Fighting
the Hepatitis B Virus
Past, Present & Future

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Universiti Putra Malaysia Press
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Selangor Darul Ehsan

Tel: 03-89468851/89468854
Fax: 03-89416172
Email: penerbit@putra.upm.edu.my
Website: www.penerbit.upm.edu.my

ISBN: 9789673446407

Professor Dr. Wen Siang Tan

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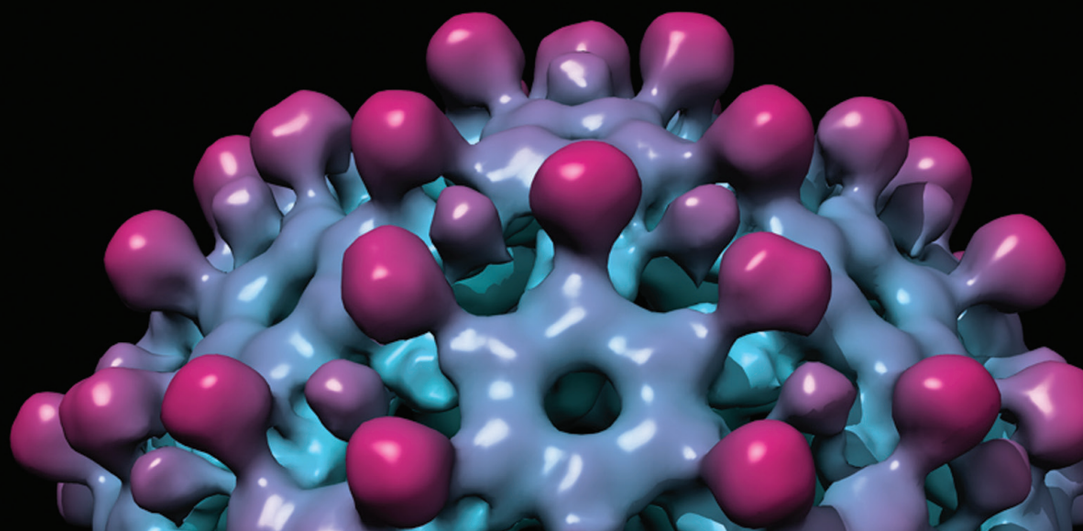

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PROFESSOR DR. WEN SIANG TAN

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the Hepatitis B Virus
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9 December 2016

Dewan Kuliah Utama
Fakulti Bioteknologi dan Sains Biomolekul
Universiti Putra Malaysia

Universiti Putra Malaysia Press
Serdang • 2016
http://www.penerbit.upm.edu.my

First Print 2016

UPM Press is a member of the Malaysian Book Publishers Association (MABOPA)
Membership No.: 9802

ISBN 978-967-344-640-7

Typesetting : Sahariah Abdol Rahim @ Ibrahim
Cover Design : Md Fairus Ahmad

Design, layout and printed by
Penerbit Universiti Putra Malaysia
43400 UPM Serdang
Selangor Darul Ehsan
Tel: 03-8946 8855 / 8854
Fax: 03-8941 6172
http://www.penerbit.upm.edu.my

Contents

ABSTRACT

Hepatitis B virus (HBV) is one of the greatest killers of human beings. The production of recombinant HBV vaccine via genetic engineering and mass immunization of the world population with the vaccine have had huge success in bringing down the number of infected people. However, until now there is no effective treatment for HBV infections. Nucleoside analogues are commonly used to treat chronic hepatitis B patients, but prolonged treatments have resulted in the selection of drug resistant strains and vaccine escape mutants. More importantly, HBV is linking arms with the human immunodeficiency virus (HIV) to kill more humans. Many dedicated scientists and highly motivated doctors have risked their lives to control the spread of HBV and search for a cure for this deadly virus. Their ultimate goal is to eradicate the virus from this planet and make the world a better place for everyone. Therefore, the main aim of this talk is to provide a comprehensive review of their contributions in the development of vaccines, diagnostic assays, therapeutics and drug delivery systems to fight HBV.

INTRODUCTION

Hepatitis B virus (HBV) has taken countless lives throughout human history. It has infected about one third of the world's population, of which 370 million are chronic carriers and about one million people continue to die each year (Michel & Tiollais, 2010). HBV causes liver cirrhosis, liver cancer (hepatocellular carcinoma) and other liver complications in humans. The virus poses a serious health problem worldwide, particularly in South East Asia, China and Africa (Figure 1), with at least 8% of the population being chronically infected. It is highly contagious. Shockingly, it is 200 times more infectious than the human immunodeficiency virus (HIV) and up till now it has killed more people than HIV. The virus is transmitted via blood or body fluids, including blood transfusion, contaminated needles and syringes, sexual contact and perinatal transmission from mother to baby during childbirth.

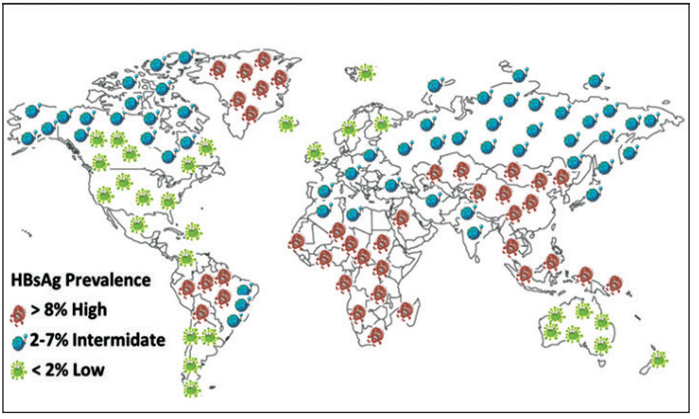

Figure 1 Geographic distribution of chronic HBV infection. Hepatitis B surface antigen (HBsAg) is a marker of chronic HBV infection. More than 7% of the population in Africa, China and South East Asia are chronically infected with HBV. (Source, CDC. Travellers' Health, Yellow Book. http,//wwwn.cdc.gov/travel/yellowbookch4-HepB.aspx).

HBV belongs to the family of *Hepadnaviridae* and the genus of *Orthohepadnavirus*. It only infects humans and other higher primates, such as, chimpanzees and orangutans. The viral infectious particle (also known as virion) was named after David S. Dane who first reported observation of the particle under a transmission electron microscope (Dane *et al*., 1970). The virion found in the blood of chronically infected patients is spherical in shape with a diameter of about 42 nm (Figure 2). It is enveloped by a lipid bilayer derived from the liver cell membranes. Embedded in the lipid bilayer are three distinct but related forms of surface antigens (HBsAg), namely S- (small), M- (middle) and L- (large) HBsAg. The viral capsid located inside the envelope is made of many copies of the core antigen (HBcAg). Within the capsid is a partially double-stranded DNA genome of about 3.2 kb which associates with the polymerase protein (P) harbouring reverse transcriptase and DNA-dependent DNA polymerase activities (Tan & Ho, 2014).

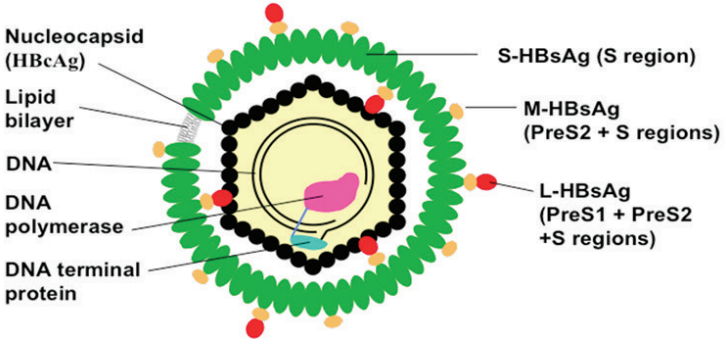

Figure 2 A schematic representation of the virion structure of HBV. HBV envelope contains three forms of surface antigen (HBsAg), namely, S- (small), M- (middle) and L- (large) HBsAg. The viral capsid located inside the envelope is made of many copies of the core antigen (HBcAg). The representations of the L, M and S forms of HBsAg have no quantitative or positional significance.

Many scientists have risked their lives to study this deadly virus. Uncountable numbers of doctors have put their lives in danger to treat and save patients infected by this virus. The ultimate goal of these unsung heroes is to eradicate the virus from this planet and make the world a better place. Therefore, the main aim of this talk is to provide a comprehensive review on the contributions of these heroes, particularly, my mentor, students and collaborators, on their endless efforts to combat HBV. This lecture is divided into four sections based on the main strategies that have been used widely to control and combat HBV: (1) Vaccination, (2) Diagnosis, (3) Drug discovery, and (4) Drug and gene delivery.

