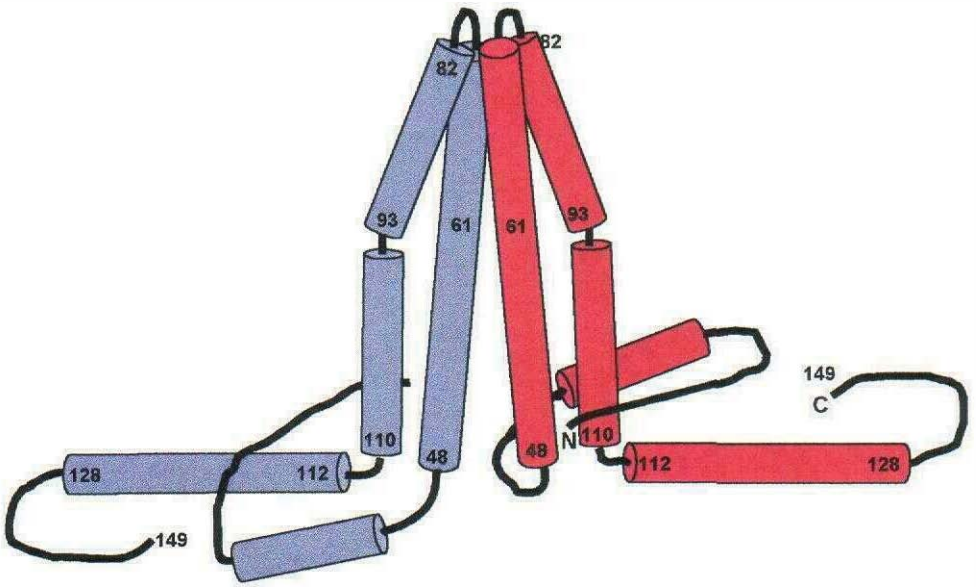



**Figure 2.9: Polypeptide folds of a capsid dimer**
The α-helical regions are indicated as cylinders and the putative N and C termini are labelled. Numbers indicate amino acid positions. Cys-61 residues of both subunits form a disulfide bridge. Cys-48 as well as others does not form disulfide bridges (modified from Wynne *et al.*, 1999).

The tip of the spike is known to be the imunodominant loop of the capsid, which corresponds to amino acids 78 to 83 (Salfeld *et al.*, 1989; Sällberg *et al.*, 1991). In the electron cryomicroscopy model, 90 and 120 spikes protrude from the shell of the assembly domain of both T=3 and T=4 particles (Crowther *et al.*, 1994). The truncated HBcAg produced in *E. coli* are able to form core-like particles and this indicating that the entire Arg-rich C-terminal region is not essential for particle assembly (Stahl and Murray, 1989; Birnbaum and Nassal, 1990). The insertion of foreign protein at N-terminal end does not interfere the particle assembly and the chimeras exhibited both the antigenic and immunogenic characteristics (Clarke *et al.*, 1987; Stahl and Murray, 1989). This finding indicates that the fused protein

buried inside the particles is accessible through the holes on the surface of the assembled particle (Crowther *et al.*, 1994).

### 2.4.3 X Protein (HBxAg)

The HBxAg translated from the first start codon on ORF X consists of 154 residues (~17 kDa). The existence of HBxAg is indicated by circulating of anti-HBxAg in patient serum (Moriarty *et al.*, 1985; Pfaff *et al.*, 1987). The X-mRNA has been detected in mammalian cells transfected with HBV genome either in prokaryotic, eukaryotic or *in vitro* system (reviewed in Rossner, 1992). Transcriptional activation by HBxAg has been assayed based on co-transfection of cultured cells with recombinant HBxAg plasmids and plasmids carrying reporter genes under the control of various different eukaryotic and cellular promoters. Transactivation by HBxAg has been demonstrated using the simian virus 40 (SV40) early promoter enhancer, HIV-1 long terminal repeat (LTR), herpes simplex virus (HSV) thymidine kinase, human T-cell lymphotropic virus-1 LTR, cytomegalovirus enhancer/promoter, and HBV promoters (reviewed in Caselmann, 1996). Promoters of cellular genes relevant for cell proliferation and transformation, which can be activated by HBxAg include *c-fos*, *c-myc*, *β*-interferon and *β*-actin (Rossner, 1992). Besides, involvement of HBxAg in hepatocarcinogenesis also has been investigated in transgenic mouse systems and development of HCC-like lesions in liver was observed (Kim *et al.*, 1991).

### 2.4.4 Polymerase Protein

The viral polymerase (*Pol*) is translated from pregenomic mRNA (Ou *et al.*, 1990). *Pol* consists of 832 residues (~93 kDa) and can be divided into three different functional regions: (i) the N-terminus encoding a genome-bound protein linked to the 5' end of the (-) strand and serves as a primer for reverse transcription (Bartenschlager and Schaller, 1988; Bosch *et al.*, 1988); (ii) RNase H activity is displayed at the 3' region and; (iii) RNA-dependent DNA polymerase coding region sandwiched between the (i) and (ii) regions. The *pol* protein could not be detected directly in infected hepatocytes, therefore, the studies of *pol* protein have been obtained from overexpression of P gene in bacterial and eukaryotic systems (Bartenschlager *et al.*, 1992; Tavis and Ganem, 1993). *Pol* also required for virus replication as shown in Figure 2.4 (Page 11).

## 2.5 Biology of Filamentous Phage

### 2.5.1 Morphology of Filamentous Bacteriophage

The Ff group of filamentous bacteriophages include strains M13, f1 and fd, are non-lytic DNA viruses that infect *E. coli* harbouring an F episome (Rasched and Oberer, 1986). M13 differs from f1 and fd only about 2% in nucleic acid sequence and the closed circular ssDNA genome consists of 6407 bases (Van Wezenbeek *et*

*al.*, 1980). The length of the filamentous phage is approximately 900 nm and 7 nm in diameter (Figure 2.10) (Glucksman *et al.*, 1992; Marvin *et al.*, 1994).

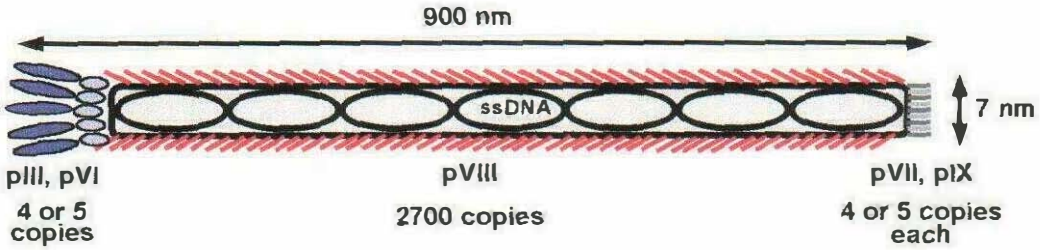



**Figure 2.10: A schematic representative of Ff phage**
The ssDNA is surrounded by 2700 copies of gpVIII proteins but the number varies with genome length. The minor coat proteins (gpVII, gpIX, gpVI and gpIII) consist of 4 or 5 copies (adapted from Wilson and Finlay, 1998).

The genome are packaged in the flexible filament comprises 2700 to 3000 copies of the major coat protein (gpVIII) and four minor coat proteins encoded by genes III, VI, VII and IX, which present in four or five copies each (Rasched and Oberer, 1986). The DNA is embedded in the cylinder along its longitudinal axis as it folded back on itself and the molecule is compact due to the extensively stacked nucleotide bases and the fixed orientation (Rasched and Oberer, 1986). A major hairpin structure near to the origins of replication is found invariably at one end of the filament regardless the length of the DNA (Ikoku and Hearst, 1981; Webster *et al.*, 1981). The DNA twisted as helix structure with a diameter of about 1.7 nm and a pitch of about 2.7 nm (Banner *et al.*, 1981).

### 2.5.2 Genomic Organisation and the Products of Filamentous Phage

The genome of filamentous bacteriophage contains 11 genes as shown in Figure 2.11. The genes are grouped based upon their functions in the life cycle of the

phages. The 'intergenic region' is a non-coding region and contains the origin (*Ori*) for the synthesis of (+) or (-) strand DNA and a packaging signal for the initiation assembly of the phage particles (Webster, 1996). The products of the genes II, V, and X are resided in cytoplasmic, which are involved in the phage DNA replication (Model and Russel, 1988) Gene X overlaps with gene II at its C-terminal portion and gpX is required for proper DNA replication and also function as an inhibitor of gpII functions (Fulford and Model, 1984)

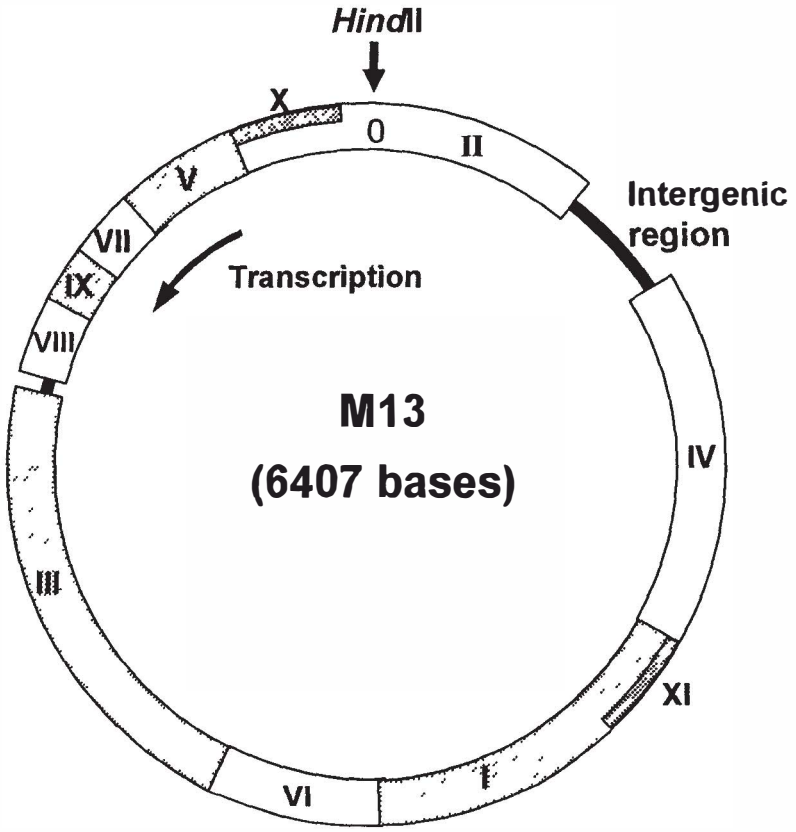



**Figure 2.11: The genome of the M13**
This single stranded DNA consists of 11 genes The unique *Hind*II site is the numbering reference point and represented as "0" for the 6407 bases genome Genes X and XI overlaps with gene II and I, respectively The packaging signal is located in the 'intergenic region' and the arrow inside the genome represents the direction of transcription (adapted from Webster, 1996)

The capsid proteins are located in the host cytoplasmic membrane prior to assembly into the phage particle (Endemann and Model, 1995). gpVIII proteins (~2700 subunits) are required to form the protein cylinder, which envelopes the phage genome. The precursor of the gpVIII consists of 73 amino acids containing 23 residues signal sequence at the N-terminal region, which is removed after the polypeptide is inserted into the membrane (Zimmermann *et al.*, 1982). The precursor for gpIII consists of 424 amino acids including 18 residues N-terminal signal sequence and the polypeptide spans the cytoplasmic membrane once and places the major portion (379 residues) in the periplasm and only 5 residues (C-terminal) are exposed in the cytoplasm (Davis *et al.*, 1985). gpVI, gpVII and gpXI proteins are synthesised in the absence of the signal sequence and the membrane insertion mechanism is unknown (Webster, 1996).

gpI, gpIV and gpXI are non-capsid assembly proteins (Russel, 1991). Moderate amount of gpI in bacterial cells may prevent loss of membrane potential and thus this suggests that gpI may interact with gpIV to form a channel in the transmembrane region through the cytoplasmic membrane (Horabin and Webster, 1988). gpXI was initially known as gpI*, is resulted from an in-frame internal translational initiation event at Met-241 of gpI (Guy-Caffey *et al.*, 1992; Rapoza and Webster, 1995). gpXI plays only a structural role in the formation of the assembly site (Rapoza and Webster, 1995). gpIV integrates into the outer membrane where it present as oligomer composed of 10 to 12 monomers (Kazmierczak *et al.*, 1994) and is thought to form a large exit port in the outer

membrane, which allows the extrusion of assembled phage particle while maintaining the integrity of the membrane (Russel, 1994). This gated channel is opened by specific interaction among the protein gpI, gpXI and gpIV after the initiation of phage assembly (Rapoza and Webster, 1995).

### 2.5.3 Replication of Filamentous Bacteriophage

The infection of *E. coli* by the filamentous phage is a multi-step process (Figure 2.12). Initially, the particle binds to the tip of the pilus via a specific interaction with the N-terminus of the gpIII proteins located at one end of the phage particle (Rasched and Oberer, 1986). Removal of N-terminal fragment (36 kDa) of gpIII protein may abolish the infectivity of phages (Gray *et al.*, 1981; Armstrong *et al.*, 1981). It is postulated that the pilus subsequently depolymerises into the inner membrane, thereby, the tip of the phage is brought to the surface of the host. The phage particle must interact with TolQ, TolR and TolA (TolQRA) proteins in order for the viral DNA to be translocated into the cytoplasm (Russel *et al.*, 1988). Lazzaroni and colleagues (1995) suggested that the transmembrane regions of these TolQRA proteins interact with each other to form complexes, which can communicate between the inner and outer membrane and also maintain the integrity of the bacterial membrane.

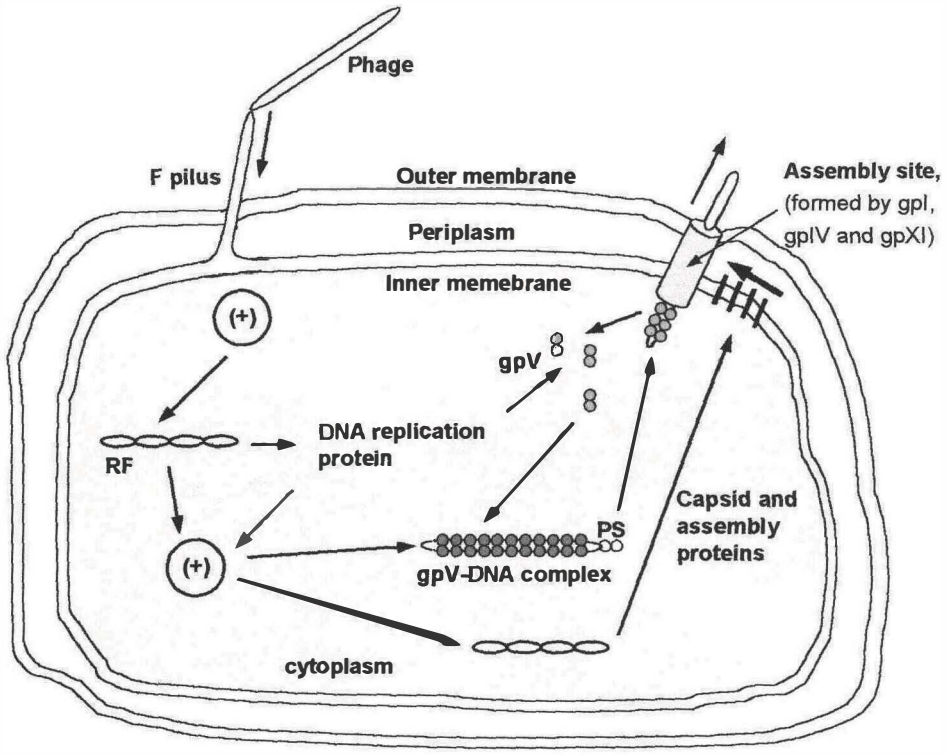



**Figure 2.12: Life cycle of filamentous phage**
The gpV-DNA complex towards the assembly site formed by gpI, gpIV and gpXI. In the extrusion process of the phage particle, gpV dimers are removed and replaced with gpVIII. The mature virion is released from the host cell. Arrow in the periplasm indicates the movement of the coat proteins into the assembly site (modified from Webster, 1996).

After the translocation of the ssDNA into the cytoplasm, host polymerase employs the (+) strand as template for the synthesis of a complementary (-) strand to yield a RF phage genome. Synthesis of (-) strand begins at *ori* (-), which located within the 'intergenic site'. The sequence of *ori* (-) resembles the –35 and –10 regions of RNA promoters and resemblance is important for primer synthesis by RNA polymerase (Higashitani *et al.*, 1997). The synthesis of (-) strand is followed by the production of coat, assembly and phage replication proteins. Phage genome replication is initiated by gpII, which initiates the cleavage of (+) strand at *ori* (+) of the RF and the freed 3' end serves as primer for rolling circle

replication on the (-) strand. The newly synthesised (+) strand is ligated by gpII to form a closed circular genome. In the early stage of infection, the new (+) strand serves as template for the synthesis of additional RF, while in the later infection, the conversion of ssDNA to RF is arrested by increasing level of gpV dimers. The gpV-DNA complexes sequestered the nascent (+) strand into the assembly particle (Webster, 1996).

Phage assembly is a membrane-associated process and the assembly site corresponds to adhesion zones or area of close contact between the outer and inner membranes (Lopez and Webster, 1985). The close contact area might form by a specific interaction among gpI, gpIV and gpXI before the assembly of the gpVII and gpIX at the end of the particle take place (Webster, 1996). Bacterial thioredoxin reductase, thioredoxin and phage encoded gpI are interacted to facilitate the phage assembly (Russel and Model, 1985). The process required ATP hydrolysis and proton motive force (Feng *et al.*, 1997). The nascent virion is extruded through the export channel with the replacement of gpV with gpVIII at the assembly site. The assembly of the phage is completed when gpVI and gpIII proteins are incorporated into the end of the filament.

## 2.6 Phage Display Technology

In 1985, Smith showed that the genome of bacteriophage f1 could be manipulated to display a foreign segment of protein on the surface of phage particle. The gene

encoding the *Eco*RI endonuclease fragments was fused into gene III of f1 and the resulting phage containing *Eco*RI-gpIII fusions could be detected by using a polyclonal antibody specific against endonuclease (Smith, 1985). This observation stimulated further research in this area and the most impressive aspects of phage display technology is the variety of uses of the technology such as display of foreign sequences on the surface protein, epitope mapping of antibody and display of random peptides on the coat proteins (Parmley and Smith, 1988; Lowman *et al.*, 1991; Tsunetsugu-Yokota *et al.*, 1991; Wang *et al.*, 1995). Two important concepts have been derived from the first phage display experiment: (i) the phage display technology should be possible to generate a large library, in which each phage displays a unique random peptide sequence and; (ii) the construction leads to a direct physical link between the encoding DNA and displayed sequence (Kay and Hoess, 1996).

### 2.6.1 Phage Display System

The coding sequences for random peptides are normally inserted into genes III or VIII of filamentous phage because the N-terminal of the coat proteins is surface-exposed. Smith (1993) has classified six different systems for displaying the foreign peptides on the surface of phage as shown in Figure 2.13. A type 3 vector has a single open reading frame bearing a single recombinant gene III (white box with hatched segment), which accepts foreign DNA inserts and display five

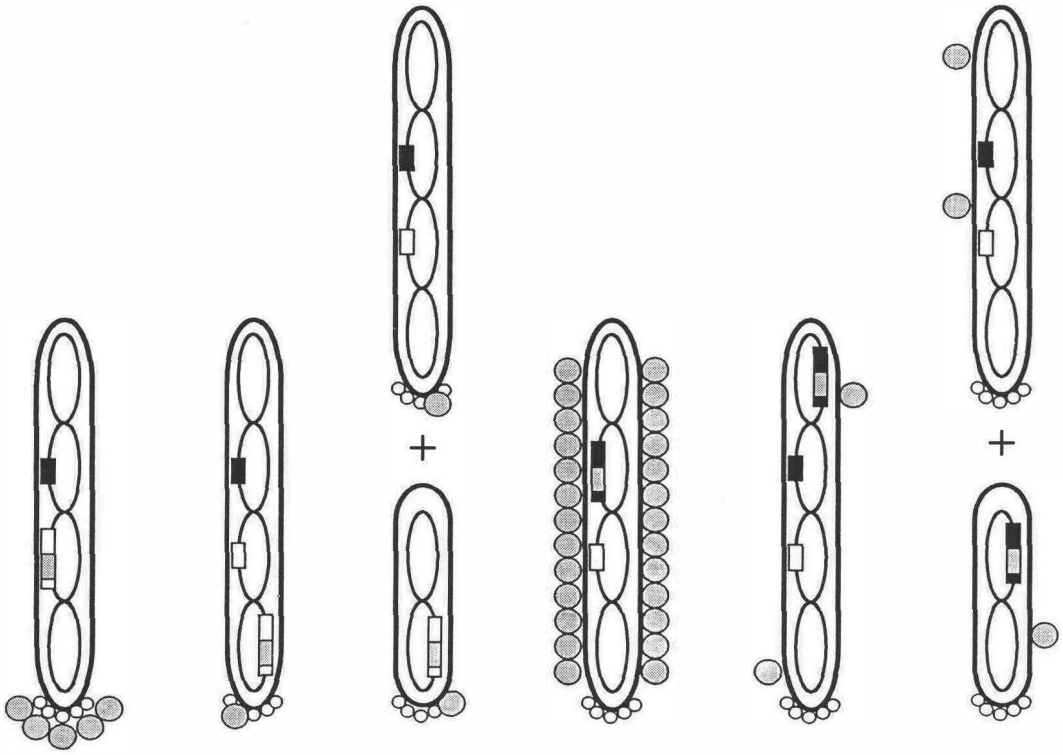



**Figure 2.13: Classification of phage display vectors**
The long and short tubes represent phage virions and phagemids, respectively. The twisted lines inside the tubes are ssDNA and the black and white bars represent the segment encoding proteins gpVIII and gpIII, respectively. The hatched bars represent the segment encoding the fusion proteins spliced into coat protein genes. The large hatched and small white circles represent the fusion proteins and the N-terminal domain of gpIII proteins, respectively. Foreign peptides that display on gpIII are either fused to N-terminal domain (Type 3) or replaced the N-terminal domain (Type 3+3 and Type33). Similarly, each copy of the gpVIII display foreign peptide in Type 8 system whereas only a minority of gpVIII display the foreign peptide in Type 8+8 and Type 88 (adapted from Smith, 1993).

copies of single type recombinant gpIII molecule (hatched circle) at the tip of the phage. Occasionally, some large foreign peptides are not suitable to be inserted in Type 3 vectors because the peptides displayed may interfere with gpIII function in virion assembly and infectivity (Smith, 1993). This disadvantage can be alleviated

by using Type 33 vector, in which two copies of gene III (wild type and recombinant) are inserted into the vector and the resulting gpIII are the mixture of wild type and recombinant molecules. If the displayed recombinant gpIII are not fully functional, the remaining wild-type gpIII proteins are sufficient for virus infectivity and assembly. Similarly, Type 88 bearing two gpVIII genes (recombinant and wild-type) in the genome and displaying a mixture of recombinant and wild-type gpVIII molecules on the surface of the virion.

Type 3+3 resembles Type 33 with two genes III in two different locations. In this case, the wild type genome is carried by a helper phage whereas the recombinant gene is on a phagemid, which is a plasmid containing the replication origin, antibiotic resistance gene and the phage intergenic region. Following the coinfection of the phagemid harbouring host cells with helper phage, yielding two different genotype phages with phagemid genome and helper genome, respectively. The mixture of wild type and recombinant gpIII are incorporated into both of the phage particles. Additionally, the phages bearing the phagemid can be titered and propagated independently since the infection conferring the antibiotic resistant gene to the host cells. Type 8+8 vector is one of the counterparts of Type 3+3 vector.

The size of the foreign inserts is the major factor that affects the efficacy of the displaying system. The peptide fused to gpIII is ranging from 6 amino acids to 38 amino acids in length (Scott and Smith, 1990; Kay *et al.*, 1993). Whereas, only

short foreign peptides coding sequence (6 to 8 residues) can be fused into gene VIII (Kishchenko *et al.*, 1994). However, foreign peptides up to 50 kDa are tolerated in Type 88 and 8+8 vectors (Smith, 1993).

### 2.6.2 Filamentous Phage Display Peptide Library

The construction of combinatorial peptide libraries is an important application of phage display technology. The filamentous bacteriophage vectors in which the foreign antigenic determinants are cloned into coat protein gene of the phage and displayed as part of the coat protein of the phage are known as "fusion phage". Parmley and Smith (1988) showed that fusion phage displaying peptide of interest could be affinity-purified from a library containing of $10^8$-fold excess of phage not carrying the target peptide and this process is known as 'biopanning'.

As illustrated in Figure 2.14, biopanning is a process of incubating a library of fusion phage particles with target molecules usually bound to a solid phase either the target molecules is attached directly onto the surface of the microtiter plates (Heiskanen *et al.*, 1999), polystyrene beads (Keller *et al.*, 1993), latex beads (D'Mello and Howard, 2001), nitrocellulose membrane (Dyson and Murray, 1995) or the target molecules are biotinylated and then immobilised on streptavidin coated plates (Parmley and Smith, 1988). phages displaying the peptides that bind to the target molecule are captured, while others are washed away. After the immobilisation of the target molecules, the library is reacted with

the target molecules. The unbound phages are washed away and the bound phages eluted by lowering the pH of the elution buffer. An attempt to decreased the elution pH gradually from pH 5 to 2 in the final round of biopanning preferentially select for high binding clones and removed a substantial number of weaker binder from the pH 2 eluates (D'Mello and Howard, 2001). The recovered phages are amplified by infecting fresh host cells and the process of binding and elution are repeated for several rounds to enrich those phages that bind to the target molecule and the binding peptides of the individual clones can be deduced by nucleotide sequencing (Parmley and Smith, 1988; Scott and Smith, 1990).

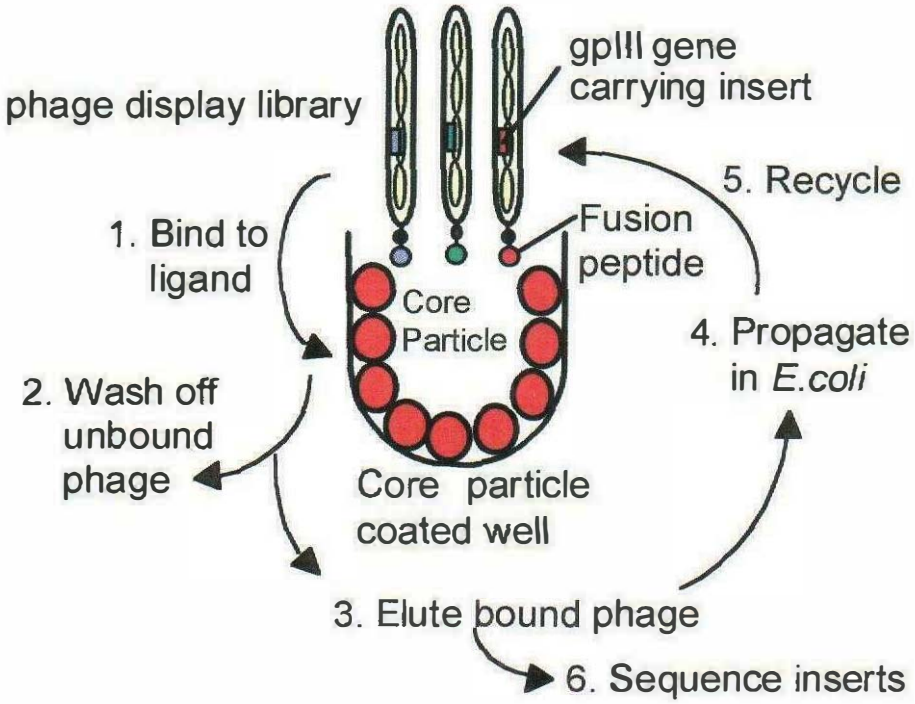



**Figure 2.14: Biopanning process**
The library of phages is incubating with target molecules that immobilised on a solid support. (1) specifically bound phages are captured by the target molecules. (2) unbound phages are washed away. (3) The bound phages are eluted and; (4) amplified by infecting fresh host cells. (5) The process 1 to 4 are repeated for several rounds and; (6) the individual clones are characterised by DNA sequencing (adapated from Yusoff and Tan, 2001).

### 2.6.3 Conformational Constrained Peptide library

In the common phage displayed peptide library, the carboxyl terminus of the peptide is fused to a capsid protein, thus there is no structural constraints imposed on the N-terminus and those peptides are free to adopt many different conformations due to high entropy (Burritt *et al.*, 1996). The resulted conformations of the unconstrained peptides are greatly different from the structure of the same amino acid sequences as it exists in native or folded forms. Consequently, the resolution of the binding motifs can be tractable only by conformational constrained peptide libraries (Burritt *et al.*, 1996). Furthermore, the fused peptides are relatively short in length compared with natural protein and therefore it is unlikely that the short peptides are sufficiently folded into stable secondary structure. In order to overcome this problem, the conformational characteristics have been artificially imposed on the peptide in order to greatly lower the entropy of such peptides and consequently reduce the range of conformational available (Smith and Petrenko, 1997). The constrained peptides library poses a more defined configuration rather than unconstrained peptides. Many proteins contain structural sensitive interaction regions that exist only in native form of the protein and these same regions may be mimicked by cyclic amino acid sequences (Burritt *et al.*, 1996).

In general, the conformational constraint peptide is imposed by flanking the random peptide sequences with two cysteine residues which able to form disulfide

bonds (Luzzago *et al.*, 1993; McLafferty *et al.*, 1993; McConnell *et al.*, 1994). The constrained peptides represent far lower complexity than unconstrained peptides (Cheadle *et al.*, 1994; Prezzi *et al.*, 1996). The fundamental constrained phage display peptide library construction is similar to linear peptide library except that an oligonucleotide encoding cysteine residue is inserted before and after the randomly generated DNA sequences and a disulfide bond is formed by oxidising during the virion morphogenesis (Smith and Petrenko, 1997). Inter-molecular disulfide bridge among the displayed peptides are unlikely to form due to the distance between the adjacent subunits are larger than 10-fold of a single disulfide bond (Smith and Petrenko, 1997).

## 2.7 Application of Phage Display Technology

### 2.7.1 Epitope Mapping and Mimicking

Phage display technology provides a simple and relatively low cost approach in mapping the specificity of the antibody binding sites. Random peptide library represents rich source of sequence combinations where the epitopes and mimotopes of monoclonal antibody can be defined (Geysen *et al.*, 1986). The epitopes of antibody might be continuous or discontinuous on the surface of a ligand. A continuous epitope comprising a few adjacent critical residues in the primary sequence whereas a discontinuous epitope comprising critical binding residues that are separated in the primary sequence but close in the native

conformation (Luzzago *et al.*, 1993). Therefore, discontinuous epitopes are generally conformational dependent because the overall protein structure is required to constrain these epitopes (Smith and Petrenko, 1997). In many cases, the epitopes are discontinuous due to the antibodies are generated by immune response against the assembled or discontinuous determinants (Geysen *et al.*, 1986). Phage peptide ligands selected against the discontinuous epitope have been no significant homology with the original antigen (Felici *et al.*, 1991, Smith *et al.*, 1993). Wrighton and colleagues (1996) selected a small peptide with no significant similarities to the primary sequence of cytokine erythropoietin but acts as full agonist of erythropoietin hormone. These peptides are collectively termed as 'mimotope' as they mimic the epitope of the natural ligands (Geysen *et al.*, 1986). Therefore, mimotopes are not much value in epitope mapping compared with those ligands that share some similarities with their natural ligands. Antibodies such as anti-HIVgp120 (Christian *et al.*, 1992), HBsAg (Motti *et al.*, 1994), anti-acetylcoline (Balass *et al.*, 1993), anti- H107-ferritin (Luzzago *et al.*, 1993) have been successfully mapped either as epitopes or mimotopes by using random phage display peptide libraries.

### 2.7.2 Identifying of Peptide Ligands

Phage display peptide libraries containing millions of foreign sequences, which represents a huge source of ligands for a variety of receptor ligands (Cortese *et al.*, 1995). Peptides selected of a ligand are often refers as novel receptors to the

existing receptors such as 18 novel domains of SH3 were successfully discovered from random peptide library (Sparks *et al.*, 1994). Additionally, peptide selection from random peptide libraries have also shown to be a powerful tool in identifying ligands for a receptor without knowing its structure ligands. Target receptors have been used in affinity selections including streptavidin (Devlin *et al.*, 1990; Kay *et al.*, 1993; McLafferty *et al.*, 1993), S fragment of RNase (Smith *et al.*, 1993), Src SH3 domains (Cheadle *et al.*, 1994; Rickles *et al.*, 1994; Sparks *et al.*, 1994), ssDNA (Krook *et al.*, 1994), plastic surface (Adey *et al.*, 1995), HBV nucleocapsid (Dyson and Murray, 1995), whole organ such as kidney and brain in living mouse (Pasqualini and Ruoslahti, 1996) and complete virion such as NDV (Ramanujam *et al.*, 2002).

### 2.7.3 Drug Discovery

Affinity selections of random peptide libraries against receptors are the potential approach toward the new drugs discovery. Peptides selected might act as agonist or antagonist or otherwise modulator for a receptor (O'Neil *et al.*, 1992; Smith *et al.*, 1993; Doorbar and Winter, 1994). Generally, peptides selected have poor pharmacological properties due to the rapid degradation in the human body by naturally occurring enzyme (reviewed in Smith and Petrenko, 1997). However, several successful cases have been reported. Wrighton and colleagues (1996) has selected small cyclic peptides from a random phage display peptide library that were able to bind and activate the receptor of cytokine erythropoietin. Therefore,

these mimetics act as full agonists and potentially substitute the larger natural hormone. Böttcher and colleagues (1998) demonstrated that in the presence of peptides selected from linear peptide library against the core particles of HBV reduced the production of virions in cell culture system.

Peptides composed of D-isomers are more resistant to degradation by cellular enzyme than L-isomers. In the study conducted by Schumacher and colleagues (1996), SH3 domains with D isomer configurations have been used for biopanning to select cyclic L-isomer peptides. The synthesised of 'mirror image' D amino acids cyclic peptides have been demonstrated to interact with natural forms (L amino acids) of SH3 domains. This approach are not restricted to genetically encoded peptide libraries (Scott and Smith, 1990; Devlin *et al.*, 1990; Cull *et al.*, 1992) but also applicable to RNA and DNA libraries since nucleic acids contain chiral centre, which may recognised by nucleases (Ellington and Szostak, 1990; Schumacher *et al.*, 1996). The approach provides a useful starting point for the design or discovery of novel drugs.

### 2.7.4 Vaccines and Diagnostics

Antigenic and immunogenic mimicry are two important terms in the context of discovery of vaccines and diagnostics by using phage display technology. Peptides selected against an antibody are antigenic mimicking the natural epitope. If the new antibodies elicited against these antigenic mimics are able to cross-

react with the antigenic determinants of the natural epitope, such peptides therefore display both antigenic and immunogenic properties (reviewed in Smith and Petrenko, 1997).

The manipulation of genetically engineered filamentous phage to obtain both antigens and immunogens for antibody productions have been reported (de la Cruz *et al.*, 1988; Greenwood *et al.*, 1991). In both cases, specific antibody could be raised against the immunisation with repeat regions of circumsporozoite of *Plasmodium falciparum* displayed on the surface of fusion phage.

Motti and colleagues (1994) demonstrated that phage displayed epitopes (phagotope) selected against monoclonal antibody specific for the human HBsAg behave as antigenic mimics of HBsAg and that particular mimotope of HBsAg displayed on filamentous phage is recognised by human immune sera. This indicates that the possible use of phagotopes as markers to detect the presence of specific antibody in the serum (Motti *et al.*, 1994). Subsequently, HBsAg displayed on phage has proved to be the immunogens, inducing reproducible and potent immunisation (Meola *et al.*, 1995). These findings demonstrated that it is possible to identify disease-specific epitopes that can be used as diagnostic reagents and as leads for the discovery of accellular vaccines (Motti *et al.*, 1994; Meola *et al.*, 1995).

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Materials

All the general materials used in this study were purchased from BDH Chemicals Ltd., Amresco, USA, Genaxis GIBCO BRL, Merck, Promega, Fermentas Inc. and Sigma Chemicals. Radioisotopes were purchased from Amersham Pharmacia.

**Table 3.1: Standard solutions and buffers**

| Solution/buffer | Components |
|---|---|
| 10x TBE | Tris-HCl (0.9 M; pH 8.0), Boric acid (0.9 M), EDTA (20 mM) |
| NET-gel | Tris-HCl (50 mM; pH 7.6), NaCl (150 mM), NP-40 (0.1% v/v), EDTA (1 mM), Gelatin (0.25% w/v), $NaN_3$ (0.02% w/v) |
| PBS | $K_2HPO_4$ (20 mM), $KH_2PO_4$ (5 mM), NaCl (150 mM) |
| STE | Tris-HCl (10 mM; pH 8.0), EDTA (1 mM; pH 8.0), NaCl (0.15 M) |
| TBS | Tris-HCl (50 mM; pH 7.6), NaCl (150 mM) |
| TE pH 7.6 | Tris-HCl (10 mM; pH 7.6), EDTA (1 mM; pH 8.0) |
| TE pH 8.0 | Tris-HCl (10 mM; pH 8.0), EDTA (1 mM; pH 8.0) |

**Table 3.2: Media**

| Media | Recipes/litre |
|---|---|
| Top layer agar | Trypticase (10 g), NaCl (5 g), Bactor-agar (10 g) |
| Minimal agar | Agar (15 g), Glucose [20% w/v; 10 ml], Vitamin B1 (625 μg), Spitzizen Salts (200 ml). Spitzizen salts contain $(NH_4)_2SO_4$ (0.2% w/v), $K_2HPO_4$ (0.14% w/v), $KH_2PO_4$ (0.6% w/v), Trisodium citrate (0.1% w/v), and $MgSO_4$ (0.2% w/v) |
| LB medium | Bacto-trypton (10 g), Bacto-yeast extract (5 g), NaCl (5 g) (adjusted to pH 7.2) |
| LB agar | Bacto-trypton (10 g), Bacto-yeast extract (5 g), NaCl (5 g), bacteriogical agar (adjusted to pH 7.2). |

**Table: 3.3 Recombinant plasmids**

| Plasmids | Description | References |
|---|---|---|
| pTacpcore (5378 bp) | This plasmid contains the coding region for amino acids 1-8 of β-galactosidase followed by 3 linker amino acids (Glu, Phe, His) and subsequently the gene encoding amino acids 3 to 183 of HBcAg. The expression of the gene is under the control of *Tac* promoter. | Stewart, 1993 |
| pR1-11E (4823 bp) | Contains amino acids 1-8 of β-galactosidase coding sequence followed by 3 linker amino acids (Glu, Phe, His) and subsequently the gene encoding amino acids 3 to 148 of HBcAg. The expression of the gene is under the control of *Tac* promoter. | Stewart, 1993 |
| pMDHBs3 (5131bp) | This plasmid contains a Cap-independent Translation Enhancer (CITE) sequence of the encephalomyocarditis virus (EMC) RNA 5' non-coding region, which function as internal entry point of eukaryotic ribosomes for initiation of translation. *In vitro* transcription is under the control of T7 promoter. It carries the gene for L-HBsAg and ampicilin resistance gene. | Dyson and Murray, 1995; Tan, 1997 |

## 3.2 Preparation and Purification of Full-length and Truncated HBcAg

This method was originally described by Murray *et al.* (1984), Dyson and Murray (1995) with some modifications by Tan (1997). Several colonies of *E. coli* strains harbouring plasmids pTacpcore and pR1-11E encoding the full-length and truncated HBcAg, respectively (Steward, 1993), were grown at 37 °C with vigorous shaking (250 rpm) in LB medium (500 ml) supplemented with ampicillin (100 μg/ml). IPTG (0.5 mM) was added when the cultures reached $OD_{600nm}$ around 0.8 to 1.0 and the incubation was continued for 16 to 20 h. The cells were harvested by centrifugation at 4,000 x*g* for 15 min at 4 °C and the pellet was suspended in lysis buffer [50 mM Tris (pH 8.0), 0.1 % (v/v) Triton; 12 ml]. $MgCl_2$ (4 mM), lysozyme (50 mg/ml; 48 μl) and DNase I (20 μg/ml; 5 μl) were then added to the suspension, and mixed by rotation for 2 h at room temperature. The cell extract was recovered by centrifugation at 11,000 x*g* for 30 min at 4 °C and the protein was precipitated by ammonium sulphate (35 % saturation). The precipitate was centrifuged with the same conditions, resuspended in TBS and dialysed against two changes of TBS (1 L) at 4 °C overnight. The dialysed solution was layered onto 8 to 40 % continuous sucrose gradient (in TBS; 12 ml) and centrifuged at 100,000 x*g* for 5 h at 4 °C (Beckman, rotor SW-41Ti). The solution was then fractionated (0.5 ml per fraction) by punching a hole at the bottom of the centrifuge tube. The Bradford assay and SDS-PAGE analysis were performed to determine the amount and the purity of the protein, and those fractions containing the HBcAg were pooled and

concentrated with a 300 kDa cut-off microconcentrator (Microsep, USA). The concentrated proteins were analysed again on SDS-PAGE and immunoblotting and the final concentration of the purified HBcAg was determined by the Bradford assay. The concentrated HBcAg preparations were supplemented with $NaN_3$ [0.02 % (w/v)] and stored at 4 °C.