VACCINE DEVELOPMENT

Hepatitis B surface antigens (HBsAg) were discovered by Baruch Blumberg in the blood of an Australian aborigine in the 1960s (Blumberg, 1964). He was awarded the Nobel Prize in Medicine in 1976. Under an electron microscope, three morphologically distinct particles are commonly observed in the sera of chronic carriers: 42 nm infectious particles which appear as spheres and another two types of non-infectious particles which look like spheres or filaments with a diameter of 22 nm (Dane *et al*., 1970). The filamentous particles appear in different lengths. The non-infectious particles are enormously abundant in the blood stream of chronic carriers, about 1,000- to 100,000-fold in excess compared to the virion (Chai *et al*., 2008). The biological function of these non-infectious particles is unclear but they are thought to serve as decoys that trap anti-HBV antibodies and thus weaken the efficacy of the human's immune response. The non-infectious particles are mainly composed of HBsAg and host-derived lipids, but they do not contain the nucleocapsid and the viral genetic materials. Baruch Blumberg and Irving Millman purified these non-infectious

particles from infected sera and applied them as a subunit vaccine against HBV infection (Blumberg, 2013). This vaccine was patented by Fox Chase Cancer Center, and approved by the Food and Drug Administration (FDA), USA (Blumberg, 2013). The vaccine was marketed by Merck & Co., Inc. in 1981, under the trade name, Heptavax B. Although this vaccine was highly protective against the viral infection, large amounts of infected sera are needed which could be contaminated by other infectious pathogens present in the sera. This vaccine was therefore discontinued in 1990 and replaced by a safer recombinant DNA vaccine (Muraskin, 1995).

Recombinant DNA Technology Reinvents Hepatitis B Vaccine

In 1969, there was a serious outbreak of HBV at the Royal Infirmary of Edinburgh which killed patients and staff in the kidney transplant unit. Kenneth Murray (known as Ken to his friends), a molecular biologist at the University of Edinburgh and also a co-founder of Biogen Inc. employed recombinant DNA technology to clone the viral DNA in a bacterial plasmid and to produce the viral proteins in bacteria (Burrell *et al*., 1979, Pasek *et al*.,1979). The experiment was a success and the first patent on recombinant DNA molecules capable of expressing HBcAg and HBsAg was filed in December 1978 (Hofschneider & Murray, 2001). Ken and his colleagues continued to produce the viral proteins in yeast by using recombinant DNA technology, and in the early 1980s the S-HBsAg was successfully produced in yeast (Valenzuela *et al*., 1982, Hitzeman *et al*., 1983, Miyanohara *et al*., 1983) and was demonstrated to protect chimpanzees from HBV challenges (McAleer *et al*., 1984, Murray *et al*., 1984). This recombinant vaccine is safer compared to the subunit vaccine purified from the

sera of infected patients as the latter may be contaminated with other infectious pathogens. Production of this recombinant vaccine is also less laborious and less time-consuming compared with the plasma-derived subunit vaccine. For commercialization, the owner of the patent, Biogen Inc., licensed the patent to SmithKline Beecham. The subsequent recombinant vaccine, Engerix-B®, was approved by the Food and Drug Administration (FDA) in the late 1980s (Frost & Reich, 2009). This vaccine, manufactured and marketed by SmithKline Beecham, is currently the best-selling recombinant hepatitis B vaccine worldwide, with sales amounting to more than US$ 1 billion per year. Following mass immunization of newborn babies with the recombinant vaccine since the 1990s, the rates of HBV carriers and liver cancer cases dropped significantly in many countries (Chen, 2009, 2010). The invention of recombinant hepatitis B vaccine has undoubtedly changed the vaccine industries and made this planet a safer place for mankind. In addition, the production of the hepatitis B vaccine via recombinant DNA technology has now become a model for many other recombinant vaccines against human and animal pathogens. The first inventor of the recombinant hepatitis B vaccine, Ken, used the royalties from the hepatitis B vaccine to support a great many charitable activities, postgraduate and undergraduate scholarships, library facilities, research grants, laboratories, conferences, science programmes for schoolchildren, lecture rooms and halls as well as building projects. Ken left not only a rich legacy but good personal values for many generations to learn.

Why there is a Continuing Need for New Hepatitis B Vaccines?

The reinvention of the HBV vaccine with recombinant DNA technology is a success story in human history and the vaccine has indeed saved countless lives worldwide. Does this mean that the human being is fully protected by the yeast derived recombinant vaccine? Why is there always a need to improve, invent and formulate new HBV vaccines? The main reason is that the virus is competing with human beings to survive on this planet. Could we totally eradicate the virus from this planet? This is a huge challenge and of course it is the ultimate goal of human beings. HBV has been mutating and immune-escape mutants with amino acid replacements, deletions and insertions across the immunodominant region or the '*a*' determinant of HBsAg have been reported widely (Zanetti *et al*., 1988, Coleman, 2006, Zuckerman & Zuckerman, 2003). Vaccine-escape mutants with nucleotide changes in their *polymerase* (*pol*) and *S* genes were found in chronic hepatitis B patients treated with nucleoside analogue drugs (Lapiński *et al*., 2012). Most frightening is that HBV and HIV co-infect millions of people worldwide, particularly in sub-Saharan Africa and East Asia (van den Berg *et al*., 2009). The patients co-infected by these two viruses have a higher rate of liver-related mortality compared to those infected only by HIV-1 or HBV alone (Thio *et al*., 2002). Furthermore, only 20-70% of patients infected by HIV produced anti-HBsAg antibodies after a standard HBV vaccination compared to 90-95% in healthy adult individuals (Laurence, 2005). All the above medical and scientific evidence, as well as the genetic diversity of HBV, strongly justify the continuing need for the development of new HBV vaccines.

Recombinant HBsAg Vaccines

The nucleotide sequence of the viral DNA has been determined (Pasek *et al*., 1979) and its translated products have been studied in detail with the invention of nucleotide sequencing methods in the 1970s. The envelope proteins, S-, M- and L-HBsAg are translated from one single open reading frame (ORF) by using three different in-frame start codons and a common stop codon (Figure 3). Thus, they have a large sequence in common at their C-termini. The largest of them, the L-HBsAg (39 kDa), contains the PreS1 region of 108 or 119 residues (depending on serotype) followed by the PreS2 amino acids of 55 residues and the S region which comprises 226 residues. The second largest protein, the M-HBsAg (31 kDa), has the PreS2 and S regions. The smallest of these proteins, S-HBsAg (24 kDa), contains only the S region. All these proteins are either glycosylated or un-glycosylated at Asn-146 of the S region. The M-HBsAg has an additional glycosylation site at Asn-4 of the PreS2 region. A myristyl group is linked to the glycine residue at the N-terminus of L-HBsAg (Persing *et al*., 1987).

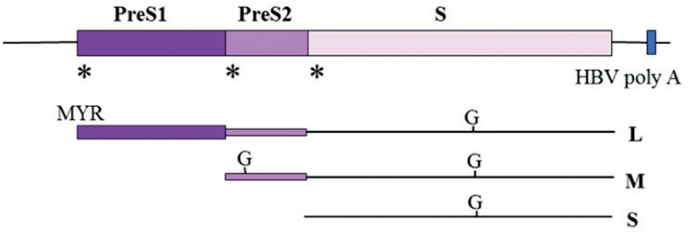

Figure 3 Envelope proteins of HBV.
The translation products of the *HBsAg* gene are shown as lines of different thickness. S-, M- and L-HBsAg are translated from a common open reading frame of the *HBsAg* gene by the use of three in-frame initiation codons (*) at the 5'-ends of the PreS1, PreS2 and S coding regions. Glycosylation is indicated by G. MYR represents the myristic acid.

The S-HBsAg can self-assemble with host lipids to form spherical virus-like particles (VLPs) with a diameter of about 22 nm. It has been produced in many eukaryotic systems via recombinant DNA technology, including *Saccharomyces cerevisiae*, *Pichia pastoris*, BHK-21 cells, CHO cells, Sf9 insect cells and tomato plants. Currently, the recombinant DNA vaccines are based on the VLPs of S-HBsAg produced in yeast cells. These vaccines are safer compared to the non-infectious particles purified from the sera of infected patients. Although the recombinant vaccines are highly effective, about 5-10% of healthy people show no or low immune responses after vaccination with three doses of these vaccines (Ottone *et al*., 2007). As the PreS regions of the L-HBsAg contain several epitopes, incorporation of these regions in current HBsAg vaccines may help improve the protective level in slow- or non-responders. A straight forward experiment to prove this concept is to produce the L-HBsAg in yeast cells and study its efficacy. Cloning of the L-HBsAg in *Pichia pastoris* was performed by Fazia (2010) who demonstrated that the purified product assembled into spherical particles closely resembling those isolated from human serum. Interestingly, apart from the L-HBsAg, the VLPs also contained the M-, and S-HBsAg. These particles are reactive against human anti-HBsAg, implying their potential as an immunogen (Yong & Tan, 2015).