### 3.2.1 The Bradford Assay

Protein samples were diluted to 40 µl with TBS (pH 7.6) in test tubes. Afterward, 1 x Bradford dye [0.01 % (w/v) Coomassie blue G-250, 4.75 % (v/v) ethanol, 8.5 % (v/v) phosphoric acid; 1 ml] was added and vortexed immediately. The mixture was incubated at room temperature for 10 min and the absorbent was measured at $OD_{595nm}$ with a spectrophotometer (Shimadzu, UV-1610; Australia). The protein concentration was determined from a standard curve obtained from measurements with BSA at various concentrations (0 to 20 µg/µl) in the same conditions.

### 3.2.2 SDS-PAGE

Proteins were separated by SDS-PAGE (Laemmli, 1970) by a discontinuous buffer system. SDS-polyacrylamide gels were prepared by using the Mini Protean 3 apparatus (BioRad). Separating gel solution [15% (w/w)] containing $dH_2O$ (3.75 ml), 4x lower buffer [1.5 M Tris-HCl (pH 8.6), 0.4 % (w/v) SDS; 3.75 ml], bis-acrylamide solution [30 % (w/v) acrylamide, 0.8 % (w/v) bisacrylamide; 7.5 ml],

10 % (w/v) ammonium persulphate (93.8 µl) and TEMED (BioRad; 7 µl) was prepared. The mixture was then dispensed into the casted gel apparatus and saturated isobutanol (~100 µl) was overlayed immediately onto the solution. The gel was allowed to polymerise for 40 min and the isobutanol layer was removed via absorption with a 3MM Whatman filter paper. Then, the stacking gel solution [5% (w/w)] containing $dH_2O$ (5.84 ml), 4x upper buffer [0.5 M Tris-HCl (pH6.8), 0.4 % (w/v) SDS; 2.5 ml], bis-acrylamide solution (1.66 ml), 10 % (w/v) ammonium persulphate (66.6 µl) and TEMED (BioRad; 7 µl) was then layered onto the resolving gel and a plastic comb was carefully inserted into the stacking gel solution and allowed to polymerise for 45 min.

Proteins samples (3 µl) were prepared by mixing with 2x loading buffer [62.5 mM Tris-HCl (pH 6.8), 25 % (v/v) glycerol, 2 % (w/v) SDS, 0.01 % (w/v) bromophenol blue, 5 % (v/v) β-mercaptoethanol; 3 µl] and boiled for 10 min. The treated samples and appropriate protein markers were then electrophoresed in Tris-Glycine [0.025 M Tris (pH 8.3), 0.192 M glycine, 1 % (w/v) SDS] at a constant current (30 mA) until the dye front reached the bottom of the separating gel (~1.5 h). The gel was stained with staining solution [0.1% (w/v) Coomassie blue R-250, 40 % (v/v) methanol, 10 % (v/v) acetic acid] for 30 min at room temperature with agitation and finally, the gels were destained with destaining solution [10 % (v/v) methanol, 10 % (v/v) acetic acid] until the bands became clear (1 to 3 h).

### 3.2.3 Western Blotting

This method was described by Towbin *et al.*, (1979) with some modifications. The polyacrylamide gel containing fractionated proteins was equilibrated in transfer buffer [25 mM Tris-Cl, 190 mM Glycine (pH 8.0), 20 % (v/v) methanol] for 10 min at room temperature. Meanwhile, a piece of nitrocellulose membrane and 6 pieces of Whatman 3MM filter paper, approximately same size with the gel, were soaked in transfer buffer in separate containers for 5 min at room temperature. The filter papers (3 pieces) were placed on the lower electrode (anode) of the semi dry blotter (Trans-Blot® SD, BioRad) and the air bubbles were removed by rolling a glass rod on top of the papers. The pre-soaked nitrocellulose membrane was placed onto the filter papers and the equilibrated gel was transferred carefully onto the surface of the membrane. Another 3 pieces of filter papers were then placed on top of the gel and the air bubbles were removed as above. The upper electrode (cathode) and the safety cover was then assembled. Afterwards, a constant current (60 mA) was applied for 1 to 2 h to transfer the proteins onto the nitrocellulose membrane. Lanes containing the molecular weight markers were cut off from the membrane and stained with staining solution for 1 min and immediately soaked in destaining solution for 1 h. The electroblotted nitrocellulose membrane was then blocked with milk diluent (KPL, USA), which was diluted 1:10 in $dH_2O$ for 2 h at room temperature. The nitrocellulose membrane was then washed 3 times with TBST [TBS (pH 7.6) supplemented with 0.05 % (v/v) Tween 20] for 5 min each wash. The nitrocellulose membrane

was incubated with a dilution 1:5000 of mAb anti-core (clone C1-5; Chemicon) in TBST for 1 h at room temperature with gentle agitation. The membrane was then washed again with TBST followed by incubating with alkaline phosphatase conjugated with goat anti-mouse antibody (1:5000) in TBST for 1 h at room temperature. Finally, the bands were developed with BCIP/NBT in alkaline phosphatase buffer [100 mM Tris-Cl (pH 9.5), 100 mM NaCl , 5 mM $MgCl_2$]. Colour development was terminated by transferring the nitrocellulose membrane to a container filled with water and the developed membrane was allowed to dry at room temperature.

### 3.2.4 Electron Microscopy of HBcAg Particles

A carbon coated grid (400-500 mesh) was touched to the surface of one drop (~20 μl) of sample and left for 5 min. The excess sample was drawn off with a filter paper and the grid was allowed to air-dry for 10 min. Following the adsorption of negative staining solution [methylamine tungstate 2% (v/v)] onto the sample coated grid for 5 min, electron microscopy was carried out with the Hitachi H-1700 and micrographs were taken at appropriate magnifications.

## 3.3 Biopanning

One set of U-shaped polystyrene well (Costar) was coated with full-length HBcAg (10 μg/ml in TBS; 120 μl) and incubated overnight at room temperature

with gentle agitation. The HBcAg coated wells were washed 3 times with PBS, and blocked with BSA (10 mg/ml in TBS, 200 µl) for 2 h at room temperature. Afterwards, the HBcAg coated wells were washed 6x with TBS (supplemented with 20 mg/ml of BSA) and phages from a disulfide constrained 7-mer phage display peptide library (New England Biolabs, USA: diluted to 2 x $10^{11}$ pfu in TBS; 100 µl) were added and incubated at room temperature for 1 h. Then, the unbound phages were removed by washing the coated well 10x with the same buffer and the bound phages were eluted from the well by incubating with elution buffer [0.1 M Glycine (pH 2.2); 200 µl] in the wells for 10 min. The eluate was transfered to a fresh microcentrifuge tube containing neutralising solution [1 M Tris-HCl (pH 9.1); 37.5 µl] and vortexed for 20 s. The eluted phages were subjected to titration and amplification. As a negative control, the HBcAg was replaced with BSA (10 mg/ml) and biopanned as above.

### 3.3.1 Phage Titration

An aliquot (10 µl) of phage mixture was transferred to a fresh microcentrifuge tube containing LB medium (90 µl), and further diluted to $10^{-4}$ or $10^{-10}$. Each dilution (10 µl) was added to a suspension of *E. coli* strain ER2537 [F' *lacI*$^{q}$ *△(lacZ)M15 proA*$^{+}$*B*$^{+}$ */fhuA2 supE thi △(lac-proAB) △(hsdMS-mcrB)5* ($r_k^- m_k^-$ McrBC$^-$); 200 µl] grown to $OD_{600nm}$ 0.8. Subsequently, the mixture was transferred to a tube containing melted top layer agar (3 ml) equilibrated to 45 °C, gently mixed and poured onto LB agar plate containing IPTG [8.3% (w/v)] and

X-gal [6.7% (w/v)]. The plates were incubated overnight at 37 °C and plaques formed were counted the next day. All titrations were performed in triplicate and the amount of phage recovered from each well was calculated as pfu.

### 3.3.2 Amplification of Phage with *E. coli*

The eluates (80 µl) recovered from the first round of biopanning experiment were added into LB medium (20 ml; in a 250 ml Erlenmeyer flask) containing ER2537 cells (100-fold dilutions of overnight culture) and propagated for 4.5 h at 37 °C with vigorous shaking. Then, the culture was centrifuged at 15,000 x*g* for 10 min at 4 °C and the supernatant was transferred to a fresh tube and precipitated with PEG/NaCl [20 % (w/v) PEG 8000 in 2.5 M NaCl; 4 ml] overnight at 4 °C. The phages were precipitated by centrifuging at 15,000 x*g* for 15 min at 4 °C and the supernatant was discarded. The pellet was then suspended in TBS (1 ml) and transferred to a fresh microcentrifuge tube. The remaining cells were removed by centrifugation at 13,000 x*g* for 5 min at 4 °C and the supernatant was transferred to a fresh microcentrifuge tube. Once again, the phages were precipitated with PEG/NaCl (200 µl) on ice for 1 h and centrifuged at 15,000 x*g* for 10 min at 4 °C. The supernatant was removed by aspiration and the pellet was resuspended in TBS (200 µl) containing $NaN_3$ [0.02 % (w/v)]. Finally, the suspension was centrifuged at 13,000 x*g* for 1 min to remove any insoluble matter and the supernatant was transferred to a fresh microcentrifuge tube and titered as above.

### 3.3.3 Small scale preparation of M13 ssDNA

A blue plaque from the plate was inoculated into LB medium (3 ml) containing log phase ER2537 cells (75µl) as host and incubated for 4.5 h at 37 °C with vigorous shaking. The culture (1.5 ml) was collected at 15,000 x*g* for 10 min at 4 °C and the supernatant was transferred to a fresh microcentrifuge tube. The phages in the supernatant were precipitated with PEG/NaCl (200 µl) for 15 min at room temperature prior to centrifuge at 15,000 x*g* for 15 min at 4 °C. The supernatant was removed by aspiration and the pellet suspended in TE buffer (pH 8.0; 100 µl). Subsequently, the pellet was extracted once with phenol, followed by chloroform and finally the DNA was precipitated with ethanol, washed, dried, and resuspended in TE (pH 8.0; 20 µl).

## 3.4 Nucleotide Sequencing

Nucleotide sequencing was performed based on the dideoxynucleotide chain termination method by Sanger and Coulson (1975) and Sanger *et al.*, (1977).

### 3.4.1 Cycle sequencing

The individual clone was characterized by cycle sequencing with the Omnibase DNA Cycle Sequencing System (Promega, USA). Four microcentrifuge tubes (0.5 ml; labelled with A, C, G and T) containing appropriate d/ddNTP mixes (2

μl) were prepared and stored on ice before being used. An aliquot of the purified ssDNA template (1 μg) was added into a fresh microcentrifuge tube (0.5 ml) containing the –55 gIII primer (5'CGTCTTTCCAGACGTTAGTAAATGAAT3'; 1.0 ρmol), [α-$^{33}$P]-dATP (10 μCi) and DNA Sequencing 5x buffer [10 mM $MgCl_2$, 250 mM Tris. HCl (pH 9.0); 5μl] were added to the same mixture. The final volume of the mixture was adjusted to 16 μl with $ddH_2O$. An aliquot of the OmniBase™ Sequencing Enzyme Mix (10 u/μl; 1 μl) was then added and briefly mixed by pipetting. The mixtures (4 μl) were then added into each tube containing the d/ddNTP mix. Mineral oil (20 μl) was then added and the tubes were centrifuged briefly. The tubes were then placed in a thermal cycler (PTC-200, Peltier Thermal Cycler) with the following profile: 94°C for 2 min; Subsequently 30 cycles of 94°C for 1 min (denaturation), 46°C for 30 s (annealing) and 72 °C for 30 s (extension). Once the thermal cycling programme was completed, the reaction was terminated by adding DNA sequencing stop solution [10mM NaOH, 95% (v/v) formamide, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol; 3 μl]. Prior to electrophoresis, the reaction mixtures were heated at 94°C for 2 min and chilled on ice immediately.

### 3.4.2 ssDNA Sequencing

The sequencing reaction using the Klenow fragment of *E. coli* DNA Polymerase I was divided into two steps: annealing of primer to its template and polymerisation. A mixture of ssDNA (285 ng/μl; 3.5 μl), primer -55 gIII (1

pmole; 1µl), 10 x TM [100 mM Tris-Cl (pH 8.5), 50 mM $MgCl_2$; 1 µl] and $ddH_2O$ (4.5 µl) was prepared in a microfuge tube and incubated at 80 °C for 5 min and allowed to cool at room temperature for 15 min. The mixture was briefly centrifuged and transferred to 4 tubes (labelled with A, C, G and T) containing nucleotide mix A, C, G and T respectively (Table 3.4; 2 µl). The Klenow reaction mix [9 mM Tris-Cl (pH 8.0), 9 mM DTT, 0.2 U Klenow fragment of *E. coli* polymerase I, 0.56 µCi α-[$^{33}$P]-dATP; 2 µl] was then added to each tube, mixed gently by tapping and incubated at room temperature for 15 min. Subsequently, a chase mix [0.25 mM dNTP in TE (pH 8.0); 2 µl] was added and the tubes were further incubated for 20 min. The reactions were terminated by adding gel loading buffer [0.5 % (w/v) bromophenol blue, 0.5 % (w/v) xylene cyanol, 10 mM EDTA in formamide; 2 µl]. Prior to electrophoresis, the reaction mixtures were heated at 90°C for 5 min and chilled on ice immediately.

**Table 3.4: ddNTP/dNTPs stock solutions for ssDNA sequencing**

| dNTPs/ddNTPs | Nucleotide mixes/µl | | | |
|---|---|---|---|---|
| | A | C | G | T |
| 0.5 mM dCTP | 500 | 25 | 500 | 500 |
| 0.5 mM dGTP | 500 | 500 | 25 | 500 |
| 0.5 mM dTTP | 500 | 500 | 500 | 25 |
| 10 mM ddATP | 1 | - | - | - |
| 10 mM ddCTP | - | 8 | - | - |
| 10 mM ddGTP | - | - | 16 | - |
| 10 mM ddTTP | - | - | - | 50 |
| TE (pH 8.0) | 500 | 100 | 1000 | 1000 |

### 3.4.3 Denaturing Gel Electrophoresis for Sequencing

The thermal cycling products were electrophoresed in polyacrylamide gel [8% (w/v)] containing urea (7 M). Prior to loading of the reaction mixtures, the gel was equilibrated to approximately 50 °C by pre-electrophoresed for 30 min in 1x TBE buffer with a constant power at 60 W using sequencing gel electrophoresis apparatus (Model S2 BRL, USA). Each reaction mixture (labelled A, C, G and T; 4µl) was loaded onto adjacent slots of the gels and electrophoresis was done at a constant power (60 W) until the first dye reached the bottom of the gel (~ 1.5 h). After electrophoresis was completed, the gel was soaked in a fixer [10 % (v/v) ethanol, 10 % (v/v) acetic acid glacial] for 20 min and subsequently transferred onto a Whatman 3MM chromatography paper. Prior to autoradiography, the gels were dried in a gel dryer (Model 543, BioRad) at 80°C for 2 h. X-ray films (Fuji) were exposed to the dried gels in dark for an indicated period of time. The autoradiographed films were developed in developer solution (Fuji) for 5 min, washed with tap water and fixed for 5 min in fixer solution (Fuji).

## 3.5 Large scale preparation and purification of M13

This method was established by Smith and Scott (1993) with some modifications. The M13 stock from DNA preparation was centrifuged at 15,000 x*g* for 5 min at 4 °C to pellet down the ER2537 cells. The supernatant (500 µl) was transferred to a universal bottle containing the host cells (2.5 ml) at log phase, mixed and

incubated at room temperature for 5 min. Then, the mixture was transferred to a flask containing LB medium (500 ml) and host cell (5 ml) at log phase and incubated at 37 °C for 4.5 h with vigorous shaking (250 rpm). The culture was distributed equally into two centrifuge tubes (250 ml) and centrifuged at 6,300 x*g* for 15 min at 4 °C (Beckman J2-MI, rotor JA14). The supernatant was transferred to a new centrifuge tube and precipitated with PEG/NaCl (0.15 vol) overnight at 4 °C. The precipitated phage was collected by centrifugation at 6,300 x*g* for 30 min at 4 °C (Beckman J2-MI, rotor JA14) and the supernatant was removed by aspiration. The precipitate was dissolved in TBS (pH 7.5, 30 ml) and centrifuged at 15,000 x*g* for 10 min at 4 °C (Beckman J2-MI, rotor JA20) to precipitate any insoluble matter. The supernatant was then transferred to a new centrifuge tube and re-precipitated with PEG/NaCl (4.5ml) for 1 h at 4 °C. Then, the precipitated phage was recovered by centrifugation at 8000 x*g* for 30 min at 4 °C (Beckman J2-MI, rotor JA20). The supernatant was removed by aspiration and the precipitate was resuspended in TBS (pH 7.5; 10 ml). The suspension was transferred to a tared vessel and TBS (pH 7.5) was added to bring up the total net weight to 32.25 g. CsCl (14.49 g) was then added to bring the density of the solution to 1.30 g/ml. The CsCl solution was transferred to a polyallomer centrifuged tube (Sorvall, USA) and centrifuged at 174,000 x*g* for 18 h at 4 °C (Sorvall, rotor T-865). After centrifugation, light-scattering band was visualised by shining a bright light downward through the tube and the translucent, nonflocculent phage band was observed lying near the middle of the tube. The phage was collected by fractionation (1 ml per fraction) and the fractions

corresponded to the phage band were pooled and transferred to a centrifuge tube for the Beckman NVT-65 rotor. The tube was filled up with TBS (pH 7.5) and centrifuged at 225,000 x*g* for 4 h at 4 °C, the supernatant was removed by aspiration and the pellet was resuspended in TBS (pH 7.5, 3 ml). The suspension was transferred to a new centrifuge tube and filled up with TBS (pH 7.5) and centrifuged at 275,000 x*g* for 90 min at 4 °C (Beckman L7-55, NVT-65). The supernatant was removed by aspiration, the pellet obtained from 500 ml of the original culture was dissolved in TBS (pH 7.5, 1 ml) and the pfu of the purified M13 was determined as described in section 3.3.1.