Multi-component Vaccines Based on the Hepatitis B Virus Capsid

Inside the viral envelope is a capsid which serves as a protective container for the viral genome. The capsid is assembled from many copies of core proteins, commonly known as core antigens (HBcAg). The full-length core protein contains 183 amino acids with a molecular mass of approximately 22 kDa. The first 144

amino acids of the HBcAg are required for self-assembly and the C-terminal region (residues 145-183) is highly basic, rich in arginine residues, and is believed to interact with the pregenomic RNA (Pasek *et al*, 1979). Advancement of molecular biology from the 1980s to 1990s facilitated the expression of HBcAg in *E. coli* (Pasek *et al*., 1979, Burrell *et al*., 1979, Edman *et al*., 1981, Stahl *et al*., 1982), tissue culture cell lines (Gough and Murray, 1982, Rossinck *et al*., 1986), yeast (Kniskern *et al.,* 1986), baculovirus (Hilditch *et al*., 1990), *Acetobacter methanolicus* (Schroder *et al*., 1991), Xenopus oocytes (Zhou and Standring, 1991) and plants (Huang *et al*., 2006, Mechtcheriakova *et al*., 2006). Interestingly, in these different hosts, HBcAg self-assembled into capsids morphologically similar to authentic nucleocapsids isolated from infected livers (Cohen and Richmond). Among these organisms, *E. coli* is the most successful expression system for the production of HBcAg in scientific research and commercial applications. In general, one litre of *E. coli* culture grown in a shake flask produces about 5 mg of HBcAg (Tan *et al*., 2003). As the C-terminal end of the HBcAg is highly rich in arginine residues and about half of them are encoded by codon AGA, supplementation of the level of AGA tRNA in *E. coli* cells by a plasmid encoding the T4 AGA tRNA gene doubled the yield of HBcAg (Tan *et al*., 2003). The optimal conditions for the production of HBcAg in a shake flask and a stirred tank bioreactor were studied intensively by Tey *et al*. (2004, 2006). Extensive mutation analysis of the HBcAg indicates that the N-terminal end of 140 residues is sufficient for self- assembly (Zlotnick *et al.,* 1996) and the yield of truncated HBcAg (tHBcAg) derivatives lacking the arginine rich region is about 5- to 10-times higher than that of the full-length HBcAg (Tan *et al*., 2003). Of course this has significantly reduced the cost of HBcAg production for downstream applications, particularly diagnosis. Due to its high

commercial value, several methods have been established for the release of HBcAg from *E. coli* cells and these include enzymatic reactions, ultrasonication (Ho *et al*., 2006), bead milling (Ho *et al*., 2008a, 2008b) and high pressure homogenization (Ho *et al*., 2008c). In addition, many methods have been established to purify the HBcAg produced in *E. coli*, which include sucrose gradient ultracentrifugation (Tan *et al*., 1999), gel filtration chromatography (Tang *et al*., 2007), expended bed chromatography (Ng *et al*., 2007, 2008, Yap *et al*. 2010), aqueous two-phase systems (Tou *et al*., 2014), agarose gel electrophoresis (Yoon *et al*., 2013) and negative chromatography (Lee *et al*., 2015, 2016).

HBcAg expressed in *E. coli* assembles into two sizes of particles: small and large, approximately 30 and 34 nm in diameter, respectively (Crowther *et al*., 1994). The large particle, which constitutes about 85% of the total population, corresponds to a triangulation number T=4 arrangement of 240 HBcAg subunits. The remaining population is the smaller sized particle, which corresponds to a T=3 shell of 180 HBcAg monomers. The factors which control the distribution of these two types of particles are not as yet understood. Furthermore, the physiological implications of these two different species of capsids in the assembly of the HBV virion in the human liver still remain an enigma. An interesting feature of the capsids is that they have holes penetrating the protein shell and it is thought that small molecules enter the capsid through these holes during the reverse transcription of pregenomic RNA to a double-stranded DNA (Crowther *et al*., 1994).

HBcAg consists of 4 α-helices (Figure 4) (Böttcher *et al.,* 1997, Wynne *et al.,* 1999). During the capsid assembly, two molecules of HBcAg interact together to form a dimer, which serves as an intermediate to assemble into the large and small particles. Apart from hydrophobic forces, Cys-61 from both monomers forms a

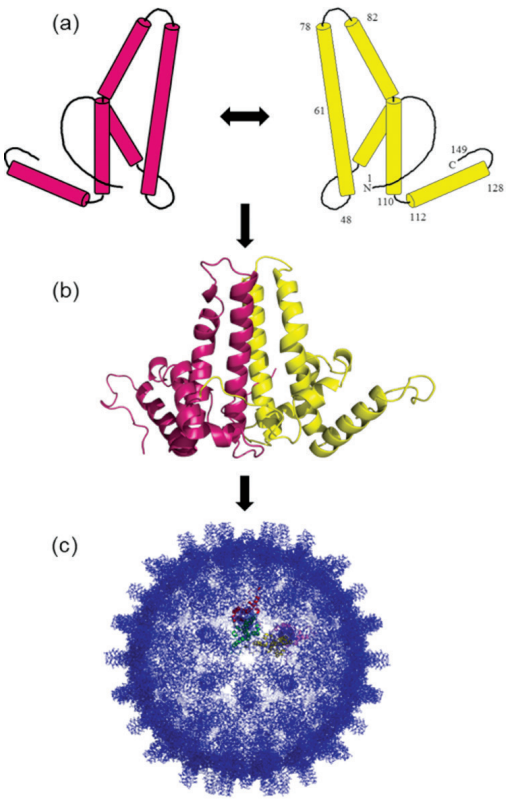

Figure 4 HBV capsid assembly.
(a) Polypeptide fold of the hepatitis B core antigen (HBcAg) monomer. Each monomer consists of 4 α-helices (indicated as cylinders). The two longest α-helices are ~42 Å long and joined by a loop corresponding to amino acids 78-82. The first long helix is roughly straight, whereas the second long helix has a kink in the middle. The numbers indicate approximate amino acid positions in HBcAg. Amino- and carboxyl-ends are indicated by N- and C-, respectively. (b) Two HBcAg monomers interact to form a dimer which acts as an intermediate to assemble into (c) capsid containing either 180 or 240 monomers. (Source, Tan *et al.,* 2007).

disulfide bridge and stabilizes the dimer and eventually the whole capsid. The four longest α-helices of the dimer (two from each monomer, Figure 4) form a protruding spike on the shell. Hence, the shell surface is spiky with 120 and 90 spikes, for the large and small particles, respectively. The immunodominant loop of the HBcAg (amino acids 74-89) is located close to the tip of the spikes.

Over the past three decades, HBV capsids have been studied extensively as a molecular carrier for foreign epitopes in the development of multi-component vaccines and diagnostic reagents. Hundreds of HBcAg fusion proteins containing insertions of foreign sequences, either at the N- or C-terminus, or the major immunodominant region located near the tip of the capsid spikes (Figure 5a), have been produced in *E. coli,* and they too assemble into particles, demonstrating the potential of HBcAg as a molecular carrier in multi-component vaccine development.

The crystal structure of the HBV capsid (Wynne *et al*., 1999, Tan *et al*., 2007) revealed that the N-terminus of the HBcAg is located on the outside of the capsid. This feature suggests that the heterologous sequence fused to the N-terminus of the monomer can be displayed on the surface of the capsid. The first attempt for N-terminal insertion was reported by Stahl *et al*. (1982), by replacing the first two residues of HBcAg with the eight residues of β-galactosidase and a linker sequence of three amino acids. This N-terminal fusion peptide was later proven to be exposed on the exterior of the capsid using X-ray diffraction analysis (Tan *et al*., 2007). An additional His-tag fused to the β-galactosidase and the tripeptide linker (Yap *et al*., 2009) was also demonstrated to be located outside the shell by cryoelectron microscopy and image reconstruction (Figures 5b & 5c) (McGonigle *et al*., 2015).

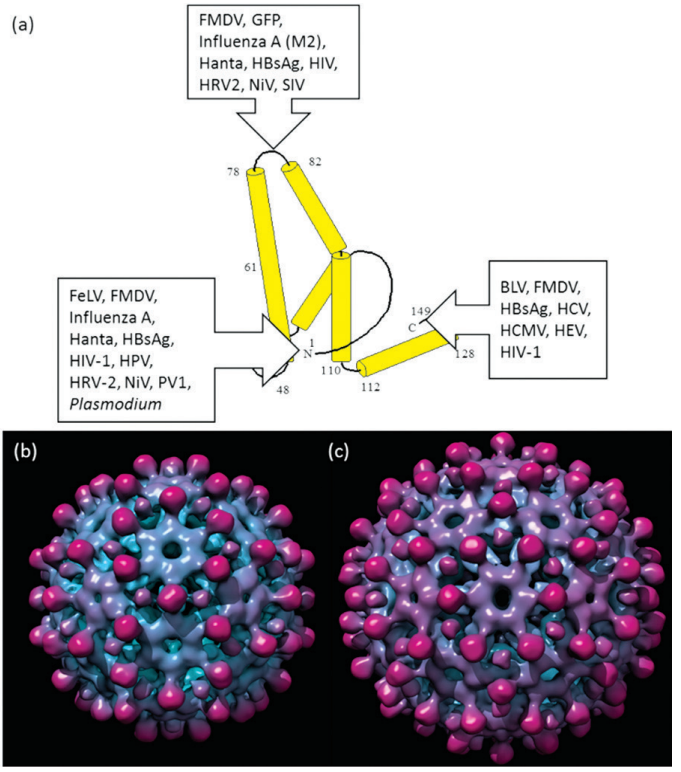

Figure 5 Display of foreign epitopes on HBV capsid. (a) Foreign polypeptides can be fused to the N- or C-terminus, or the major immunodominant region (residues 78-82) of the hepatitis B core antigen (HBcAg). His-tag fused to the N-terminal end of HBcAg protrudes from the surface of *T*=3 (b) and *T*=4 (c) HBV capsids (Courtesy of Dr. David Bhella, University of Glasgow). BLV (bovine leukemia virus), FeLV (feline leukemia virus), FMDV (foot-and-mouth disease virus), HBsAg (hepatitis B virus surface antigens), HCV (hepatitis C virus), HEV (hepatitis E virus), HIV-1 (human immunodeficiency virus type 1), HPV (human papillomavirus), HRV-2 (human rhino virus type 2), PV1 (poliovirus type 1), SIV (simian immunodeficiency virus), NiV (Nipah virus), SIV (simian immunodeficiency virus).

Several studies showed that foreign sequences fused to the N-terminal ends may impair the formation of capsids unless a flexible linker is incorporated immediately preceding the N-terminus of HBcAg (Clarke *et al*., 1987, Yap *et al*., 2009). Foreign epitopes linked to the N-terminus of HBcAg include those from the foot-and-mouth disease virus (FMDV; Beesley *et al*., 1990), feline leukemia virus (FeLV; Clarke *et al*., 1990), human rhino virus type 2 (HRV-2; Brown *et al*., 1991), poliovirus type 1 (PV1; Clarke *et al*., 1990), simian immunodeficiency virus (SIV; Yon *et al*., 1992), Bordetella pertussis (Charles *et al*., 1991), hepatitis B virus surface antigens (HBsAg; Schödel *et al*., 1992), human immunodeficiency virus type 1 (HIV-1; Isaguliants *et al*., 1996), human influenza A virus (Neirynck *et al*., 1999), Hanta virus (Koletzki *et al*., 2000) and the Nipah virus (Yap *et al*., 2011). These derivatives assembled into highly immunogenic capsid-like particles. One of these chimeric particles carrying the external domain of human influenza A matrix 2 (M2) protein conferred 100% protection against flu challenge in mice (Neirynck *et al*., 1999) and has successfully completed a phase I clinical trial (De Filette *et al*., 2008).

X-ray crystallography and cryoelectron microscopic analyses revealed that the C-terminal end of HBcAg is located inside the capsid (Conway *et al*., 1997, Wynne et al., 1999, Watts *et al.*, 2002), thus insertion of a foreign epitope to this end is most likely to be hidden inside the capsid and as a result has a lower immunogenicity. Many studies have shown that the arginine rich region at the C-terminal end can be replaced by foreign sequences of varying length, up to 720 amino acids (Yoshikawa *et al*., 1993), without impairing particle formation. A series of foreign epitopes (Figure 5a) from HBsAg (Stahl and Murray, 1989), HIV-1 (von Brunn *et al.*, 1993), bovine leukemia virus (BLV; Ulrich *et al*., 1992), human cytomegalovirus (HCMV; Tarar *et al*., 1996), Hanta virus (Ulrich

et al., 1998), HEV (Touze *et al*., 1999), FMDV (Nekrasova *et al*., 1997) and HCV (Yoshikawa *et al*., 1993), have been fused to the C-terminal end of HBcAg. All these chimeric particles assembled into particles. Some of these foreign epitopes, which are believed to be located inside the assembled particles, can be detected by specific antibodies. It is believed that the inserted foreign epitopes emerge through or are located near the edge of the capsid holes, and thus become accessible to antibodies (Crowther *et al*., 1994, Kenney *et al*., 1995). Another possibility is that these chimeric particles are less stable, disrupted in the preparation and reaction conditions used and thus exposed to antibodies.

Another attractive position for displaying foreign sequences is the immunodominant region of HBcAg (residues 78-82) located at the tip of the capsid spike (Figure 5a). This position appears to have the capacity to accommodate longer heterologous sequences and has been shown to induce strong B cell and T cell immune responses. For instance, the whole molecule of green fluorescent protein (GFP) of 238 amino acids was successfully displayed on the HBcAg particles and fluoresced (Kratz *et al*., 1999). The chimeric particle also elicited a strong humoral response against the native GFP. In addition, a range of fusion proteins at the immunodominant region has also been reported. These fusion proteins harbour the epitopes from human rhinovirus type 2 (HRV2; Brown *et al*., 1991), simian immunodeficiency virus (SIV; Yon *et al*., 1992), FMDV (Chambers *et al*., 1996), human papillomavirus (HPV; Tindle *et al*., 1994), HBsAg (Schödel *et al*., 1990), Hanta virus (Ulrich *et al*., 1998) and the Nipah virus (Yap *et al*., 2012).

Hepatitis B Vaccine Based on Phage Particles

Apart from the HBV capsid, phage particles provide an alternative means to display immunogens. Tan *et al.* (2005) fused the highly conformational immunodominant region of HBsAg (residues 111–156), or denoted as '*a*' determinant, to the C-terminal end of the 10B capsid protein of the T7 phage (Figure 6). The recombinant phage, namely T7–HBsAg111–156, displayed 415 copies of the foreign epitope on one particle and about 10^{16} copies of reasonably pure '*a*' determinant can be produced and purified from a 1-litre culture within 6 hours. Rabbits immunized with the recombinant phage mounted an immune response which was as good as that of a human-derived HBsAg, demonstrating the potential of the T7–HBsAg111–156 phage particle as a vaccine candidate.

The filamentous phage, M13 (Figure 7), has been widely used to display HBV immunogenic epitopes. The M13 phage contains about 2700 copies of the pVIII coat protein which allows a very high copy number to be displayed on the surface of the viral particle. A 12-residue epitope, HBsAg28-39, was fused successfully to the pVIII coat protein and the recombinant phage particles were used to immunize BALB/c (H-2d) mice in the absence of an adjuvant. After 8 days of immunization, an MHC class I restricted HBsAg specific cytotoxic T lymphocytes (CTL) response was observed, demonstrating the potential of the recombinant M13 phage particles as potent immunogens without an adjuvant. For a low copy number display, in general 3 to 5 copies, foreign epitopes are fused to the pIII protein located at the tip of the filamentous phage. The PreS regions (163 residues) of L-HBsAg were fused to the pIII protein but the expression level of the fusion protein was very low (Kok *et al*., 2002). This could be due to the low copy number of the pIII protein. Nevertheless, the fusion protein, PreS-g3P, was shown

to be highly antigenic (Kok *et al*., 2002). In addition, the HBcAg (about 180 residues) fused to the pIII protein was shown to be highly immunogenic in BALB/c mice (Bahadir *et al*., 2011).

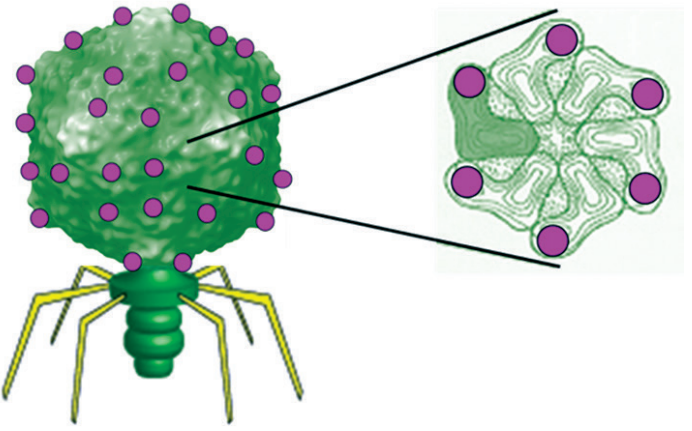

Figure 6 Display of the immunodominant region of HBV on bacteriophage T7.
Purple circles represent residues 111–156 of small hepatitis B surface antigens (S-HBsAg) fused to the C-terminal end of the 10B capsid protein of T7 phage and displayed on the surface of the phage particle. The phage can display 415 copies of foreign peptides up to 50 residues.