## 3.6 Antibody-Phage Competition Assay

Microtiter plate wells were coated with full-length HBcAg (300 nM in TBS; 120 µl) overnight at room temperature in a humidified environment with gentle agitation. Phage (2 x $10^9$ pfu/ml in TBS supplemented with 0.2 mg/ml of BSA; 50 µl) was added with a series of different concentrations (0 to 0.17 µg/µl in the same buffer; 50 µl) of anti-HBcAg monoclonal antibody (mAb C1-5, Chemicon, USA) in microcentrifuge tubes. An aliquot of these mixtures (100 µl) was transferred to the HBcAg coated wells and incubated for 1 h at room temperature with gentle agitation. Afterward, the wells were washed 6x with TBS (containing 0.2 mg/ml of BSA). Bound phage was eluted and titered as described in section 3.3.1.

## 3.7 Phage Binding Assay

The strength of interactions between the selected fusion phages with HBcAg was determined by two assays: (i) phage binding assay on a solid phase whereby phages were allowed to interact with HBcAg coated on a microtiter plate well and, (ii) phage binding assay in solution to determine the relative dissociation constant ($K_d^{rel}$).

### 3.7.1 Phage Binding Assay on a Solid Phase

A microtiter plate was coated with HBcAg as described in the biopanning experiment (section 3.3). The purified phages ($2 \times 10^{11}$ pfu; 100 µl in TBS) were then incubated with HBcAg coated wells for 1 h at room temperature. Bound phages were then eluted with glycine buffer [0.1 M (pH 2.1); 200 µl] and neutralised with Tris-HCl [1 M (pH 9.1); 37.5 µl]. The eluted phages were titered as described in section 3.3.1 and their relative binding affinities to HBcAg were expressed in pfu.

### 3.7.2 Phage Binding Assay in Solution

This method was initially described by Friguet *et al.*, (1985) and modified by Dyson *et al.*, (1995) to measure $k_d^{rel}$ values of fusion phages. The full-length or truncated HBcAg at various concentrations (0 - 1500 nM; 180 µl) was mixed with

a constant amount of phage (1 x $10^9$ pfu/ml; 180 µl) in TBS containing BSA (0.2 mg/ml) in siliconised tubes and incubated at 4 °C for 18 h. An aliquot (100 µl) of each mixture was then transferred to U-shaped,. polystyrene wells (Costar, USA) that had been coated with full-length HBcAg [2 µg/ml in TBS (pH 7.6)] as described above. After 1 h of incubation, the wells were washed 6x with TBS containing BSA (0.2 mg/ml). Then, the bound phages were eluted, neutralised and titrated as described previously (section 3.3.1). All assays were performed in triplicate.

## 3.8 Inhibition of L-HBsAg Binding to HBcAg by Synthetic Peptides

### 3.8.1 Minipreparation of Plasmid DNA Containing L-HBsAg gene

This method was modification from those described by the methods of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). It was used to extract small quantities of plasmid from bacterial cells mainly for the identification of recombinant clones.

A well isolated single colony of bacteria was inoculated into LB medium (5.0 ml) supplemented with amplicilin (100 µg/ml) and incubated overnight with shaking at 250 rpm at 37 °C. The overnight culture (1.5 ml) was then transferred to a microcentrifuge tube and the cells were collected by centrifugation at 4,000 x*g* for 15 min at 4 °C. The pellet was resuspended in ice-cold Solution I [50 mM

glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0); 100 µl]. Solution II [0.2 M NaOH, 1 % (w/v) SDS; 200 µl] was then added, mixed and incubated on ice for 5 min. Ice-cold solution III [5 M potassium acetate, 11.5 % (v/v) glacial acetic acid; 150 µl] was added and incubated on ice for another 5 min. The mixture was centrifuged at 15,000 x*g* for 5 min at 4 °C, and the supernatant was transferred to a fresh microcentrifuge tube. After that, the solution was extracted once with phenol, phenol:chloroform:isoamylalchohol (25:24:1), and chloroform, consecutively. An equal volume of organic solvent was added to the aqueous DNA solution and the mixture was vortexed vigorously for 2 to 3 min, followed by centrifugation at 15,000 x*g* for 2 to 3 min to separate the organic and the aqueous phase. The upper aqueous solution was transferred to a new microcentrifuge tube and the DNA was then precipitated with absolute ethanol (-20 °C; 2 vol) for 20 min or overnight at -20 °C. The plasmid was recovered by centrifugation at 15,000 x*g* for 10 min at 4 °C, washed with 70 % (v/v) ethanol, dried and dissolved in TE buffer (pH 8.0; 20 µl).

### 3.8.2 Large Scale Preparation and Purification of Plasmid

This method was employed to prepare large quantities (~ 0.5 mg) of pure plasmid, pMDHBs3, carrying the L-HBsAg gene for *in vitro* transcription (Dyson and Murray, 1995; Tan, 1997). This method can be divided into two major steps: preparation of crude lysate by the alkaline lysis method (Sambrook *et al.,* 1989) and plasmid purification by PEG precipitation (Lis and Schlreif, 1975; Lis, 1989).

An Overnight culture of *E. coli* strain CJ236 harbouring pL-HBsAg (500 ml; LB medium) were harvested by centrifugation at 4,000 x*g* for 15 min at 4 °C and resuspended in ice-cold STE buffer (100 ml). The cells were precipitated under the same conditions and the pellet was resuspended in ice-cold solution I (18 ml). Cells were lysed by adding lysozyme [10 mg/ml in 10 mM Tris-HCl (pH 8.0); 2 ml] and freshly prepared solution II (40 ml). The mixture was incubated at room temperature for 10 min, followed by addition of ice-cold solution III (20 ml), and incubated on ice for 10 min, and the white precipitate was precipitated by centrifugation at 5,000 x*g* for 15 min at 4 °C. The supernatant was then filtered through 4 layers of cheese cloth and absolute isopropanol (0.6 vol) was added, and the mixture was incubated at room temperature for 10 min. The pellet was collected by centrifugation at the same conditions, washed with ethanol [70 % (v/v)] and resuspended in TE buffer (pH 8.0; 3 ml). An equal volume of ice-cold LiCl (5 M) was added, mixed well and centrifuged at 11,000 x*g* for 10 min at 4°C. The supernatant was then transferred to a new centrifuge tube and isopropanol (equal volume) was added and centrifuged at 11,000 x*g* for 10 min at room temperature. The pellet was rinsed with 70 % (v/v) ethanol, dried and resuspended in TE buffer (pH 8.0; 0.5 ml) containing DNAase-free pancreatic RNAase (20 µg/ml). The solution was incubated at room temperature for 30 min, PEG 8000 [13 % (w/v) in 1.6 M NaCl; 0.5 ml] was added and the mixture was centrifuged at 13,000 x*g* for 10 min at 4 °C. The DNA pellet was then dissolved in TE buffer (pH 8.0; 400 µl), extracted once with phenol, phenol:chloroform:isoamylalchohol (25:24:1) and followed by chloroform.

Ammonium acetate (10 M; 100 µl) and ethanol (2 vol) were added to precipitate the plasmid. The pellet obtained from centrifugation at 15,000 x*g* for 5 min at 4°C was washed, dried and dissolved in TE buffer (pH 8.0; 0.5 ml).

### 3.8.3 Quantification of DNA

The quantity of DNA was measured by using a spectrophotometer measurement of the optical density (OD) at 260 nm. An $OD_{260nm}$ of 1.0 represents a concentration of 50 µg/ml for dsDNA and 40 µg/ml for ssDNA. The purity of nucleic acid in solution was estimated as a ratio $OD_{260nm}/OD_{280nm}$, and the ratio of 1.8 indicates a reasonably pure DNA preparation.

### 3.8.4 *In vitro* transcription and translation

This method was described by Dyson and Murray (1995) with some modifications by Tan (1997). Restriction enzyme digestion with *Sal I* was first performed to linearise plasmid pL-HBsAg, then the linearised plasmid was purified with phenol, phenol:chloroform (1:1), chloroform extraction and precipitated overnight with sodium acetate (3 M, pH 5.2; 0.1 vol) and ethanol (2 vol.). The purified plasmid (1 µg) was used as template for *in vitro* transcription containing rNTPs (7.5 mM) and T7 RNA polymerase (Promega, USA). The mixture was incubated at 37 °C for 2 h and the synthesised RNA (2 µl aliquots) was immediately kept at −80 °C. For the translation reaction, the RNA was

diluted 10-fold and an aliquot (1 µl) was added into a microcentrifuge tube containing 6 x cocktail mix [amino acid mixture minus Met (0.12 mM), [$^{35}$S]-methionine (1 µCi/µl; Pharmacia), $Mg(OAC)_2$ (3.75 mM), KCl (0.72 M), DTT (12 mM); 1.5 µl], rabbit reticulocyte lysate (Promega, USA; 5 µl), RNasin (Promega, 7.2 u/µl; 1.0 µl), and nuclease free $dH_2O$ (0.5 µl). The mixture was incubated at 30 °C for 2 h and the reaction (1 µl) was analysed on SDS-PAGE. The gel was dried in a gel dryer (Model 543, BioRad) and autoradiographed against an X-ray film (Fuji). After overnight exposure, the film was developed by soaking in a developer (Kodak) for 5 min, washed and fixed for 5 min in a fixer (Kodak).

### 3.8.5 Preparation of Synthetic Peptides

Peptides WSFFSNI, WPFWGPW, C-WSFFSNI-C and C-WPFWGPW-C were synthesised by Genemed Synthesis, USA. Peptide LEDPASR was synthesised by the Department of Chemistry, University of Edinburgh. All the lyophilised peptides were dissolved in $ddH_2O$ to give a stock concentration of 20 mM. Peptides were further diluted with NET-gel buffer to the appropriate concentrations for the *in vitro* assay. For the peptides that did not dissolve in water, they were dissolved in methanol [50% (v/v)] or by adding dimethylformamide until the lyophilised powder completely dissolved. The peptide solutions were kept in –20 °C freezer prior to use.

### 3.8.6 Inhibition Assay

One set of U-shaped polystyrene strip plate (Nunc, Denmark) was coated with full-length HBcAg (10 μg/ml) overnight at room temperature as described in section 3.3. A constant concentration of [$^{35}$S]-L-HBsAg (100-fold dilution of a translation reaction) was mixed with a serial dilutions of peptides ranging from 0 to 1000 μM. The mixtures were then transferred to HBcAg coated wells and incubated for 24 h at 4 °C. In the following day, the wells were washed 6x with NET-gel buffer supplemented with DTT (2 mM) and the radioactivity of bound [$^{35}$S]-L-HBsAg was determined with a scintillation counter (Beckman, LS 6500; USA).

# CHAPTER IV

# RESULTS AND DISCUSSION

## 4.1 Preparation of Core Particles from Full-Length and Truncated HBcAg

HBcAg consists of 183 amino acids and capable of self-assembles into core-like particles when expressed in *E. coli*. These particles appear to be similar to the viral nucleocapsids isolated from chronic carriers of HBV when observed under an electron microscope (Cohen and Richmond, 1982). The shortest polypeptide of HBcAg required for self-assembly is 140 amino acids from the N-terminus while the arginine-rich C-terminal region (amino acids 149-183) is required for nucleic acid binding (Gallina *et al.*, 1989; Zlotnick *et al.*, 1996).

In this study, the full-length and truncated HBcAg, respectively, were produced in *E. coli* strain W3110IQ harbouring plasmids pTacpcore and pR1-11 (Steward, 1993; Tan, 1997). The molecular mass of full-length and truncated HBcAg encoded by plasmids pTacpcore and pR1-11 are approximately 22 and 17 kDa, respectively (Steward, 1993; Tan, 1997). The proteins were expressed upon induction by IPTG. Subsequently, the proteins were precipitated by ammonium sulphate and fractionated on sucrose gradient centrifugation. Fractionated samples were analysed on SDS-polyacrylamide gel and the positive fractions were combined and concentrated by microconcentrator. A dominant band of

approximately 22 and 17 kDa, were observed in full-length and truncated HBcAg preparations, respectively [lanes 4 to 11 of Figure 4.1 (a) and; lanes 4 to 13 of Figure 4.1 (b)]. These bands correspond to the molecular mass of the full-length and truncated HBcAg, respectively. Thus, the result indicates that the core particles formed by full-length and truncated HBcAg were present in the corresponding fractions. No band was observed in lanes 12 to 19 in Figure 4.1 (a), while lanes 21 to 23 contained other protein with size approximately 40 kDa. Although lanes 14 to 23 of Figure 4.1 (b) contains protein with ~17 kDa but these are believed to be the premature assembled core particles and as a result they were migrated slower than assembled core particles.

The concentration of the protein in each fraction was determined by the Bradford assays (Figure 4.1) and fractions 4 to 9 and 4 to 13, respectively, of the full-length and truncated HBcAg preparations, were separately pooled and concentrated, using 300 kDa cut-off microconcentrator. The concentrated proteins were separated with SDS-PAGE and proteins were immunoblotted with the anti-core monoclonal antibody (Figure 4.2). An intense band corresponds to 22 kDa and 17 kDa (Figure 4.2: Lanes 2 and 3) respectively, were observed in the full-length and truncated HBcAg. Nevertheless, less intense smear bands below the intense band with sizes less than 22 kDa were also detected in full-length HBcAg preparation. The smear bands were reported by Tan (1997), who further demonstrated that the first six amino acids of the N-terminus of the polypeptides in the smear and intense bands contained the same amino acid sequence, TMITDS. This sequence

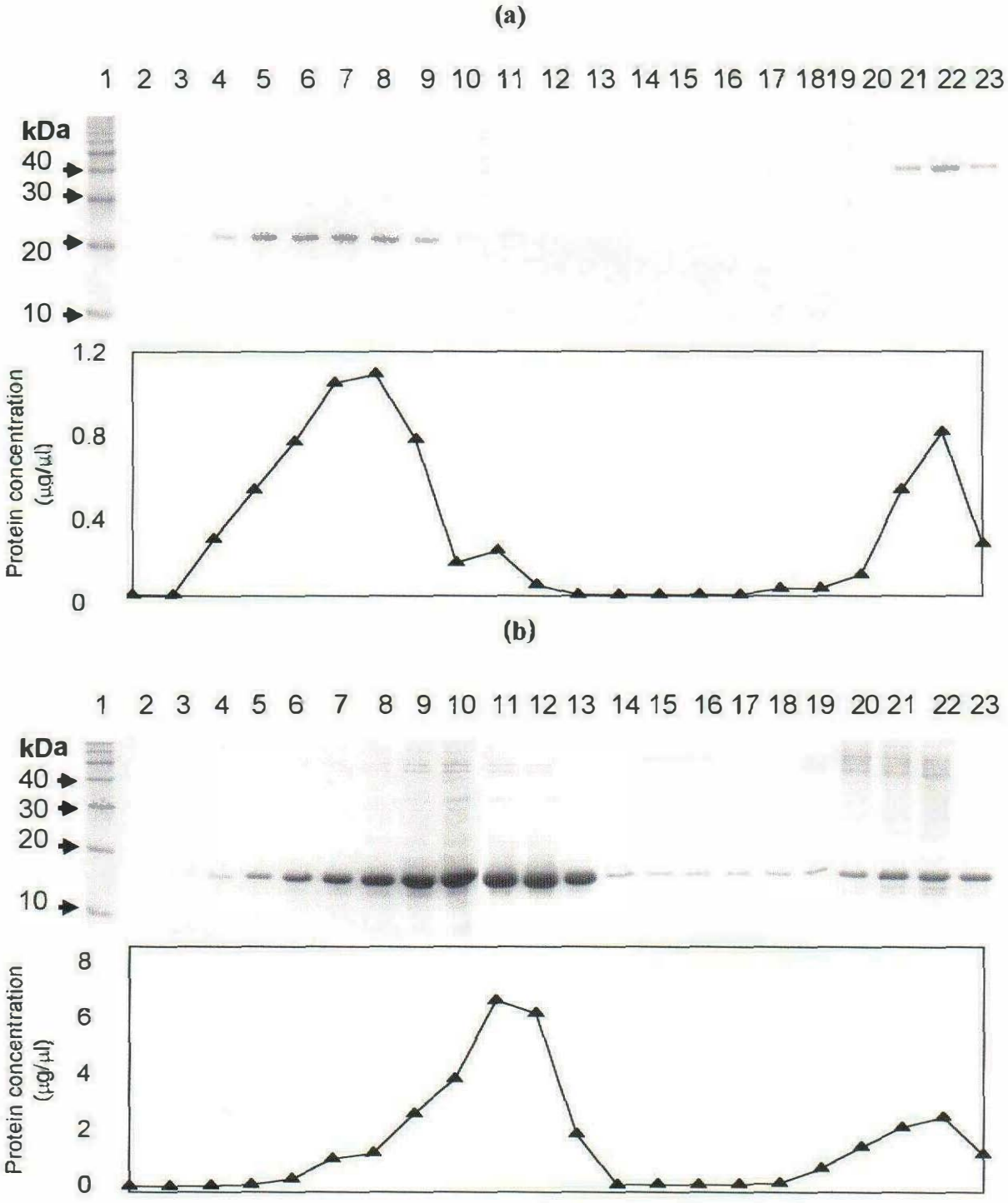



**Figure 4.1: SDS-PAGE of full-length and truncated HBcAg fractionated on sucrose gradient centrifugation.** Lanes 2 to 23 are 22 fractions collected from sucrose gradient centrifugation. Lane 1 is protein marker in kDa. (a) full-length and, (b) truncated HBcAg preparations. Both gels were stained with Coomassie blue.

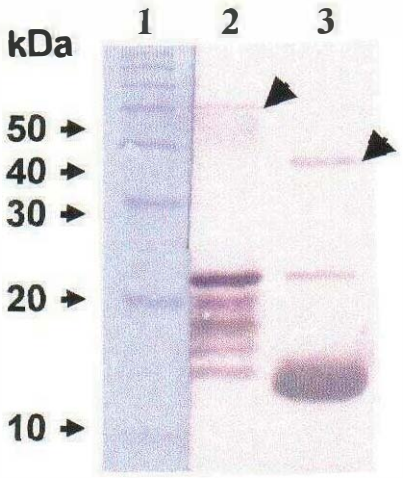



**Figure 4.2: Immunoblot of concentrated HBcAg**
Lane 2: full-length HBcAg with a dominant band of approximately 22 kDa and other smear bands less than 22 kDa. Lane 3: truncated HBcAg gave rise to a dominant band of approximately 17 kDa. Lane 1 is protein marker in kDa. The separated proteins were transferred onto nitrocellulose membrane and blotted against the anti-core mAb C1-5 (Chemicon, USA). Arrow-heads show the dimers of full-length and truncated HBcAg with molecular mass of approximately 45 and 35 kDa, respectively.

corresponds to amino acids 2 to 7 of the β-galactosidase sequence fused to the N-terminus of HBcAg (Tan, 1997). Besides, protein bands with molecular mass approximately 45 and 35 kDa were also detected by the western blot (Figure 4.2; lanes 2 and 3), and they were thought to be the dimers of full-length and truncated HBcAg, respectively. Dimer formation is partially contributed by Cys-61 of HBcAg, which forms a disulfide bridge between two monomers. However, these disulfide bonds are not essential for particle formation (Nassal *et al.*, 1992; Zhao and Standring, 1992). The present of dimers could be due to incomplete denaturation of the samples by boiling for 5 min prior to electrophoresis or insufficient amount of mercaptoethanol or DTT, which act as a reducing agent.