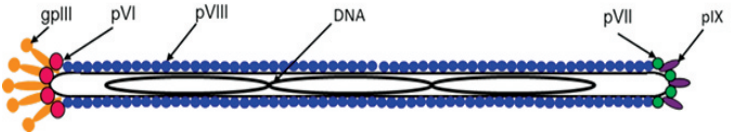

Figure 7 A schematic representative of bacteriophage M13. The virion contains a ssDNA surrounded by approximately 2700 copies of the major coat protein (pVIII). The minor coat proteins (pIII, pVI, pVII & pIX) with 3 to 5 copies are located at either end. Fusion of foreign oligonucleotides into the genes encoding the coat proteins results in the corresponding peptides being displayed on the surface of the phage particle.

VACCINE DEVELOPMENT

Past, ➢ **HBV vaccines save millions of lives.**

➢ **Recombinant DNA technology reinvents HBV vaccines.**

Present, ➢ **Virus-like particles are potential carriers for multicomponent vaccines.**

Future, ➢ **Co-display of HBV and HIV functional epitopes on a single virus-like particle.**

DIAGNOSIS OF HEPATITIS B MARKERS

Having infected about one-third of the world's population, HBV's impact on the global economy has always been the driving force for the development of streamlined diagnostic assays. The determination of HBV infections is mainly through detection of the presence of viral nucleic acids, antigens, as well as the antibodies produced against these antigens by the human body. The appearance of these serological and genetic markers during acute, chronic and occult infections is summarized in Figure 8.

For detection of HBV infection, the clinically used serological markers include both viral antigens and host antibodies produced against these viral antigens. These include HBsAg, HBeAg, anti-HBsAg, anti-HBeAg and anti-HBcAg. About 90-95% of healthy adults infected by HBV will mount an effective immune response against the virus, thereby terminating the infection. For acute infections, the first detectable marker is the HBsAg, followed by the HBeAg. At around 3 months post-infection, anti-HBcAg can be detected in the serum samples of patients infected by HBV. Anti-HBsAg and anti-HBeAg can be detected after 7 months. Overall, acute infections can be determined via detection of HBsAg during infection, and anti-HBcAg after about 6 – 7 months, where the HBV is resolved (Figure 8). Upon virus clearance, anti-HBsAg and anti-HBcAg remain present and provide protection to the individuals against re-infection by HBV.

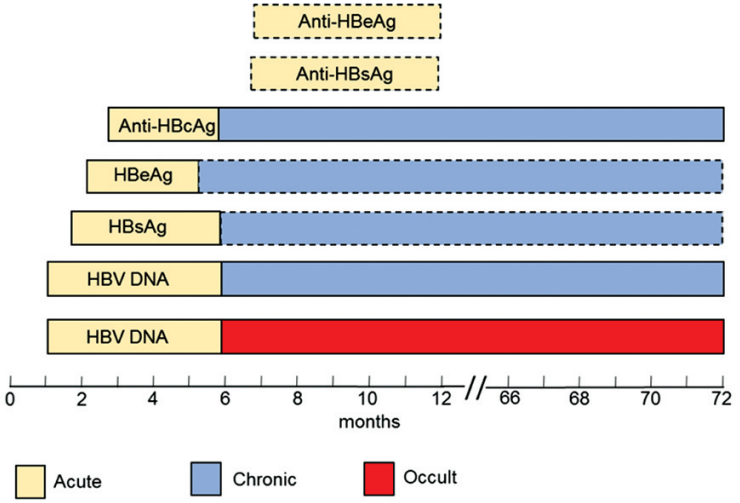

Figure 8 HBV markers in sera during acute, chronic and occult infections.
The period in months is indicated below the boxes containing the markers. The presence of the markers indicated in boxes with broken lines varies in individuals. Occult infection is characterized by the presence of HBV DNA but in the absence of HBsAg.

However, not everyone is fortunate enough to have undergone an acute infection. Approximately 5 – 10% of healthy adults, 50% of young children and 90% of infants fail to respond efficiently against HBV infection, which results in persisting infection or known as chronic infection. For chronic infection, both the HBsAg and anti-HBcAg persist after 6 – 7 months post-infection, and can last a whole life-time. Thus to determine if the infection is chronic, a test to detect the presence of HBsAg 6 – 7 months post-infection has to be carried out. The HBeAg and anti-HBeAg titers, on the other hand, are to measure the level of viral infectivity and seroconversion status (Hatzakis *et al*., 2006).

The third type of infection, known as occult infection, is characterized by the presence of viral DNA but in the absence of detectable HBsAg (Said, 2011). Unlike acute and chronic infections with the presence of antigens or antibodies, detection of occult HBV infection often requires sensitive PCR assay targeting the viral DNA. Apart from detecting occult infection, the quantity of viral DNA is also used to monitor HBV replication, disease progression, as well as to investigate the host's responses to drug treatments (Hatzakis *et al*., 2006).

To detect the serological markers mentioned above, a highly sensitive and specific radioimmunoassay (RIA) was developed in the early 1970s. RIA involves the use of a solid phase as a platform for antigen-antibody interaction, in which one of the antibodies is labelled with radioisotope. It has been used for the detection of HBsAg and anti-HBsAg (Lander *et al*., 1971, Ling and Overby, 1972, Purcell *et al*., 1973). However, the use of radioisotopes requires special precautions, licensing and equipment. Thus, it was quickly replaced by the enzyme-linked immunosorbent assay (ELISA), which utilizes enzyme-substrate reactions instead of radioisotopes. Since then, ELISA has been used widely for detecting HBV serological markers such as the HBsAg (Wolters *et al*., 1976), HBcAg (Bredehorst *et al*., 1985), HBeAg (van der Waart *et al*., 1978, von der Waart *et al*., 1978), anti-HBcAg (Gerlich and Luer, 1979) and anti-HBeAg (Korec *et al*., 1990). A more recent method which utilizes quantitative real-time PCR (qPCR) is able to monitor the replication of HBV DNA and quantify its amount at different stages of the viral infection (Jardi *et al*., 2001). In the advancement of these methods, we believe that phage display can be used to generate antibodies and diagnostic reagents. Therefore, the main aim of our study was to detect hepatitis B markers with phage display technology.

Phage Displayed Peptides as Diagnostic Reagents

Diagnostic assays, particularly ELISA, involve molecular interactions between the antigen and antibody. More precisely, the paratope of an antibody interacts with an epitope or a mimotope (discontinuous epitope) on the surface of an antigen. These paratopes and epitopes can be formed from less than 10 amino acid residues. Therefore, random short peptide sequences which interact strongly with these epitopes or paratopes can be selected from a random phage displayed peptide library.

Phage display technology is a powerful tool that can be used to rapidly select novel peptides which bind to a wide range of antigens, ranging from macromolecules, for instance proteins, lipids, and carbohydrates, to whole cells such as parasites, bacteria and viruses. Through recombinant DNA technology, any peptide can be fused to the major or the minor coat proteins of bacteriophages and be displayed on the surface of the bacteriophages (Smith and Petrenko, 1997). The vast peptide libraries displayed on bacteriophages can then be prepared and screened for specific ligands which bind to targeted protein via a process known as biopanning (Scott and Smith, 1990). Biopanning is illustrated in Figure 9, where an immobilized target protein is exposed to the phage displayed peptide library. Recombinant phages displaying peptides that interact with the target protein will remain attached, while others are removed during washing. The bound phages can then be eluted and propagated in *E. coli*. The propagated phages can then be used for another round of screening to select only specific ligands which interact strongly with the target protein. The nucleotide sequences of the selected clones are then determined to deduce the amino acid sequences of the ligands.

Through biopanning against recombinant HBV capsid, a paratope mimic displayed on the M13 phage was isolated from a

disulfide constrained random heptapeptide library (Ho *et al*., 2003). This paratope with the sequence WSFFSNI was used as a diagnostic reagent for detecting HBcAg released from the virion in HBV positive serum samples (Hasmoni *et al*., 2005). Phage-ELISA, dot blot assay and immunoprecipitation assay were developed based on this paratope mimic (Hasmoni *et al*., 2005). In a phage-ELISA and dot blot assay, the recombinant phage binds HBcAg with a K_D of 2.5 nM, and was able to detect a minimum amount of 10 ng HBcAg.

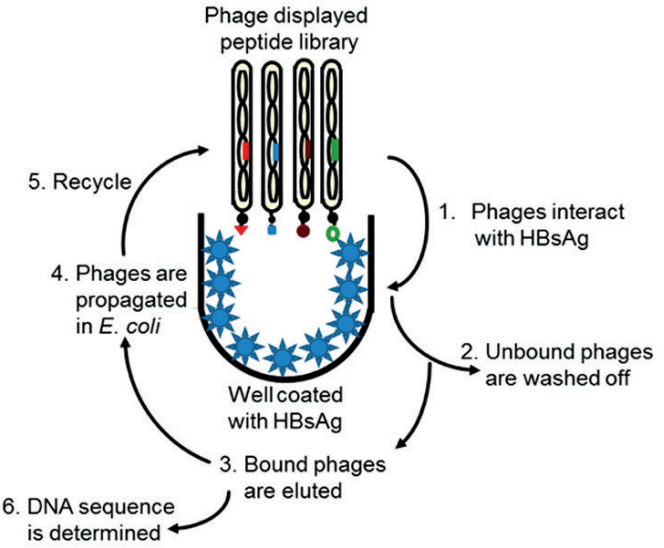

Figure 9 Processes in biopanning of a phage displayed peptide library against a target protein.
Purified HBsAg is coated on a microtiter plate well. A phage displayed peptide library is added and phages are allowed to interact with HBsAg. Unbound phages are washed off. Bound phages are eluted and propagated in *E. coli.* The selection process is repeated for 3 to 4 cycles and the identity of the selected phages is determined by sequencing the *gpIII* gene carrying the insert.

Tan *et al*. (2005) isolated a phage-displayed cyclic peptide, bearing the sequence ETGAKPH, that interacts strongly with the immunodominant region of HBsAg through biopanning. The paratope mimic, ETGAKPH, was selected from a disulfide constrained random heptapeptide library displayed on the pIII of phage M13 (5 copies per phage) by panning against human plasma-purified HBsAg. The recombinant phage harbouring ETGAKPH binds strongly to HBsAg with a K_D of 2.9 nM. Hence, the whole phage was used as a diagnostic reagent to establish both direct and indirect phage-ELISA for detection of HBsAg in HBV infected patients, as shown in Figure 10. Both direct and indirect phage-ELISA were able to detect HBsAg down to 1 pg/ml.

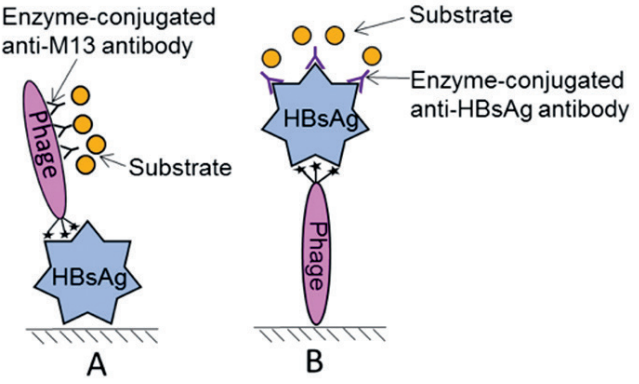

Figure 10 Phage-ELISA for detecting hepatitis B virus surface antigen (HBsAg).
(A) The major steps used in an assay for detecting HBsAg immobilized on a solid support using a phage carrying the HBsAg-binding peptide. (B) The major steps used in an assay for detecting HBsAg by immobilizing a phage carrying HBsAg-binding peptide on a solid support. Anti-M13 or anti-HBsAg antibodies conjugated to an enzyme is added to interact with the bound phage or HBsAg, then substrates for the enzyme are added to produce a measurable signal.

Most recently, Muhamad *et al*. (2013) determined the three-dimensional structure of ETGAKPH with nuclear magnetic resonance (NMR) and demonstrated its interaction with the '*a*' determinant of HBsAg. Collectively, these data justify the use of phage-displayed peptides as a sensitive diagnostic reagent, instead of the conventional use of antibodies. The sensitivity of the phage-based diagnostic assay was further enhanced by Monjezi *et al.* (2013) by incorporating qPCR to phage-ELISA, as shown in Figure 11. Amazingly, the resultant phage display mediated immuno-PCR (PD-IPCR) is 10,000 times more sensitive than the phage-ELISA (Monjezi *et al*., 2013).

Compared to the conventional ELISA which uses viral antigens or antibodies against these antigens, the isolation of phage born peptides is cheaper, faster and easier as immunization of animals is not required. The overall affinity of a selected peptide towards an antigen or antibody can be further improved by displaying the peptide at high copy number on the major coat protein of a bacteriophage, such as the pVIII in the filamentous phage, or 10B of phage T7. Phage born peptides have also been employed vastly as diagnostic reagents for the Newcastle disease virus (Lee *et al*., 2006, Ramanujam *et al*., 2004), Nipah virus (Eshaghi *et al*., 2005), cucumber mosaic virus (Gough *et al*., 1999), *Salmonella enterica* serovar Typhi (Tang *et al*., 2003, Thong *et al*., 2007) and HIV-1 (Palacios-Rodriguez *et al*., 2007), all of which demonstrate the potential of phage displayed peptides as highly sensitive diagnostic reagents for the detection of etiological reagents. The phage-based diagnostic assays that can be developed are not limited to ELISA, but also other diagnostic technology such as flow cytometry, proximity ligation assay and lateral flow strip assays.

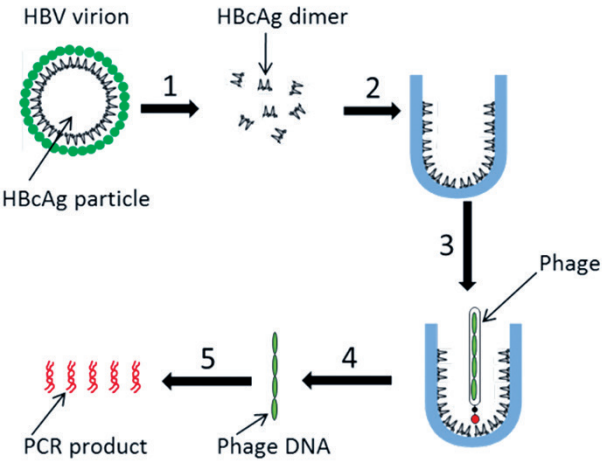

Figure 11 Phage display mediated TaqMan real-time immuno-PCR. HBV positive human serum sample is heated to release the HBcAg from the virion (1). The HBcAg is immobilized on a microtiter plate well (2) and interacted with the recombinant phage displaying HBcAg-binding peptide (3). The well is heated up to release the phage DNA (4). The presence of HBcAg is then confirmed by qPCR of the recombinant phage DNA (5).

DIAGNOSIS

Past,	➢ Traditional methods such as agar gel diffusion (Ouchterlony), TEM and radioimmunoassay (RIA) were used.
Present,	➢ ELISA is used for detecting HBV antigens and antibodies. ➢ PCR and qPCR are employed for detecting HBV DNA.
Future,	➢ Produce HBV antigens and antibodies using phage display technology. ➢ Point-of-care diagnostic methods can be linked to a mobile device for detecting HBV serological markers and nucleic acids.

DRUG DISCOVERY

The worldwide mass vaccination programme on infants has greatly reduced incidents of chronic infection and liver cancer. However, mutations of the viral genome have lowered the efficiency of the current HBV vaccines. Currently, there are approximately 370 million chronic HBV carriers waiting to be cured. Nucleotide and nucleoside analogues have been used to treat chronic HBV infection, which suppress the virus replication. Prolonged administration of nucleotide and nucleoside analogues have however given rise to mutants that are resistant to these anti-viral drugs. Moreover, HBV and HIV co-infection further complicates the situation. Treatments for these co-infections have further hastened the selection of resistant mutants, lowering the effectiveness of anti-viral drugs. All of the above evidence has led us to believe in the need for continuing development of new therapeutic agents in the effort to combat HBV, particularly the newly emerging mutants.

Traditionally, drugs and therapeutic agents were discovered through application of crude extracts from sources such as plants and fungi, followed by isolation of active ingredients contributing to the desired effects. In modern drug discovery however, structural bioinformatics allow *in silico* screening for chemical compounds which interact with a targeted compound, be it a protein, nucleic acid, carbohydrate or lipid, with a computer linked to virtual chemical libraries. In practice, however, *in silico* selected compounds often end with disappointment due to poor solubility, affinity and specificity.