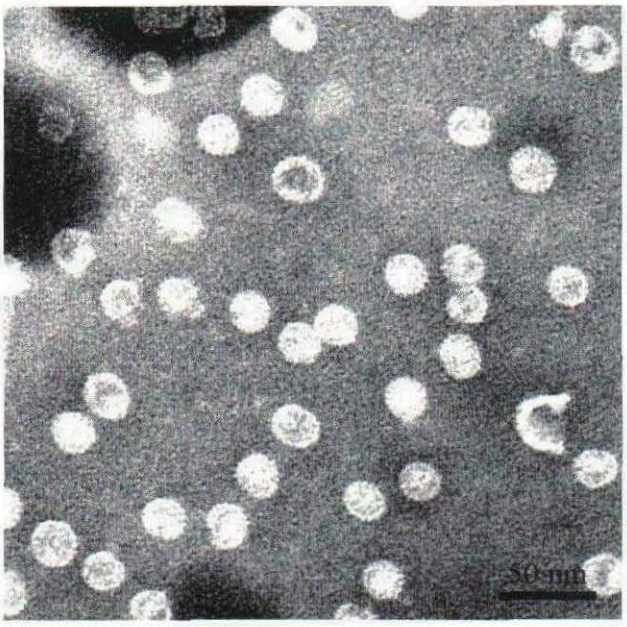
As HBcAg is capable to form particles, sucrose gradient centrifugation provides an efficient method to separate the particles from other host proteins. Figure 4.1 shows the typical protein profile after a single round of sucrose gradient centrifugation [8 to 40% (w/v)]. The sedimentation rate depends upon particle mass, early fractions of the peak contained exclusively large particles as it migrated faster in the sucrose gradient and the later portions mainly composed of small particles. As demonstrated by Tan (1997), the early fractions and the last fractions of the peak were pooled and concentrated separately. Subsequently, these preparations were subjected to second round of centrifugation. Two separated peaks with some overlapping on the edges were obtained. As examined by light scattering, the early fractions of the first peak and the later fractions of the second peak were composed exclusively large and small particles, respectively

(Tan, 1997). In this study, however, the large and small particles were not separated because the HBcAg samples were subjected to only one round of sucrose gradient centrifugation. Although both particles are different in dimensions but the structure of the protruding spikes, which corresponds to the immuno-dominant region of HBcAg exposed on the exterior surface, is similar in both large and small particles (Wingfield *et al.*, 1995; Zlotnick *et al.*, 1996). Hence, the separation of the large and small particles was not necessary in this study.

HBcAg is highly enriched in Arg residues particularly in the C-terminal region. Arg codons in the HBcAg mRNA are mainly encoded by triplet AGA, which is the rarest codons in the gene of *E. coli* (Aota *et al.*, 1988). Furthermore, direct measurement of the level of AGA t-RNA have shown that it exists in a low concentration in all phases of cell growth (Saxena and Walker, 1992; Emilson *et at.*, 1993). The higher expression level of truncated HBcAg in this study (Figure 4.2), which lacks the Arg-rich C-terminal region, is much greater than full-length HBcAg agreed with this factor (Zheng *et al.*, 1992; Stewart, 1993).

In order to investigate the assembled core-like structures, dialysed HBcAg preparations were negatively stained and observed under an electron microscope. Figure 4.3 shows the electron micrographs of core particles formed by full-length and truncated HBcAg. This finding is in good agreement with that reported by

(a)




(b)

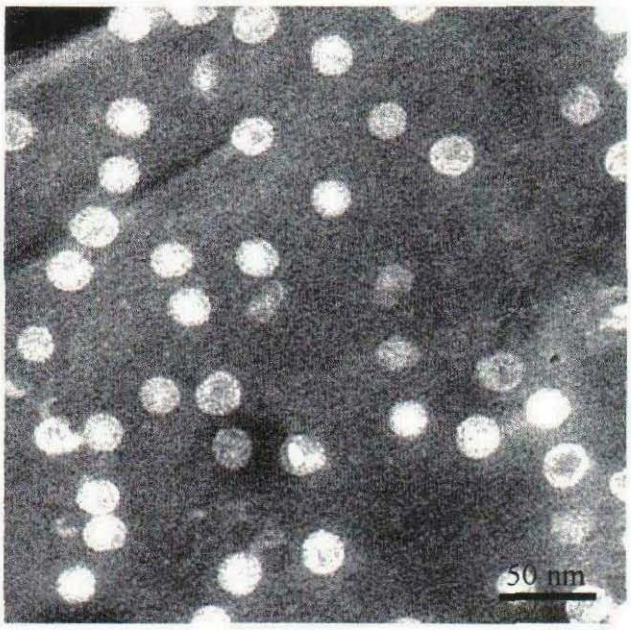



**Figure 4.3: Electron micrograph of core particles**
Purified core particles were dialysed overnight and negatively stained with methylamine tungstate. (a) full-length and, (b) truncated HBcAg preparations.

Cohen and Richmond (1982). The ratio of large to small particles depends upon the length of the HBcAg gene truncations. As reported by Kenney *et al.*, (1995), the ratio of large to small particles in the liver of an infected patient is ~13 to 1. In order to obtain a close ratio of large to small particles as in infected human liver, the core particles of full-length HBcAg preparation were used as substrate in the following biopanning experiment.

## 4.2 Biopanning of Constrained Peptide Library against Core Particles

In this experiment, the core particles were coated on U-shaped polystyrene wells overnight at room temperature. The HBcAg coated wells were blocked with BSA and subsequently incubated with the phage library. After 1 h incubation, the unbound phages were removed and the bound phages were eluted from the well. The eluted phages were amplified prior to subsequent round of panning and the individual phage clone was characterised by nucleotide sequencing. In order to select high affinity binding phage clones, the stringency was increased by (i) the selection was performed at room temperature (25°C), (ii) washing the wells thoroughly (10 times) to remove the low affinity binding phages, and (iii) reduced the time to 1 h to select for ligands with rapid on rates (Tan *et al.*, 2001). The biopanning procedures adopted in this study were different from the method used by Dyson and Murray (1995) as listed in Table 4.1. Therefore, the peptide sequences screened from this study were obviously different from the

hexapeptides isolated from a linear hexapeptide library (as reported by Dyson and Murray, 1995).

**Table 4.1: Differences in biopanning procedures**

| Differences | Dyson and Murray (1995) | In this study |
|---|---|---|
| Substrate | Truncated HBcAg | Full-length HBcAg |
| Coating surface | Nitrocellulose membrane | U-shaped polystyrene well |
| HBcAg coating conditions | HBcAg was washed through the membrane | HBcAg was incubated on microtiter plate overnight at room temperature |
| Blocking condition | The HBcAg coated membrane was blocked with BSA (10 mg/ml) and Tween [0.02% (v/v)] overnight at 6°C | The HBcAg coated wells were blocked with BSA (10 mg/ml) for 2 h at room temperature |
| Phage display peptide library | Phage display linear hexapeptide library | Phage display disulfide constrained heptapeptide library |
| Incubation period | The HBcAg coated membrane was incubated with the phage library for 4 h at 6°C | The phage library was added into the well for 1 h at room temperature |

The phage display peptide library used in this study is a combinatorial random heptapeptide library that carrying the foreign peptide sequences at the N-terminus of the gpIII proteins of M13, which is flanked by a pair of cysteine residues. Under non-reducing conditions, recombinant phages able to display the cyclic heptapeptides on their gpIII proteins (Smith and Petrenko, 1997). Cyclic heptapeptide screened from the library are more specifically and tightly interact with highly conformational epitopes displayed on the core particles rather than phage clones selected from a linear peptide library, because the higher entropy of the linear fusion peptide reduced the specificity and affinity of the peptide toward conformational epitopes.

In order to prevent mutations during amplification, individual blue plaques were picked from unamplified eluates that had been plated on LB agar. Each plaque represents an individual phage clone, which carries a single type of fusion peptide. The genome of *E. coli* ER2537 was genetic engineered to confer the ability of α-complementation, which carried the *LacZ⊿M15* fragments capable to encode the defective M15 polypeptide (Maloy *et al.*, 1994). The phage carries a *Hinc*II fragment of the *lac* operon containing the lac promoter, an operator and a short N-terminal region of the *LacZ* gene required for α-complementation. When the phage infects the host cells, both the complementary segments of β-galactosidase assemble to form a functional tetramer in the presence of IPTG and X-gal, which causes cells appear in blue colour. If white plaques are present in the agar, it is most likely that the pool of phage became contaminated with environmental wild-type M13, which lacks the *lac* operon. In order for blue-white screening to work, the bacterial strain must be capable of α-complementation. The plaques would be formed in ~7 h incubation and total incubation time should not more than 18 h as deletions may occur. In order to avoid picking up more than one individual phage from the neighbouring plaques, well isolated blue plaques were randomly picked from a plate less than 100 plaques as shown in Figure 4.4.

All together 15, 19 and 40 individual clones, respectively, from first, second and third rounds of panning, were isolated and the coding regions of the fused peptides were sequenced. The deduced amino acid of each clone is shown in Table 4.2. In the first round of panning, 40% of phages screened carried the

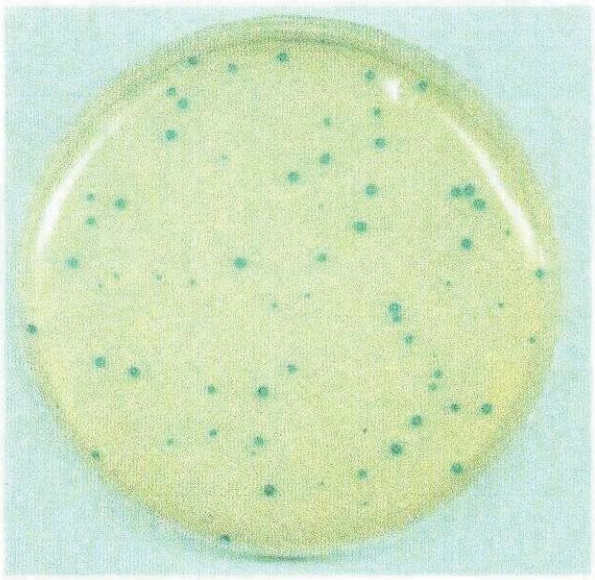


**Figure 4.4: Plaque assay showing blue recombinant M13 plaques**
Less than 100 plaques were evenly separated at an appropriate dilution.

**Table 4.2: Cyclic heptapeptides obtained from three rounds of biopanning**
Number in the parentheses represents the number of isolated phage clones bearing the same peptide sequence.

| Rounds of Biopanning | Deduced amino acid Sequences | Frequency of Sequences (%) |
|---|---|---|
| 1st round | C-PNTLLGS-C | 40.0 (6) |
| | C-PNTPSQW-C | 6.7 (1) |
| | C-KWPQWLL-C | 6.7 (1) |
| | C-ARVPPAF-C | 6.7 (1) |
| | C-KSDGLYT-C | 6.7 (1) |
| | C-LKTSSTV-C | 6.7 (1) |
| | C-PMQEFKQ-C | 6.7 (1) |
| | C-QKEEINA-C | 6.7 (1) |
| | C-SPPDLKE-C | 6.7 (1) |
| | C-TTNKSTE-C | 6.7 (1) |
| 2nd round | C-WPFWGPW-C | 42.1 (8) |
| | C-WSFFSNI-C | 31.6 (6) |
| | C-WSFFKLY-C | 5.3 (1) |
| | C-LLNTGLR-C | 5.3 (1) |
| | C-MAHCSTC-C | 5.3 (1) |
| | C-NLAQTTT-C | 5.3 (1) |
| | C-TDRSGHT-C | 5.3 (1) |
| 3rd round | C-WSFFSNI-C | 65.0 (26) |
| | C-WPFWGPW-C | 10.0 (4) |
| | C-RPFWGPW-C | 10.0 (4) |
| | C-QPWHWPP-C | 5.0 (2) |
| | C-WSFFKLY-C | 2.5 (1) |
| | C-DILHYSK-C | 2.5 (1) |
| | C-NELRSGL-C | 2.5 (1) |
| | C-NSVRVYS-C | 2.5 (1) |

peptide sequence C-PNTLLGS-C and the other sequences composed approximately 60%. Another two related peptide sequences were also screened in the first round. The peptide sequences C-PNTPSQW-C shares the first three residues with C-PNTLLGS-C, while the amino acids P and LL in the peptide C-KWPQWLL-C were also found in C-PNTLLGS-C. Interestingly, the residues WP and W in the peptide sequence C-KWPQWLL-C were also found in peptide C-WPFWGPW-C that screened from the subsequent rounds of panning. When the washing stringency was increased in the second round of panning, 42 and 32% of the phages screened carried the fusion sequence C-WPFWGPW-C and C-WSFFSNI-C, respectively. The latter sequence increased to ~65%, while the former sequence reduced to 10% in the third round of panning. An additional sequence, which related to the sequence C-WPFWGPW-C was also appeared with the same frequency (10%), but the first W was substituted by R. The most consensus sequences screened from the third round of panning were C-WSFFSNI-C (~65%), followed by C-WPFWPW-C (10%) and C-RPFWGPW-C (10%). The phage bearing the sequence C-WSFFKLY-C in which the first four amino acids are similar to sequence C-WSFFSNI-C appeared once in second and third rounds of panning. Biopanning of the phage library against BSA was carried out as a negative control and no recognisable consensus sequence was observed in the 15 phage clones isolated from the third round of panning (Table 4.3).

The most consensus fusion heptapeptide sequence obtained in the first round of biopanning was C-PNTLLGS-C, which relates to a linear hexapeptide LLGRMK

**Table 4.3: Peptide sequences obtained from third round of biopanning against BSA**
Number in the parentheses represents the number of isolated phage clones bearing the same peptide.

| Phage Clones | Deduced amino acid Sequences | Frequency of Sequences (%) |
|---|---|---|
| BSA1 | C-RYPSSWT-C | 6.7 (1) |
| BSA2 | C-LLVAKTN-C | 6.7 (1) |
| BSA3 | C-DTTTAFY-C | 6.7 (1) |
| BSA4 | C-TDGEKTH-C | 6.7 (1) |
| BSA5 | C-LELNPYR-C | 6.7 (1) |
| BSA6 | C-LHPSHPH-C | 6.7 (1) |
| BSA7 | C-TGFAQQT-C | 6.7 (1) |
| BSA8 | C-LRMQLPP-C | 6.7 (1) |
| BSA9 | C-LRAHTHY-C | 6.7 (1) |
| BSA10 | C-TIMPFLK-C | 6.7 (1) |
| BSA11 | C-LPHFALP-C | 6.7 (1) |
| BSA12 | C-HYQVTTR-C | 6.7 (1) |
| BSA13 | C-KNKRSPA-C | 6.7 (1) |
| BSA14 | C-SRASLSP-C | 6.7 (1) |
| BSA15 | C-SDVPYGT-C | 6.7 (1) |

isolated by Dyson and Murray (1995) using truncated HBcAg as substrate. The three hydrophobic amino acids, LLG, at the N-terminus of the linear hexapeptide LLGRMK appear in position 4 to 6 of the constrained heptapeptide in the sequence C-PNTLLGS-C. Although both sequences share some similar amino acid sequence, but the conformational state that associates with the core particles may differ. This is due to the fact that the conformation of a constraint peptide associates with the target molecule is more defined than a linear peptide, but a linear peptide is freely to adopt any configuration in a solution (Kay and Hoess, 1996).

Phage bearing sequence C-LLNTGLR-C that isolated from the second round of panning shares some similar amino acid sequence with C-PNTLLGS-C and the linear hexapeptide LLGRMK. However, only 1 out of 19 phage clones screened in the second round carried C-LLNTGLR-C, which was not found in the first round of panning show some similarities with C-NELRSGL-C isolated from the third round of panning. The amino acid residues LL and GXR of the C-LLNTGLR-C are found in LLGRMK. Besides, the amino acid residues L and GL within C-LLNTGLR-C were also found in C-NELRSGL-C. Therefore, these peptide sequences share some similarities.

The heptapeptide sequences obtained in the second round were not found in the first round of panning. This could be due to the low frequency of each individual phage clone in the first round except phage carrying the sequence C-PNTLLGS-

C, in which phages bearing some amino acid sequence homology appeared in the second (C-LLNTGLR-C) and third (C-NELRSGL-C) rounds. However, phage bearing the sequence C-NELRSGL-C was selected once in the third round of panning as the phages screened predominantly carrying the peptide C-WSFFSNI-C and C-WPFWGPW-C. This suggests that the selection stringency in this study preferred the isolation of phages bearing these amino acid sequences, which were obviously different from hexapeptides isolated by Dyson and Murray (1995).

Two predominant sequence motifs can be observed in the third rounds of panning: C-WSFFSNI-C (65%) and C-WPFWGPW-C (20%) and their derivatives (C-WSFFKLY-C and C-RPFWGPW-C, respectively). The amino acid sequence SNI in C-terminus of C-WSFFSNI-C was substituted with KLY in C-WSFFKLY-C. The frequency of phages carrying the peptide C-WSFFSNI-C increased about 33% in third round of panning. While the phage carrying the peptide sequence C-WPFWGPW-C reduced approximately 32% in third round of panning. The screening of cyclic peptide library against the full-length HBcAg core particles favoured the isolation of phage bearing sequence C-WSFFSNI-C than C-WPFWGPW-C. The fusion peptide C-WSFFSNI-C contains two hydroxylic side chains (S) and one amino acid bearing amide group (N), suggesting that hydrogen bonding plays an important role for the selection of this particular peptide.

Peptides can be classified into hydrophobic or hydrophilic based upon the characteristic of the amino acid side chains. A primary structure of a protein is

composed of a linear chain of amino acid and each amino acid has a measured hydrophobicity value that reflects its attraction to or repulsion from a water environment (Tanford, 1978). Based upon the characteristic of amino acid side chains, the genetically encoded 20 amino acids are divided into four categories: (i) negatively charged and hydrophilic: aspartate acid (D) and glutamate acid (E); (ii) positively charged and hydrophilic: lysine (K), arginine (R) and histidine (H); (iii) uncharged and hydrophilic: asparagines (N), glutamine (Q), serine (S), threonine (T) and tyrosine (Y); and (iv) uncharged and hydrophobic: alanine (A), glycine (G), valine (V), leucine (L), isoleucine (I), proline (P), phenylalanine (F), methionine (M) and tryptophan (W). F, W and Y are also jointly classified as aromatic amino acids.

Generally, the peptides isolated from the disulfide constrained heptapeptide library are mostly composed of non-polar and uncharged amino acids. None of the amino acid in peptides C-PNTLLGS-C, C-WPFWGPW-C and C-WSFFSNI-C bearing a charged side chain. However, R, possessing a basic side chain is present in peptide C-LLNTGLR-C, C-TDRSGHT-C, C-NELRSGL-C and C-NSVRVYS-C (Table 4.2) but these sequences appeared once in their corresponding round of panning. The peptide sequence C-WSFFSNI-C consists of 4 non-polar amino acids (underlined) and 3 uncharged hydrophilic amino acid (S and N). Whereas all amino acids in the peptide C-WPFWGPW-C are composed of hydrophobic amino acids. In the peptide C-PNTLLGS-C, 4 out of 7 residues are non-polar amino acid (underlined) and the remaining bearing uncharged polar

side chain. Non-polar amino acids do not contain significant dipole or capacity for forming hydrogen bonds. Hence, these amino acids are not interacting with water molecules but amino acids S and T bearing hydroxylic side chain, while N bearing an amide side chain are able to form hydrogen bond with other hydrophilic amino acids (Zubay *et al.*, 1995). Non-polar amino acids in the selected peptides greatly increased the hydrophobicity of these peptides. However, it does not affect the solubility of the fusion peptide carrying by phage because the average hydrophobic values of the peptide are insignificantly affected the totalled hydrophobicity of the system. Therefore, the peptides displayed on the gpIII proteins are dissolved in water and freely to be captured as ligands by their receptor, where the ligands are naturally composed of hydrophobic residues in the native structure. The cyclic peptides C-WSFFSNI-C and C-WPFWGPW-C also contain respectively, 3 and 4 amino acids bearing the aromatic side chains (underlined). Both peptide sequences were not discovered in the affinity selection using a linear random hexapeptide library (Dyson and Murray, 1995).