Phage-displayed peptide libraries have been employed widely for the selection of antiviral peptides against many viruses over the last 20 years (see review in Castel *et al*., 2011). Viral proteins which play major roles in HBV replications, such as, the HBsAg, HBcAg and DNA polymerase are potential targets for drug discovery. As in

most other viruses, the binding of specific ligands toward key players of the HBV life cycle would interfere with the virus replication, either extracellularly or intracellularly. Extracellularly, peptides or antibodies which bind specifically to the virus or the viral-host cell receptor can potentially neutralize the infectious virion. Multiple groups of researchers have isolated the Fab fragments of antibodies by panning phage-displayed Fab fragment libraries against HBsAg, and successfully demonstrated their capabilities in neutralizing infectious HBV particles (Bose *et al*., 2005, Jia *et al*., 2008, Kim *et al*., 2002, Park *et al*., 2005, Tiwari *et al*., 2009, Yang *et al*., 2007, Zhang *et al*., 2004, Zhang *et al*., 2006). Another popular target for panning with phage display peptide libraries, in the effort to discover novel anti-viral drugs, is the PreS1 region, due to its important roles in both viral assembly and infectivity (Deng *et al*., 2005a, Deng *et al*., 2005b, Deng *et al*., 2007, Tan *et al*., 2005, Wang *et al*., 2011). As for intracellular inhibition, HBcAg is a common target in the discovery of HBV inhibitors, due to its role in forming the viral nucleocapsid as well as its interactions with the PreS region during the viral assembly (Dyson and Murray, 1995, Ho *et al*., 2003). Conversely, extracellular inhibition of the viral cycle is relatively straightforward and less complicated, where penetration of the inhibitors across the membrane of liver cells is irrelevant, unlike the intracellular inhibitors.

Intracellular Targeting

HBcAg is a vital component of HBV. It assembles into nucleocapsids by encapsidating the viral pre-genomic RNA (pgRNA) in the host cytoplasm (Bruss, 2004). Through reverse transcriptase, the pgRNA is reverse transcribed into partially double stranded genomic DNA within the core particle. The HBcAg then interacts with HBsAg embedded in the membrane of the endoplasmic reticulum (ER),

thereby providing the virus with its envelope needed for infection. This matured HBV can then be released from the host through secretion via Golgi apparatus. However, not all nucleocapsids are secreted out of the host cell. Some of the nucleocapsids are recycled back into the nucleus, followed by disassembly of these nucleocapsids, thereby unleashing the viral DNA (Kann *et al*., 2007, Rabe *et al*., 2003). Therefore, many of the intracellular inhibitors are centered around the disruption of HBcAg's interactions among themselves or with the HBsAg. The replication cycle of HBV with potential choke points for intracellular inhibitors is illustrated in Figure 12.

HBcAg also functions as a transcriptional activator, where its presence contributes to a high level of viral replication (Nassal, 1999). Hence, anti-viral agents have been designed to target HBcAg, including single-chain variable fragment intrabodies (Yamamoto *et al*., 1999), aptamers (Butz *et al*., 2001) and heteroaryldihydropyrimidines (Deres *et al*., 2003). Through phage display technology, intrabody Fab fragments capable of neutralizing HBcAg within liver cells were isolated from phage display antibody libraries (Serruys *et al*., 2009, Serruys *et al*., 2010, Tan *et al*., 2007, Walsh *et al*., 2011). Further, peptides which inhibit interaction of HBcAg with HBsAg were also selected from phage display peptide libraries (Dyson and Murray, 1995, Ho *et al*., 2003).

HBV was the first virus used in the selection of peptide inhibitors from a 6-mer linear phage display peptide library (Dyson and Murray, 1995). In this study, a phage displaying peptide, LLGRMK, was selected through panning against immobilized HBcAg particles. The affinity between the recombinant phage and HBcAg was measured, with a K_D of 0.17 μM. The corresponding synthetic peptide, with the sequence ALLGRMKG, was shown to inhibit the association of HBV nucleocapsid and L-HBsAg *in vitro*

(Figure 13). Through transfection of cell culture with replication-competent HBV plasmid, Böttcher *et al*. (1998) demonstrated the ability of the synthetic peptide ALLGRMKG to reduce HBV replication significantly, and that the peptide binding site was located at the tips of the spikes formed by amino acids 78-82 (78DPASR82) of HBcAg. Mutations of the acidic amino acid of HBcAg (E77 or D78) to alanine greatly reduced the binding affinity of the peptide to the mutated HBcAg (Böttcher *et al*., 1998). Eventually, it was proven by Tang *et al*. (2007) that these residues are essential for the peptide-HBcAg interaction, through a cross-linking study. Cryo-electron microscopy was performed to solve the 3-dimensional structure of the HBcAg-peptide complex but due to insufficient resolution, the detailed binding mechanism remained inconclusive (Böttcher *et al*., 1998).

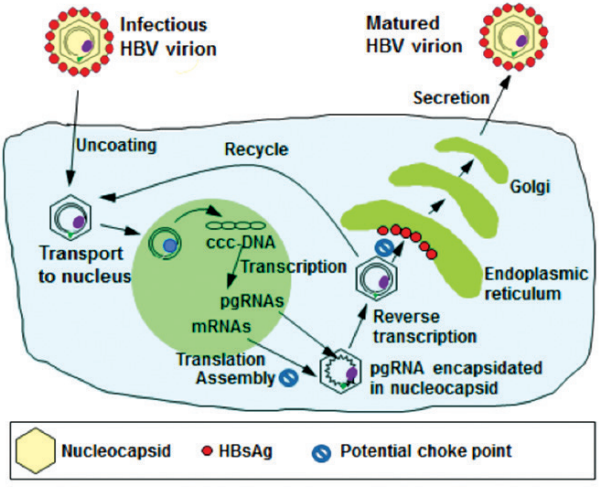

Figure 12 The replication cycle of HBV.
The viral replication can be disrupted through intracellular inhibitors which prevent capsid assembly or interaction of the nucleocapsids with HBsAg.

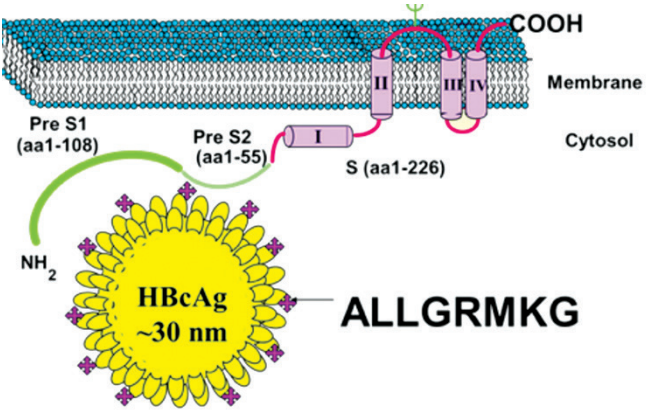

Figure 13 Peptide ALLGRMKG inhibits interaction of HBcAg with L-HBsAg.
A peptide with the sequence LLGRMK, that interacts with HBcAg, was selected from a phage displayed peptide library. A synthetic peptide with the sequence ALLGRMKG (A & G at both ends of the peptide are from the gpIII protein of the fusion phage) inhibits the association of HBcAg particles with L-HBsAg containing PreS1, PreS2 and S regions.

In 2003, two cyclic peptides, CWSFFSNIC and CWPFWGPWC, were isolated through biopanning with a disulfide-constrained phage displayed peptide library against HBcAg. These cyclic peptides bind HBcAg with more than 10-fold higher strength compared to the linear peptide LLGRMK. The corresponding cyclic peptides synthesized chemically were shown to inhibit the association of HBcAg and L-HBsAg. It also blocked the binding of C1-5, a monoclonal antibody which binds HBcAg at amino acids 78-83 (Ho *et al*., 2003). The interactions between the cyclic peptides and HBcAg are believed to be hydrophobic, as both the conformational constraint cyclic peptides and the immunodominant region of HBcAg are mainly composed of hydrophobic amino acids. Due to the hydrophobicity of these cyclic peptides, the 3-dimensional

structures of the cyclic peptides in complex with HBcAg have not been solved. Thus, further modifications on the peptides are required to improve their solubility.

Extracellular Targeting

For an infectious HBV to replicate, the first step involves its interaction with the host cell receptor, followed by integration of the nucleocapsid into the cell. Hence, molecules which mimic the structure of these receptors can potentially bind to HBV, preventing further interaction of the virion with the hepatocyte, thereby halting the infection. The study of HBV infection remains challenging until today, due to its narrow host range as well as the absence of a cell line suitable for HBV infection and propagation (Beck and Nassal, 2007). Thus, most of our understanding regarding HBV infection comes from studies on duck HBV (DHBV) which revealed several glycoproteins as potential receptors for DHBV, such as the gp180/p170 and GST-PreS polypeptides (Schultz *et al*., 2004). These glycoproteins have been classified as prototype members of the membrane bound carboxypeptidase D (CPD) (Kuroki *et al*., 1995, Tong *et al*., 1995). Urban *et al*. (1999) showed that the PreS region of DHBV binds to duck CPD with a K_D of 0.46 nM, and that they play vital roles in the virus attachment and entry via carboxypeptidase-like (A and B domains) and non-enzymatic (C domain) domains, respectively (Urban *et al*., 2000). Even with the DHBV host receptors identified, no promising inhibitor has been reported so far.