## 4.3 Interaction between Fusion Phages with Hepatitis B Core Particles

Following the selection of the recombinant phages from the disulfide constrained peptide library, the next challenge was to measure the strength of interaction, or known as binding affinity, between the recombinant phages and core particles. Two assays were adapted to examine the binding affinities of the phages to the core particles; (i) the first binding assay on the solid phase was carried out to

relatively compare the affinities among the phages to the core particles coated on the U-shaped polystyrene microtiter plate, and (ii) the phage binding assay in solution was carried out in order to measure the affinities to core particles in solution in terms of relative dissociation constant (Friguet *et al.*, 1985; Dyson and Murray, 1995; Dyson *et al.*, 1995).

In order to study the interaction between selected phages and the core particles, the fusion phages bearing the sequence C-WSFFSNI-C (clone WSFFSNI), C-WPFWGPW-C (clone WPFWGPW), C-PNTLLGS-C (clone PNTLLGS) and C-LLNTGLR-C (clone LLNTGLR) were scaled up and purified with CsCl centrifugation (Smith and Scott, 1993). Peptide sequences C-PNTLLGS-C, C-WPFWGPW-C and C-WSFFSNI-C were chosen because they were the most consensus sequence screened from the first, second and third rounds of panning, respectively. Besides, phage bearing the C-LLNTGLR-C was added in this assay because the sequence shares some homologies with LLGRMK and PNTLLGS. After ultracentrifugation, the phages were concentrated at one particular band at the middle of the centrifuge tube (Figure 4.5). The collected phages were titered and their concentrations were found to be around 1-3 x $10^{12}$ pfu/ml (Table 4.4). The amino acid sequences that fused to the N-termini of gene III of filamentous phages were confirmed again by DNA sequencing (Figure 4.6), and the results show that the amplified phages carried the expected fusion peptides.

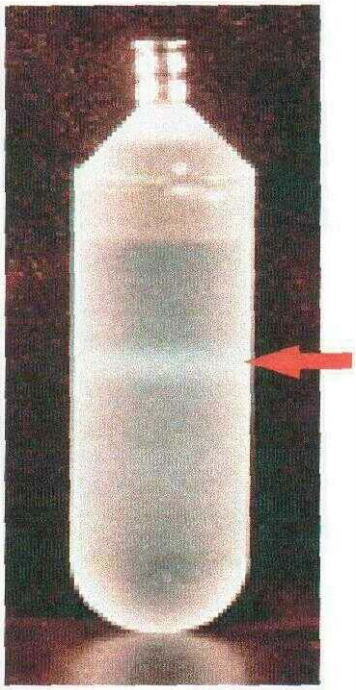


**Figure 4.5: CsCl centrifugation of M13**
After being centrifuged for 18 h at 174,000 *xg* at 4 °C in CsCl solution. The filamentous phage sequence concentrated at a band located near the middle of the centrifuge tube as pointed by the arrow.

**Table 4.4: Final concentration of purified phages**

| Phage clone | gpIII fusion | Average pfu/ml |
|---|---|---|
| WSFFSNI | C-WSFFSNI-C | $1.11 \times 10^{13}$ |
| WPFWGPW | C-WPFWGPW-C | $2.75 \times 10^{13}$ |
| PNTLLGS | C-PNTLLGS-C | $2.55 \times 10^{13}$ |
| LLNTGLR | C-LLNTGLR-C | $2.58 \times 10^{13}$ |

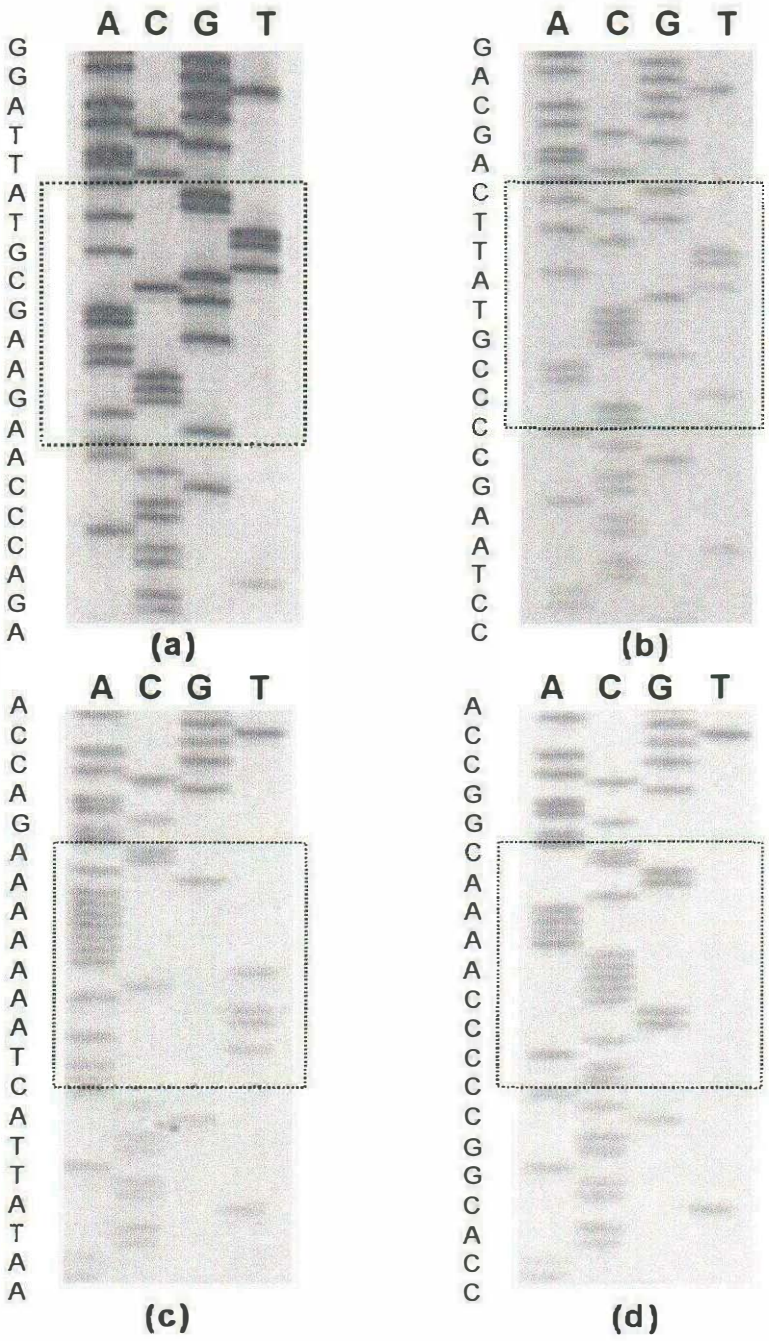



**Figure 4.6: Nucleotide sequencing of the ssDNA of the selected fusion phages**
All sequences of the non-coding strand were read from left to right, ACGT, and from bottom to top 5' to 3'. Nucleotide sequences encoding fusion peptides (a) PNTLLGS; (b) LLNTGLR; (c) WSFFSNI and (d) WPFWGPW are shown at the left of the autoradiographs.

### 4.3.1 Binding Assay on Solid Phase

Four phage clones have been selected from the pool of phage isolated from biopanning. In order to further eliminate the weaker binder to core particles, binding assay on solid phase was performed. The selected phages were reacted with core particles coated on microtiter plate wells. The pfu of the input phages (2 x $10^{11}$ pfu) as well as other parameters were remained the same as described in biopanning (refer to Materials and Methods, page 45). The output pfu ($pfu_x$) was determined following the titration of the eluted phages. Hence, the $pfu_x$ of the phages were compared qualitatively and phages with high $pfu_x$ represent tighter binders whereas weaker binders show low $pfu_x$.

Phage clone WSFFSNI showed the greatest binding affinity followed by clone WPFWGPW. The $pfu_x$ for phage clones WSFFSNI and WPFWGPW were 1.05 x $10^7$ and 1.43 x $10^5$, respectively, as shown in Figure 4.7. While the number of phage clones LLNTGLR and PNTLLGS bound to the core particles were 352 and 650 pfu, which were substantially lower than WSFFSNI and WPFWGPW. In other words, the $pfu_x$ of phage clone WSFFSNI on solid phase is approximately 2000-fold higher than phage clones PNTLLGS and LLNTGLR. The phage clone PNTLLGS contains the sequence LLG, which correspond to residues 63-65 in PreS1 region (Dyson and Murray, 1995) showed a higher (~2-fold) $pfu_x$ than LLNTGLR as shown in the inset of Figure 4.7. The phage bearing amino acid

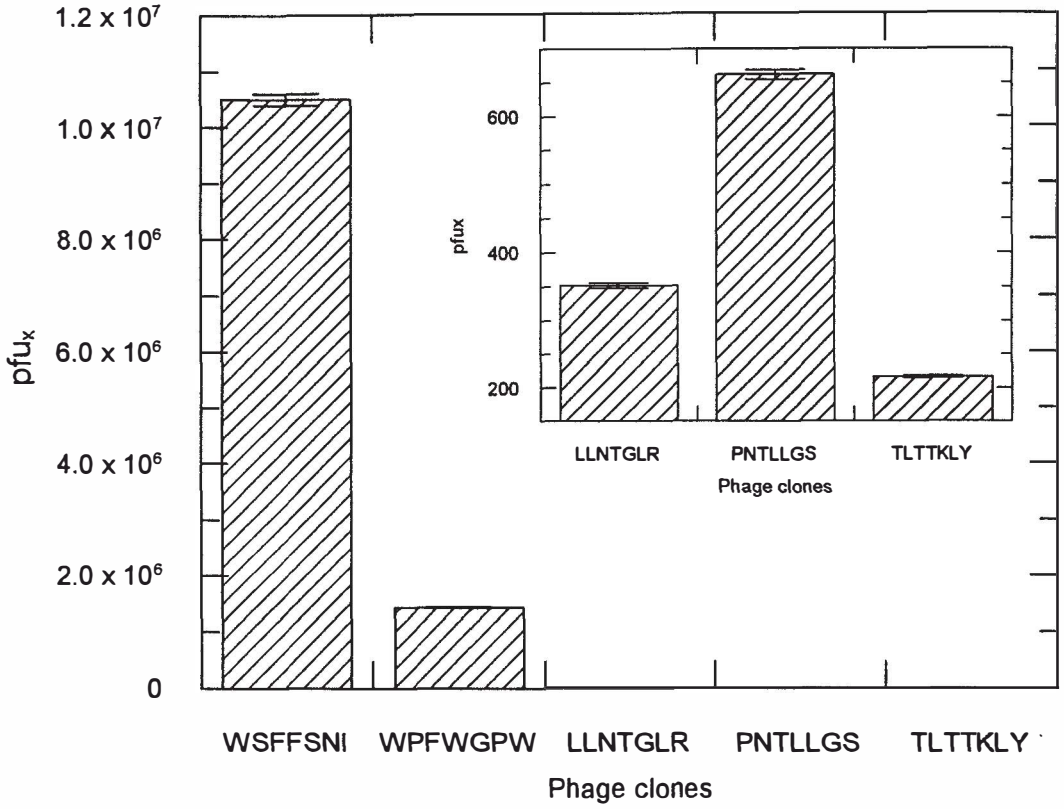



**Figure 4.7: Phage binding assay on a solid phase.**
Phage clones carrying different fusion peptides were incubated with immobilised full-length HBcAg. $pfu_x$ indicates number of phages eluted from the well. The inset represents the enlarged scale for the phages bearing the sequence C-LLNTGLR-C, C-PNTLLGS-C and C-TLTTKLY-C. Phage carrying the peptide sequence C-TLTTKLY-C that binds tightly to Newcastle disease virus (NDV) was served as negative control (Ramanujam *et al.*, 2002).

sequence C-TLTTKLY-C, which recognises Newcastle disease virus (NDV) (Ramanujam *et al.*, 2002) showed the lowest number of bound phage to the HBV core particles, which served as a negative control in this experiment. Relatively, the numbers of phage bound to core particles, assayed on solid phase are WSFFSNI> WPFWGPW> PNTLLGS> LLNTGLR. The result indicates that the phage clones bearing the cyclic peptide C-WSFFSNI-C and C-WPFWGPW-C exhibit significant high binding affinities to the core particles that immobilised on the microtiter plate. Phage clones PNTLLGS and LLNTGLR were not included in the following experiments due to the substantially low affinities to the core particles compared with clones WSFFSNI and WPFWGPW.

The binding assay on solid phase does not reflect the actual binding affinity of ligands to its substrate due to two factors: (i) the immobilised proteins and the phage was separated into two phases, which does not permit the measurement of true dissociation constant, and (ii) the protein may undergo conformational changes when it was immobilised on a solid phase (Friguet *et al.*, 1984). Thus, another method was established for measuring the relative dissociation constant quantitatively in solution (Friguet *et al.*, 1985).

### 4.3.2 Binding Affinities in Solution

The method for the determination of dissociation constant between the fusion phage and substrate molecules in solution is based upon the measurement of

affinity constant between antigens and antibodies in an equilibrium solution (Friguet *et al.*, 1985). The method has been modified for determination of relative dissociation constant in fusion phage-substrate complexes (Dyson and Murray, 1995) and HBsAg-HBcAg interaction (Tan *et al.*, 1999; Tan and Dyson, 2000). Basically, in this assay a constant concentration of fusion phage was incubated with varying concentrations of substrate until equilibrium was reached. The number of free phage was quantitated by incubating a small amount of the mixture in a microtiter plate well coated with the substrate. The well was washed and bound phage was then eluted and titrated. The relative dissociation constant measured by this method is valid if the following criteria are fulfilled. These criteria are: (i) the association-dissociation equilibrium is reached (ii) the equilibrium is undisturbed, and (iii) the temperature is remained the same throughout the assay.

### 4.3.3 Determination of Relative Dissociation Constant

The binding data were analysed with the Scatchard plot and the non-linear hyperbolic curve fitting method (Dyson *et al.*, 1995; Tan, 1997; Tan and Dyson, 2000). Relative dissociation constants determined in the equilibrium binding assay are quoted as relative rather than absolute quantities because of the complexity of HBcAg and its association with phage are multivalents (Dyson and Murray, 1995; Tan *et al.*, 1999).

### 4.3.4 The Scatchard Plot

In an equilibrium solution, the core particles and phages can be presented as follows:

$$\mathbf{C + P \rightleftharpoons CP} \qquad (1)$$

Where C represents free core particles; P represents free phage and CP represent complex of core-phage.

The reactions are presumed to be bimolecular and reversible, and the system is defined to have one identical set of binding site. Therefore, the dissociation constant can be derived as:

$$\mathbf{K_d = [C]\,[P]/[CP]} \qquad (2)$$

The fraction, Y, of the bound phage is given by:

$$\mathbf{Y = [CP]/([CP] + [P])} \qquad (3)$$

Therefore, the Scatchard plot equation can be derived by combining equations (2) and (3) as follows:

$$\mathbf{Y/[C] = (1\text{-}Y)/K_d} \text{ or;} \qquad (4)$$

$$\mathbf{Y/[C] = -(1/K_d)Y + 1/K_d} \qquad (5)$$

The gradient of a plot of Y/[C] against Y is equal to $-1/K_d$ (equation 5). Table 4.5 shows the binding data between phages and HBcAg and Figure 4.8 shows the Scatchard plots for phage clones WSFFSNI and WPFWGPW binding to full-length and truncated HBcAg. $K_d^{rel}$ values obtained from linear regression analysis of the plots are presented in Table 4.6.

**Table 4.5: Binding data of the interaction between phage clones and HBcAg**

(a) full-length HBcAg and phage clone WSFFSNI

| [C] (μM) | $pfu_x$ | $Y = pfu_o\text{-}pfu_x/pfu_o$ | $Y/[C]$ ($\mu M^{-1}$) |
|---|---|---|---|
| 0 | 328 ± 32.0 ($pfu_o$) | - | - |
| 0.005 | 246 ± 30.5 | 0.250 ± 0.093 | 50 ± 18.500 |
| 0.01 | 217 ± 0.0 | 0.340 ± 0.000 | 34 ± 0.010 |
| 0.025 | 144 ± 13.1 | 0.560 ± 0.040 | 22.4 ± 1.600 |
| 0.05 | 138 ± 10.1 | 0.580 ± 0.031 | 11.6 ± 0.616 |
| 0.1 | 94 ± 5.2 | 0.710 ± 0.016 | 7.1 ± 0.159 |
| 0.5 | 70 ± 7.1 | 0.786 ± 0.022 | 1.6 ± 0.043 |
| 0.75 | 68 ± 9.3 | 0.793 ± 0.028 | 1.1 ± 0.038 |
| 1.5 | 65 ± 6.3 | 0.802 ± 0.019 | 0.5 ± 0.013 |

(b) full-length HBcAg and phage clone WPFWGPW

| [C] (μM) | $pfu_x$ | $Y = pfu_o\text{-}pfu_x/pfu_o$ | $Y/[C]$ ($\mu M^{-1}$) |
|---|---|---|---|
| 0 | 66.5 ± 2.5 ($pfu_o$) | - | - |
| 0.005 | 57.5 ± 0.5 | 0.135± 0.008 | 27.1± 1.504 |
| 0.025 | 34 ± 2.0 | 0.489± 0.030 | 19.6 ± 1.203 |
| 0.0625 | 22.3 ± 3.7 | 0.665± 0.055 | 10.6 ± 0.886 |
| 0.1 | 16.5 ± 0.5 | 0.752± 0.008 | 7.5 ± 0.075 |
| 0.5 | 10 ± 3.0 | 0.850± 0.045 | 1.7 ± 0.090 |
| 1.0 | 8 ± 2.4 | 0.880± 0.037 | 0.9 ± 0.037 |
| 1.5 | 4.5 ± 1.2 | 0.932± 0.019 | 0.6 ± 0.013 |

(c) truncated HBcAg and phage clone WSFFSNI

| [C] (μM) | $pfu_x$ | $Y = pfu_o\text{-}pfu_x/pfu_o$ | $Y/[C]$ ($\mu M^{-1}$) |
|---|---|---|---|
| 0 | 174 ± 4.0 ($pfu_o$) | - | - |
| 0.00125 | 121.5 ± 1.5 | 0.302 ± 0.009 | 241.4 ± 6.896 |
| 0.005 | 95 ± 12.0 | 0.460 ± 0.069 | 92.0 ± 13.790 |
| 0.01 | 43 ± 3.3 | 0.664 ± 0.019 | 75.3 ± 1.880 |
| 0.025 | 58.5 ± 7.5 | 0.753 ± 0.043 | 26.6 ± 1.720 |
| 0.1 | 34 ± 2.0 | 0.805 ± 0.011 | 8.0 ± 0.115 |
| 0.2 | 21 ± 3.0 | 0.879 ± 0.017 | 4.4 ± 0.086 |
| 1.0 | 20 ± 1.0 | 0.885 ± 0.006 | 0.9 ± 0.006 |
| 1.5 | 15 ± 1.0 | 0.914 ± 0.006 | 0.6 ± 0.004 |

(d) truncated HBcAg and phage clone WPFWGPW

| [C] (μM) | $pfu_x$ | $Y = pfu_o\text{-}pfu_x/pfu_o$ | $Y/[C]$ ($\mu M^{-1}$) |
|---|---|---|---|
| 0 | 290 ± 10.0 ($pfu_o$) | - | - |
| 0.00125 | 196.7 ± 10.5 | 0.322 ± 0.036 | 257.4 ± 28.960 |
| 0.0025 | 125 ± 25.0 | 0.569 ± 0.086 | 227.6 ± 34.484 |
| 0.005 | 120 ± 20.0 | 0.586 ± 0.069 | 117.2 ± 13.790 |
| 0.01 | 96.7 ± 9.4 | 0.667 ± 0.033 | 66.7 ± 3.251 |
| 0.0625 | 60 ± 10.0 | 0.793 ± 0.034 | 12.7 ± 0.552 |
| 0.1 | 50 ± 0.0 | 0.828 ± 0.000 | 8.3 ± 0.000 |
| 0.2 | 40 ± 10.0 | 0.862 ± 0.034 | 4.3 ± 0.172 |
| 0.5 | 40 ± 0.0 | 0.862 ± 0.000 | 1.7 ± 0.000 |
| 1.5 | 25 ± 5.0 | 0.914 ± 0.017 | 0.6 ± 0.012 |

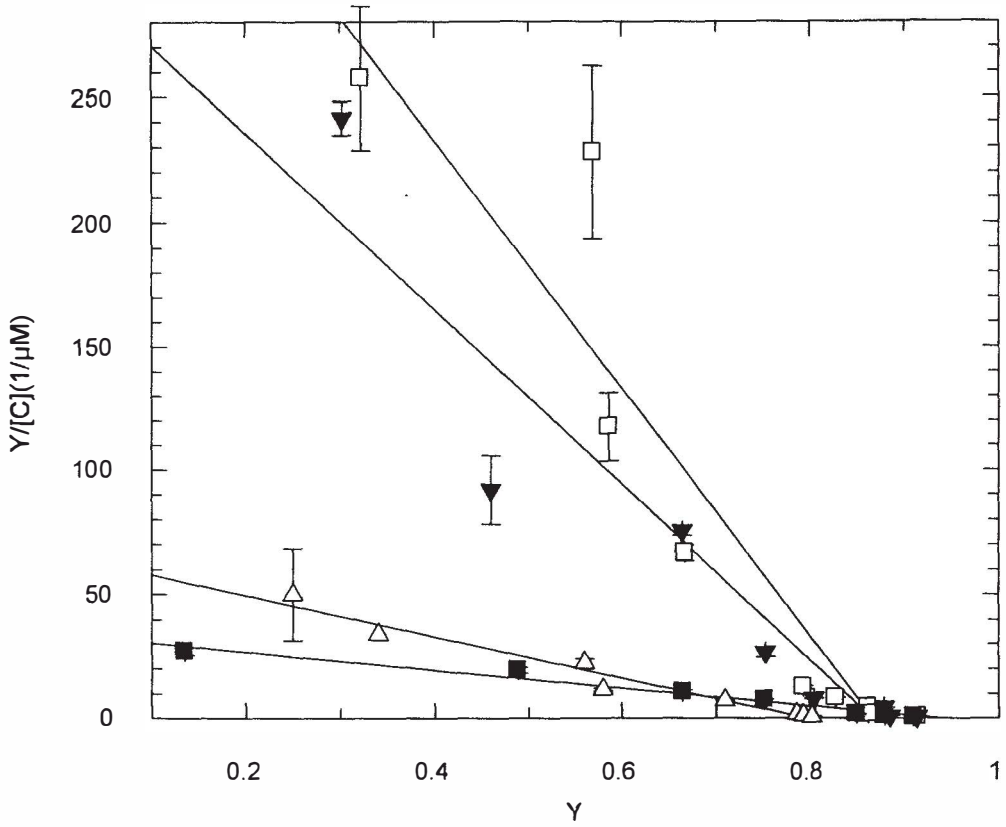



**Figure 4.8: Scatchard plots of phages binding to full-length and truncated HBcAg.** The binding data were fitted with straight lines. The slopes representing the $K_d^{rel}$ were culcalated by using the equation $y = m\,x + c$ ($Y/[C] = -(1/K_d)Y + 1/K_d$). Where, Y: fractions of bound phages; and C: free core particles in the solution. (Δ) represents the interaction of phage clone WSFFSNI with full-length; (▼) represents the interaction between phage clone WSFFSNI with truncated HBcAg; (■) represents the interaction between phage clone WPFWGPW with full-length HBcAg; and (□) represents the interaction between phage WPFWGPW and truncated HBcAg.

Generally, Scatchard plot analysis is only suitable for evaluation of binding constant for a single binding site, where the points could be fitted with a straight line and the slopes can be calculated easily. However, if the phage or its substrate possesses more than one binding site, the Scatchard plot analysis is unsuitable due to the presence of different slopes at the high and low concentrations of the substrate. Scatchard analysis of the binding data produced linear plots with many of the data points at high concentration of HBcAg were squeezed into small region of the figure near the intercept of the Y axis. In order to present the binding data uniformly, the fraction of free phage ($pfu_o/pfu_x$) in the solution was plotted against the $\log_{10}[C]$ and the curve was then fitted to a hyperbolic curve by using an appropriate binding equation, which was suggested to be a more reliable method for experimental binding data analysis (Tan, 1997).

### 4.3.5 Curve Fitting of Hyperbolic Equations

The $K_d^{rel}$ values for the binding data in Table 4.5 were also calculated with non-linear hyperbolic curve fitting method (Dyson *et al.*, 1995; Tan and Dyson, 2000). The equation (4) or (5) can be rearranged to give a hyperbolic curve equation:

$$\mathbf{Y = [C]/[C] + K_d} \qquad (6)$$

Y equal to $pfu_x/pfu_o$, where $pfu_x$ represents the amount of free phage and $pfu_o$ represents the amount of input phage. The hyperbolic functions start at 0 when [C] = 0, (all phage remain free in solution, $pfu_x/pfu_o = 1$). When half of the phage are engaged in the core-phage complexes,

$pfu_x/pfu_o = ½$, then $K_d = [C]$.

The hyperbolic relationship between $pfu_x/pfu_o$ and [C] is shown in Figure 4.9. The $K_d^{rel}$ values were calculated by curve fitting the binding data and summarised in Table 4.6.

**Table 4.6: Relative dissociation constants of the phage-HBcAg complexes**

| Phage clone | gpIII fusion | Specificity | $K_d^{rel}$ (nM)[a] | $K_d^{rel}$ (nM)[b] |
|---|---|---|---|---|
| WSFFSNI | C-WSFFSNI-C | full-length HBcAg | 11.5 ± 0.9 | 13.5 ± 2.1 |
| WPFWGPW | C-WPFWGPW-C | full-length HBcAg | 26.6 ± 2.1 | 22.0 ± 1.7 |
| WSFFSNI | C-WSFFSNI-C | truncated HBcAg | 2.5 ± 0.4 | 4.9 ± 1.6 |
| WPFWGPW | C-WPFWGPW-C | truncated HBcAg | 1.8 ± 0.2 | 1.9 ± 0.4 |

[a] $K_d^{rel}$ values were calculated by Scatchard plot analysis of the data in Figure 4.8.
[b] $K_d^{rel}$ values were calculated by curve fitting method of the data in Figure 4.9.

The $K_d^{rel}$ values calculated by linear regression of the Scatchard plots are very similar to those obtained by non-linear curve fitting method using the same data, in which, the variations are within the standard errors. In the Scatchard plots, the data were fitted with straight lines indicating that both phages possess a single binding site on the full-length and truncated HBcAg.

In general, the $K_d^{rel}$ values for the interaction between the full-length HBcAg and both phages are higher than those of the truncated form of HBcAg. The $K_d^{rel}$ values for the full-length HBcAg are about 3-fold and 10-fold higher than those of the truncated HBcAg for phage clones WSFFSNI and WPFWGPW, respectively. Both full-length and truncated HBcAg are capable to assembly into core-like

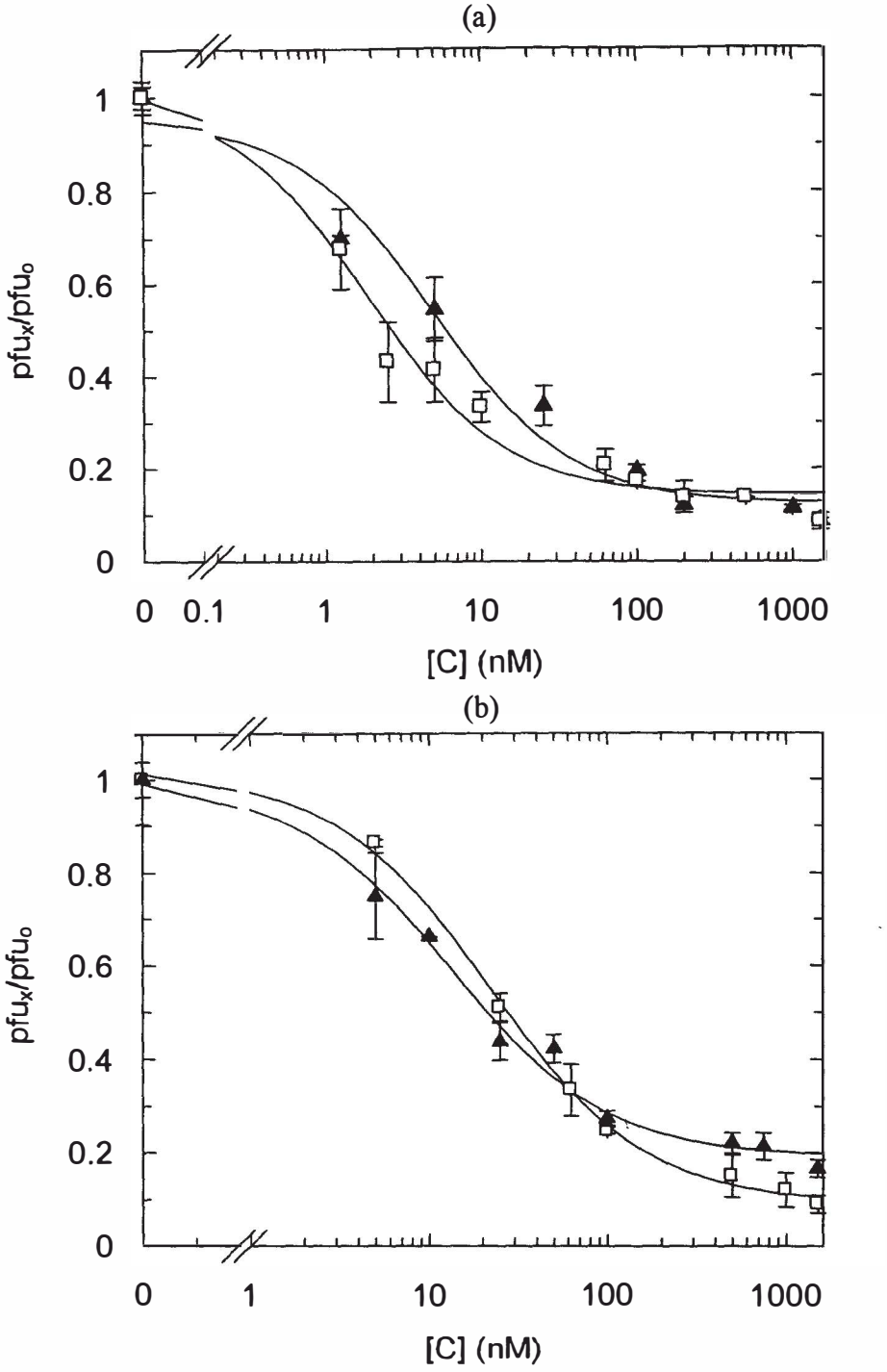



**Figure 4.9: Non-linear hyperbolic curves of the fusion phages binding to core particles.** A constant concentrations of phages were incubated with varying concentration of HBcAg for 18 h at 6 °C. A portion of the equilibrium solution was then transferred to HBcAg-coated wells, and bound phages were determined by titering eluted pfu. $pfu_x$, number of phage eluted in the presence of HBcAg, $pfu_o$, number of phage eluted in the absence of inhibitor. Phage carrying the peptide C-WSFFSNI-C (▲) and C-WPFWGPW-C (□) with various concentrations of (a) full-length HBcAg and (b) truncated HBcAg.

particles as observed by electron microscopy (Cohen and Richmond, 1982). The number of spikes for T=3 and T=4 shells are 90 and 120, respectively (Crowther *et al.*, 1994). The arrangement of dimers around their icohedral five-fold axes is almost identical, whereas the quasi-six-fold arrangements of dimers are different between these two particles (Zlotnick *et al.*, 1996). The arrangement of dimers could be the main factor that affects the formation of T=4 and T=3 shells. The T=4 shells decreased progressively as the protein construct was shortened from its C-terminus of HBcAg (Zlotnick *et al.*, 1996). Therefore, the ratio of T=4 to T=3 particles are higher in full-length HBcAg than truncated HBcAg preparations. As shown in antibody inhibition assay (section 4.4), the selected phages may bind or partially bind to immunodominant regions of HBcAg that constitutes the protruding spikes at the tips of shell domains. As a result, a substantial number of phage might capture by a single core particle. From the $K_d^{rel}$ calculation, each phage recognised only a single binding site on HBcAg. Therefore, if each spike attracted two phage particles, a total of 180 and 240 phage particles could be captured on a single truncated and full-length HBcAg formed particles, respectively. However, each phage contains 3 to 5 copies of gpIII protein (Kay and Hoess, 1996), therefore it is unknown whether a single phage particle with its gpIII proteins would associates with either one or more binding sites on the spike composed of two monomers. Nevertheless, the relative affinities of truncated HBcAg higher than full-length HBcAg could be due to the fact that different ratios of T=4 to T=3 in both preparations which subsequently affect the distribution of phage in a given volume. The distribution of T=3 and T=4 particles

in both preparations was different in a given volume. In the excess of HBcAg, as used in the equilibrium binding assay, a larger number of small particles provides more surfaces for association with phage in an equal amount of protein for a given volume and thus increased the efficiency of phage-HBcAg association.

Although the $K_d^{rel}$ values are different for both phages binding to full-length and truncated HBcAg, respectively, but all values are within nanomolar range. The $K_d^{rel}$ values for both phages to truncated and full-length HBcAg are less than 10 and 30 nM, respectively, which are substantially lower than the phage carrying linear peptide LLGRMK (Dyson and Murray, 1995). The $K_d^{rel}$ values of phage bearing LLGRMK are 170 (±10) and 220 (±10) nM with the truncated and full-length HBcAg, respectively. Therefore, the $K_d^{rel}$ values for phage clones WPFWGPW and WSFFSNI are at least 17- and 7-fold, lower than phage bearing LLGRMK with truncated and full-length HBcAg, respectively. All data obtained from both studies are valid and comparable due to fulfilment of following criteria in the assay: (i) the temperature was kept constant (6 °C) throughout the experiment; (ii) the binding equilibrium is reached; and (iii) the concentration of titrated phage is proportional to the free phage in the equilibrium mixture. Furthermore, the phage clone WSFFSNI contains two Ser residues might involve in hydrogen bonding with the exterior surface of HBcAg, which provides another factor that WSFFSNI showed the highest affinity to full-length HBcAg among the isolated phages. However, phage clone WPFWGPW exhibits the strongest

affinity to truncated HBcAg among the phages including phage bearing peptide LLGRMK isolated by Dyson and Murray (1995).

## 4.4 Inhibition of Phages from Binding to Hepatitis B Core Particles with Antibody

Recombinant phages screened from the phage display peptide library may be used to substitute antibody for the detection of HBcAg in a sample. As shown in above experiment, both the recombinant phages exhibit $K_d^{rel}$ values in nanomolar range. These values indicate that the interaction strength of the selected phages with core particles is strong enough as in antibody-antigen interaction. In principle, the phage should be able to compete with an antibody that binds to the same site on the core particles. Monoclonal antibody, mAb C1-5 (Chemicon; USA) interacts with *E. coli* derived recombinant HBcAg subtype ayw (Bichko *et al.*, 1993) was used in this study. It reacts with a linear epitope on the surface of assembled core particles and also binds to denatured HBcAg in immunoblotting (Bichko *et al.*, 1993). The epitope for mAb C1-5 was mapped at amino acid positions 78-83 on the surface of assembled core particles (Pushko *et al.*, 1994). Therefore, the immunodominant loop of HBcAg (amino acid positions 78-83) is overlapping with the epitope of the mAb C1-5 on the core particles (Salfeld *et al.*, 1989; Sällberg *et al.*, 1991; Conway *et al.*, 1998).

The mAb C1-5 was used as a competitor for the selected fusion phages from binding to their binding sites on the surface of core particles. In this experiment, a

constant amount of phage was incubated with varying concentration of mAb C1-5 on microtiter plate wells coated with HBcAg. The wells were then washed and bound phages eluted by a low pH elution buffer and the eluates were titrated as output pfu ($pfu_x$). Figure 4.10 shows that both phage clones WSFFSNI and WPFWGPW, inhibited by the antibody with an $IC_{50}$ value approximately 0.01 μg/μl for both phages. Apsalons and Bichko (1994) showed that mAb C1-5 binds to HBcAg capsids with a $K_d$ value of 0.2 nM, which is at least 10-fold stronger than the phage-HBcAg interaction identified in this study. mAb anti-preS2 against the PreS2 region of denatured HBsAg employed as a negative control did not inhibit the phages from binding to core particles (Figure 4.10).

An advantage of conformationally constrained peptides is that the binding entropies are lowered, thereby increasing both the affinity and selectivity of the peptides for a receptor (O'Neil *et al.*, 1992; Gho *et al.*, 1997). The sequences of the cyclic peptides selected in this study are not identical to those isolated from a linear peptide library (Dyson and Murray, 1995). The linear peptide sequences that recognised the binding site are not necessary homology with the cyclic peptide sequences that react with the same receptor (McConnell *et al.*, 1994). Constraining a linear peptide that recognises a receptor may reduce the affinity to that particular receptor and consequently abolish the interactions in some cases. As shown by McConnell and colleagues (1994), constraining the linear consensus sequence that recognised mAb against angiotensin II (AII) and the resulting cyclic peptide showed a 100-fold weaker affinity to the antibody. Interestingly,

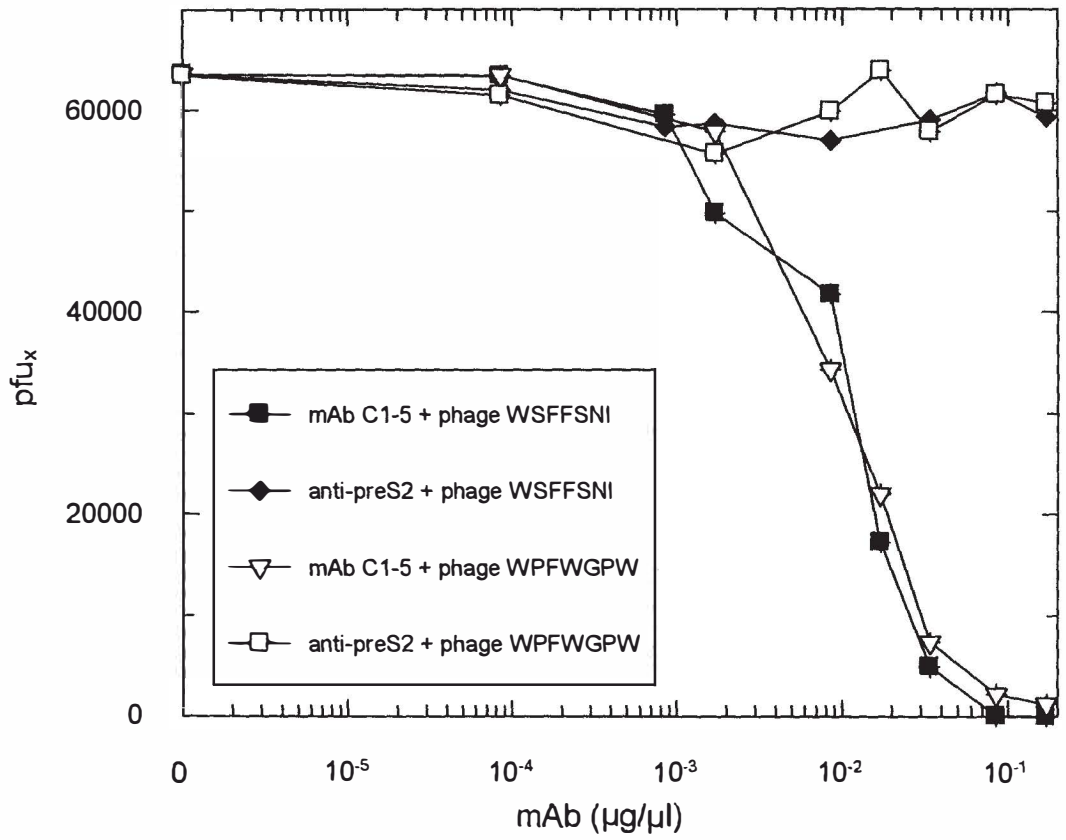



**Figure 4.10: Inhibition of phages from binding to core particles with mAb** Phages were incubated with various concentrations of mAb C1-5 against core particles and the concentrations of the bound phages were titrated. $pfu_x$ indicates number of phage eluted in the presence of antibody. The symbols representing the phages are indicated in the box located inside the graph. mAb anti-PreS2 served as a negative control.

constrained peptide with a different amino acid sequence isolated from a disulfide constrained peptide library shows the highest affinity to the same receptors (McConnell *et al.*, 1994). Aparts from the parameters such as phage-HBcAg interacting temperature, incubation period, immobilised surface and washing stringency that potentially affect the selected peptide sequences, choosing of phage display peptide library is another important factor that determines the isolated peptide sequences. Most importantly, peptide sequences screened from an appropriate peptide library show a high affinity to their receptors. Hence, the main objective of this study was achieved, whereby peptide sequences obtained are different from those isolated from a linear peptide library, and furthermore the affinities of the isolated phages to the core particles are stronger than the linear peptides isolated by Dyson and Murray (1995).

## 4.5 *In vitro* transcription and translation of L-HBsAg

Rabbit reticulocyte lysate, an eukaryotic cell system, was chosen as an expression system to produce L-HBsAg because no one has successfully produced the polypeptide in *E. coli* or other prokaryotic systems. Although overproduction of HBsAg in yeast and animal cells have been reported (Valenzuela *et al.*, 1982; Murray *et al.*, 1984), the recombinant proteins tend to aggregate into particles similar to those secreted by virally infected hepatocytes, which would not be suitable for the study of the interaction between HBcAg and HBsAg (Tan *et al.*, 1999). The rabbit reticulocyte lysate system offers several advantages: (i) the

incorporation of [$^{35}$S]-methionine into the L-HBsAg during translation allows the detection of translation products by autoradiography and the measurement of radioactivity by scintillation counting, and (ii) formation of disulfide bonds among the translation products can be avoided by adding reducing agent such as DTT into the rabbit reticulocyte lysate (Tan, 1997; Tan and Dyson, 2000) because it has been shown that S-HBsAg forms dimers and oligomers in the secreted subviral particles (Huovila *et al.*, 1992; Wunderlich and Bruss, 1996). Although only small amount (~ $10^{-15}$ mole per µl of reticulocyte) of product is synthesised by this method but the radioactivity technique provides a sensitive method for quantitative measurement (Tan and Dyson, 2000).

In rabbit reticulocyte lysate, *in vitro* translated L-HBsAg and its mutants formed complexes with HBcAg that could be immunoprecipitated with anti-HBcAg polyclonal antibody and this finding indicates that the PreS regions are involved in the interaction with HBcAg (Dyson and Murray, 1995; Tan *et al.*, 1999). Mutagenesis study showed that two segments of HBsAg are involved in the association with HBcAg. These segments are located between residues 24 and 191 and the other between residues 191 and 322 of the L-HBsAg, suggesting that the PreS regions alone are not sufficient for efficient association, but contact regions within S domain are also critical for the interaction of HBsAg and HBcAg (Tan *et al.*, 1999).

In order to produce the L-HBsAg in rabbit reticulocyte, its gene was cloned into pCITE-2c vectors, which contains the 5' non-coding region of the encaphalitis viral RNA that enhances translation with eukaryotic ribosomes. Figure 4.11 shows that the insert of approximately 1200 bp was successfully released from the recombinant plasmid pMDHBs3 (Dyson and Murray, 1995; Tan, 1997) after being digested with *Eco*RI and *Sal*I. Circular and linearised (digested with *Sal*I) plasmids were used as templates for *in vitro* transcription by T7 RNA polymerase. The RNA transcripts were then translated to L-HBsAg in rabbit reticulocyte supplemented with [$^{35}$S]-Met, in the absence of microsomal membrane. The translation of L-HBsAg from RNA transcripts produced protein bands (Figure 4.12, lanes 2 and 3) of approximately 39 kDa, corresponding to the unglycosalated form of L-HBsAg (Dyson and Murray, 1995). Although the amount of translation product encoded by circular and linearised plasmids almost the same but it does not reflect the efficiency of transcription of both circular and linearised plasmids unless the amount of mRNA was quantitated. The addition of DTT in the translation mixture was to prevent the formation of disulfide bridges among the translated products.

## 4.6 Inhibition of the Association of L-HBsAg and Core Particles with Synthetic Peptides

The PreS regions of L-HBsAg have been found disposed on the external surface of HBV and also in the cytosolic side of cellular membrane vesicles (Heermann *et al.*, 1984; Kuroki *et al.*, 1990; Ostapchuk *et al.*, 1994; Prange and Streeck *et al.*,

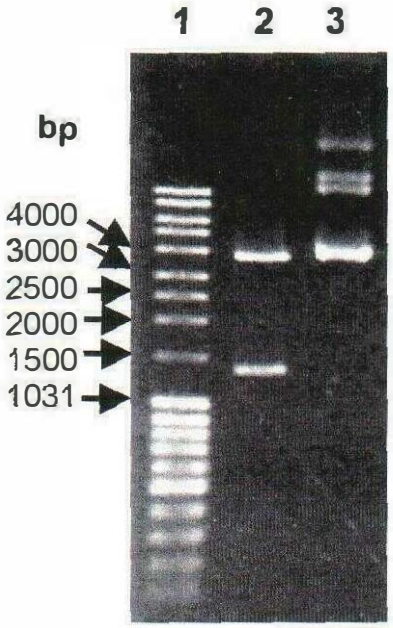



**Figure 4.11: Restriction enzyme digestion of pMDHBs3**
Recombinant plasmids were digested with *Sal*I and *EcoR*I (Lane 2), and *EcoRI* (Lane 3). Lane 1 is DNA marker in bp. Digested plasmids were analysed on 1% (w/v) agarose gel.

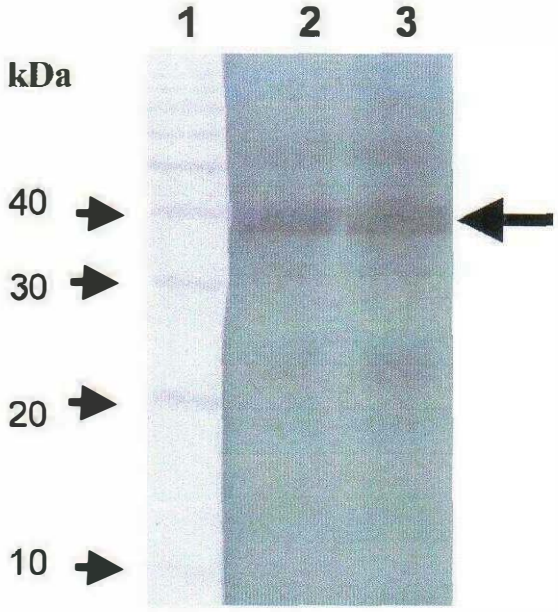



**Figure 4.12: Autoradiograph of [$^{35}$S]-L-HBsAg *in vitro* translation products** Approximately 39 kDa protein band is indicated by an arrow. Lane 2: product encoded by linear form of pMDHBs3; Lane 3: product encoded by the circular form of pMDHBs3. Lane 1 is protein marker in kDa.

1995), suggesting two important roles for these regions: (i) interaction between viral envelope and nucleocapsid during virion assembly and (ii) attachment to virus-specific receptors on hepatocytes. Disruption of either interaction is thought to play an important role to prevent virus morphogenesis. Thus, small inhibitors that block the specific interaction between the core particles and L-HBsAg may disrupt the viral assembly.

In order to evaluate the inhibitory activities of conformational constrained and linear peptides carrying the same amino acid sequence, based upon the fusion peptides isolated in this study, two linear peptides with amino acid sequence WSFFSNI and WPFWGPW, and two cyclic peptides CWSFFSNIC and CWPFWGPWC were synthesised. Peptides were cyclised by a head-to-tail disulfide bond formed by two cysteine residues that flanked both termini of the synthetic peptides. Varying concentration of synthetic peptides were mixed with a constant amount of *in vitro* translated $^{35}$[S]-L-HBsAg. The mixtures were then added into microtiter plate wells coated with core antigen and incubated for 24 h at 4 °C. The unbound $^{35}$[S]-L-HBsAg and synthetic peptides were washed away and the radioactivity was measured by scintillation counting. The whole process was carried out in the presence of DTT.

Synthetic peptides WPFWGPW and CWPFWGPWC comprise hydrophobic amino acids did not dissolve in the solution that used in the assay and as a result their inhibitory activity could not be determined. However, the linear

heptapeptides WSFFSNI containing three basic amino acids exhibited a half maximal inhibition ($IC_{50}$) of approximately 9.8 μM (Figure 4.13), which was about 10-fold lower than that of LLGRMKG peptide (Tan, 1997). Introduction of two cysteine residues into the hydrophobic heptapeptides greatly reduced the solubility of CWSFFSNIC in water-based buffer, which showed an $IC_{50}$ of about 200 μM (Figure 4.13). As a result, the unconstrained peptide WSFFSNI showed a better inhibitory activity approximately 20-fold higher than that of the disulfide constrained C-WSFFSNI-C. Based upon the $K^{d}_{rel}$ values of the interaction of phage and HBcAg, in principle, the inhibitory effects of constrained peptide should be higher than the linear peptide with the same peptide sequence, due to its more defined and specific conformation. This suggests that the established *in vitro* assay is not an appropriate system to access the inhibitory effect of highly hydrophobic peptides. Therefore, alternative system has to be developed in order to improve the present assay. Nevertheless, a peptide that binds to core particles with a better inhibitory activity was successfully identified. As a negative control, synthetic peptide with amino acid sequence LEDPASR, which corresponds to the residues at the tip of the nucleocapsid spike (residues 76-82 of subtype adyw) did not inhibit the interaction between the $^{35}$[S]-L-HBsAg and core particles even at the highest concentration (1 mM), in accord once with that reported by Tan, (1997).

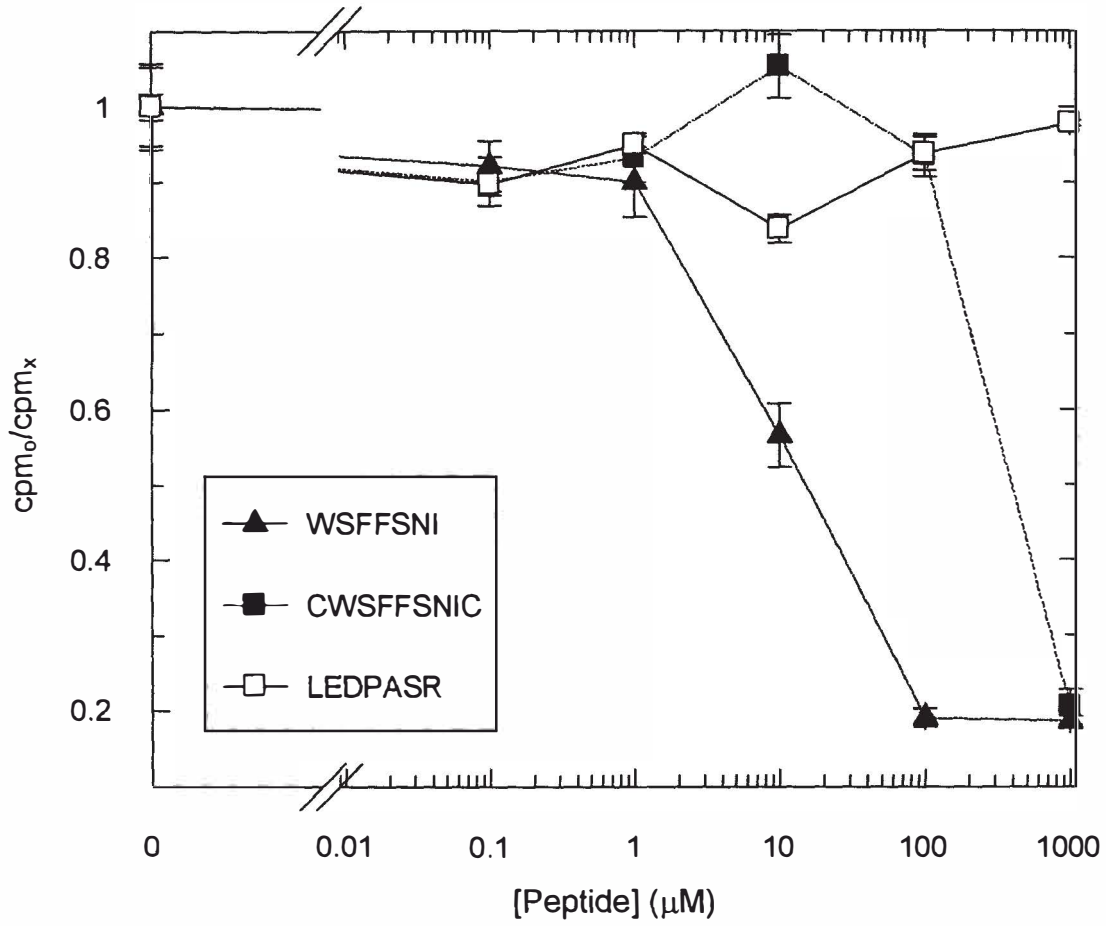



**Figure 4.13: Inhibition of L-HBsAg binding to core particles with synthetic peptides.** The radiolabelled L-HBsAg was incubated with synthetic peptides bearing the amino acid sequences WSFFSNI (▲) and cyclic peptide C-WSFFSNI-C (■) ranges from 0 to 1000 μM. The synthetic peptide LEDPASR (□) corresponds to amino acids 76-82 of the nucleocapsid spike was used as negative control. $cpm_x$ indicates the radioactivity in the presence of peptide and $cpm_o$ indicates the radioactivity in the absence of peptide.

The presence of DTT in the NET-gel buffer is needed in order to prevent the formation of disulfide bonds among translated $^{35}$[S]-L-HBsAg as well as other components in the rabbit reticulocyte lysate in the assay. The absence of DTT in a control experiment showed that the components of the reaction tend to precipitate and as a result, the amount of $^{35}$[S]-L-HBsAg involved in the association with HBcAg reduced significantly. This suggests that DTT is an essential ingredient in an *in vitro* translation reaction in order to prevent aggregation of reactants in the assay.

The major problem encountered in the peptide inhibition study was the solubility of the synthetic peptides. White precipitates were observed when the linear peptide WPFWGPW and two cyclic peptides CWSFFSNIC and CWPFWGPWC were suspended in water, in which, these peptides composed mainly of amino acids bearing hydrophobic side chains. However, no precipitation occurred for linear peptides WSFFSNI and LEDPASR containing three and four hydrophilic amino acids, respectively (underlined). As an effort to dissolve the peptides, methanol [50% (v/v)] and dimethyformamide (DMF) were used and the peptides C-WSFFSNI-C, C-WPFWGPW-C and WPFWGPW were successfully dissolved. However, gel-like aggregates were observed for peptides C-WPFWGPW-C and WPFWGPW [in methanol (50% v/v) or DMF] upon diluted in NET-gel buffer supplemented with DTT. The gel-like aggregate was not observed in the dilution of peptide C-WSFFSNI-C with NET-gel buffer. These difficulties greatly reduced the inhibitory effects of the hydrophobic synthetic peptides. As a result, the

inhibitory effects of the peptides C-WPFWGPW-C and WPFWGPW have not been evaluated due to the insolubility of these peptides in water-based buffer.

The solubility of the peptides can be improved by integrating the hydrophobic peptides WPFWGPW, CWPFWGPWC and CWSFFSNIC into a large protein domain such as *E. coli* enterotoxin, which can be used to deliver an antiviral peptide into virally infected cells (Marcello *et al.*, 1994; Loregian *et al.*, 1999) or a carrier containing amino acids bearing the charged side chains such as Lys, Arg, His, Asp or Glu, where the total hydrophobicity in the small peptide can be neutralised by other hydrophilic amino acids in the host protein (Kyte and Doolittle, 1982).

## 4.7 Docking Sites of Fusion Peptides on HBcAg

Using electron cryomicroscopy and imaging reconstruction, Bötcher *et al.*, (1998) showed that a decapeptide, GSLLGRMKGA, containing the LLGRMK motif bound to the tips of the spikes formed by two subunits of HBcAg. Substitution of either of the acidic residues (Glu77 or Asp78) close to the tip of the core protein to Ala (uncharged amino acid) greatly reduced the affinity of the peptide LLGRMK to the altered core particles. Phage bearing the peptide C-WSFFSNI-C and C-WPFWGPW-C were demonstrated to inhibit the binding of $^{35}$[S]-L-HBsAg to core particles *in vitro* and the binding site of peptides C-WSFFSNI-C and C-WPFWGPW-C were briefly located at amino acid position 78 to 83 of HBcAg at

the protruding spikes of core particles based upon the observation that the phage bearing these sequences were inhibited by mAb C1-5 that also binds to the same region. This implies that the docking site of the heptapeptides was also located at the tips of spikes of core particles. Nevertheless, large molecules such as immunoglobulins may cause steric hindrance over a considerable distance on any surface to which they bind. Electron cyromicroscopic analysis of the newly identified heptapeptides would ultimately identify the exact docking sites of the peptides on core particles. Alternatively, crystallisation of the core particles together with the synthetic peptides may provide another solution to this problem.

# CHAPTER V

## SUMMARY AND CONCLUSION

Taking together the observation of immunoblotting and electron microscopy of genetic engineered core-like particles, the full-length and truncated HBcAg were successfully produced in *E. coli*. Fusion phages bearing the cyclic peptide sequences that interact specifically with the core antigen were identified from the disulfide constrained phage display peptide library. The isolated phage clones were demonstrated to be tightly bound to the core particles with affinities higher than phages bearing linear hexapeptide. An equilibrium binding assay in solution showed that the phages bearing the peptides C-WSFFSNI-C and C-WPFWGPW-C associate tightly to truncated HBcAg with $K_d^{rel}$ values approximately 5 and 2 nM, respectively.

mAb C1-5, which binds to the immunodominant region of HBcAg, was able to inhibit the binding of phage bearing the sequence C-WSFFSNI-C and C-WPFWGPW-C onto the core particles with a half maximal inhibition value ($IC_{50}$) of approximately 0.01 μg/μl.

The cyclic and linear peptides with the sequence CWSFFSNIC and WSFFSNI were able to block the interaction between the *in vitro* translated L-HBsAg and core particles with the half maximal inhibition concentration of about 10 μM and

200 μM, respectively. Therefore, these short peptides can serve as small inhibitors that inhibit the viral morphogenesis in virally infected hepatocytes. Due to the hydrophobicity of peptides WPFWGPW and its cyclic form, CWPFWGPWC, were not totally dissolved in water-based buffer and thus the inhibitory effects of these peptides could not be evaluated. An appropriate delivery system or recombinant carrier is required to improve their solubility and subsequently to deliver the peptides into virally infected cells.

The phages bearing the sequence C-WSFFSNI-C and C-WPFWGPW-C are potentially developed into an immunodiagnostic marker that could be used in diagnostic systems such as immunoblotting and ELISA in terms of "phage antibody" (Chiswell and McCafferty, 1992) against the core antigen. The recombinant phages may potentially replace the monoclonal antibodies available in the market. The advantages of the phage-based diagnostic reagent are: (i) low cost in production compared with monoclonal or polyclonal antibody raising in cell lines or animals; (ii) the recombinant M13 can be rapidly propagated within hours while the production of monoclonal antibodies are time consuming, which normally takes months and; (iii) the purification of phage particles involves lesser steps compared with laborious steps needed in monoclonal antibody purification. The attachment of synthetic peptides to a recombinant carrier will facilitate the peptides enter to the virally infected cells and specifically inhibit HBV replication. Hence, the synthetic peptides may serve as an useful agent for therapeutic treatment of individuals infected by HBV and also act as a

prophylactic vaccine for preventing of HBV infection (Tan *et al.*, 2001). These findings support the hypothesis that the small peptide-based reagents may be effective for antiviral activity or provide a useful leads for antiviral agent.

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# VITAE

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