On the other hand, the PreS1 region of HBV has been in the spotlight for anti-viral studies through phage display technology, due to its function in viral assembly and infectivity (Deng *et al*., 2007, Tan *et al*., 2005, Wang *et al*., 2011). A cyclic peptide, with the sequence CETGAKPHC, was selected through panning of

disulfide-constrained phage display peptide library against the plasma purified HBsAg (subtype *ad*). We have demonstrated that CETGAKPHC binds to the immunodominant region of HBsAg (amino acids 111-156) with K_D of 2.9 ± 0.9 nM and 0.83 ± 0.63 nM, respectively (Tan *et al*., 2005). Years later, through a nuclear magnetic resonance (NMR) study, Muhamad *et al*. (2013) revealed that this peptide in fact adopts cis/trans conformations due to isomerization of the proline residue. In addition, *in silico* analysis has also revealed two binding sites that correspond to the first (amino acids 107-137) and second (amino acids 138-149) loops of the immunodominant region of HBsAg (Muhamad *et al*., 2013). As blocking of HBsAg can potentially neutralize the infectivity of HBV, this peptide can be further developed into a therapeutic agent targeting the extracellular virion.

Instead of using the plasma purified HBsAg, Deng *et al*. (2007) used thioredoxin-PreS fusion protein as a bait to isolate peptides which interact with the PreS regions of HBsAg from a phage display cyclic peptide library. In this experiment, thioredoxin-PreS fusion protein was used, due to its high solubility and stability over the PreS region expressed alone (Deng *et al*., 2005a). The isolated peptides have a common sequence motif of WTXWW (X, flexible), and were demonstrated to interact with the PreS1 region at amino acids 21 to 47 ($PreS1_{21-47}$). Mutational analyses were performed on these PreS1 binding peptides. It was found that the mutation of tryptophan to alanine but not phenylalanine abolished the binding of the peptides to the PreS1, leading the authors to speculate that the tryptophan/phenylalanine, due to their amphipathic characteristics, may mediate interaction of protein and lipid/aqueous interfaces during the viral attachment (Deng *et al*., 2007). A homology search and subsequent analysis have revealed a lipoprotein lipase (LPL), a key enzyme involved in lipoprotein metabolism, containing amino acid sequence SWSDDWWS at its C-terminal region, as a potential

HBV receptor, where the interactions between LPL with $PreS1_{21-47}$ and HBV particles have been demonstrated through *in vitro* binding, virus capture and cell attachment assays (Deng *et al*. 2007). Apart from the findings of Deng *et al*. (2007), several other potential HBV receptors on HepG2 cells were also reported to have interacted with $PreS1_{21-47}$ (Dash *et al*., 1992, De Falco *et al*., 2001). These PreS1 binding peptides which mimic the binding site of potential HBV receptors can be exploited to reduce viral infectivity by blocking the viral attachment to a hepatocyte.

Deng *et al*. (2005b) and Wang *et al*. (2011), respectively, isolated a cyclic peptide (CSRLLYGWC) and a linear peptide (KHMHWHPPALNT) from phage display peptide libraries selected against the PreS1. However, there was no consensus sequence observed between these peptides and those isolated by Tan *et al*. (2005) and Deng *et al*. (2007). This observation suggests that with different phage libraries and selection methods, different peptides targeting different parts of HBV may be discovered. On the other hand, the diversity of the selected peptide sequences indicates that various cellular proteins are involved during the viral attachment. Therefore, targeting these proteins, be it viral proteins or host cell receptors, could one day reduce the viral infectivity in humans.

DRUG DISCOVERY

Past,	➢ Corticosteroids and immunosuppressive drugs were used to treat chronic hepatitis B.
Present,	➢ Interferon-α and nucleoside analogues are used to treat chronic hepatitis B.
Future,	➢ Combination therapy with drugs targeting the HBV replication cycle. These drugs are delivered specifically to liver cells.

DRUG AND GENE DELIVERY

Most liver cancer cases are caused by prolonged HBV infection. Liver cancer is the world's fifth most common cancer, accounting for 5.6% of all human cancers and ranks third among cancer-related mortalities (Thomas *et al*., 2010). In recent years, gene therapy has gained popularity amongst researchers working on an effective treatment for cancer, and virus-based nanoparticles, particularly bacteriophages, have been proposed as gene and drug delivery vectors. The use of phages comes with many advantages. Their coat proteins provide additional protection for drugs and genetic materials packaged within the particles, keeping the cargo intact for an extended period of time. Apart from being physically stable in a wide range of pH and temperatures (Jepson and March, 2004), the phage propagation can easily be scaled up with a relatively low budget. As bacteriophages lack tropism for eukaryotic cells, they are generally safe to be used as vessels for gene and drug delivery.

Bacteriophages as Drug and Gene Delivery Vectors

The specific targeting and internalization of recombinant bacteriophage displaying integrin-binding peptides into mammalian cells were demonstrated by Hart *et al*. (1994). In another independent study performed by Yokohama-Kobayashi and Kato (1994), the transfer of genetic materials into a mammalian cell was successfully performed through bacteriophage in the presence of DEAE dextran and lipopolyamine. Five years later, Larocca *et al*. (1999) delivered a reporter gene encoding the green fluorescent protein (GFP) into a mammalian cell, through the use of recombinant M13 phage displaying fibroblast growth factor on its surface.

HBV infection is believed to be initiated by interaction of its envelope protein, particularly the PreS regions of HBsAg, with a

specific host receptor located on the surface of the liver cell. In order to develop a gene delivery system, the PreS regions were fused to the pIII protein of phage M13 (Kok *et al*., 2002) and used for transfection of HepG2, a human hepatocarcinoma cell line (Tang, 2008). Although the recombinant phage M13-PreS was shown to transfect HepG2 cells, the efficiency was well below expectations, probably due to the low copy number of pIII (5 per phage). To increase transfection efficiency, Tang *et al*. (2009) divided the PreS1 region into 2 shorter fragments, residues 1-47 and 60-108. These fragments were fused separately to the 10B protein of phage T7, in which 415 copies of fragments per phage were displayed. In comparison, the recombinant phage displaying amino acids 60-108 was shown to be the most effective in transfecting and internalizing into HepG2 cells, in a time- and dose-dependent manner (Tang *et al*., 2009).

Phage delivery based on the PreS regions targets both normal and cancerous liver cells. Thus, the cargo should not contain any materials harmful to normal liver cells. For delivery of anti-tumor drugs such as doxorubixin, which is cytotoxic towards both normal and cancerous cells, a more specific navigating ligand is required. Therefore, Zhang *et al*. (2007) selected a ligand that specifically targets the cancerous liver cell through panning of the phage-peptide library against HepG2 and subtraction with L-02 (normal) cell lines. Through an *in vivo* study with BALB/c mice, they demonstrated that the recombinant phage displaying the ligand FQHPSFI bound to tumor cells following an intravenous injection. This further confirms the potential of phage display in drug or gene delivery systems to target hepatocellular carcinoma (HCC). However, before a phage can be applied in humans, the specificity of the phage towards human microflora must be studied in depth, to avoid changes of microbiome patterns in a healthy individual.

Virus-like Particles as Drug and Gene Delivery Vectors

Similar to phage particles, recombinant virus-like particles such as the HBV capsid can also be used for targeted delivery of drugs and genes. The HBV capsid contains 180 or 240 copies of HBcAg, which form small and large icosahedral structures with triangulation number of *T*=3 *and T*=4*,* respectively (Crowther *et al.,* 1994).

The HBV capsid has been studied thoroughly as a molecular carrier for foreign epitopes in the development of multivalent vaccines, over the past 30 years (for reviews, see Murray and Shiau, 1999, Pumpens and Grens, 2001, Whitacre *et al.,* 2009). In addition to its popular use in vaccine development, Lee and Tan (2008) demonstrated that the HBV capsid can dissociate into HBcAg dimers in the presence of urea, and the dimers re-associate back into capsid upon the removal of denaturants through dialysis. By exploiting this feature, Lee and Tan (2008) successfully packaged GFP within the inner cavity of the HBV capsid, thereby opening the window of opportunity for its further development into a targeted nanoparticle delivery system.

Via dissociation and re-association of the HBV capsid, Lee *et al.* (2012) packaged fluorescein labelled oligonucleotides within the HBV capsid. HBV capsids are spiky, where each of the spikes is formed from a bundle of four α-helical polypeptides of HBcAg dimer, two from each monomer. As mentioned earlier, the ligand LLGRMK was isolated from a phage displayed peptide library panned against the HBV capsid (Dyson and Murray, 1995). Cryo-electron microscopy (Böttcher *et al.,* 1998) and mass-spectrometry (Tang *et al.,* 2007) revealed that the binding site for this ligand to the HBV capsid is located precisely at the tip of the spike. Based on these findings, Lee *et al.* (2012) designed a 24-mer peptide comprising cell-internalizing peptides (CIP) at the N-terminus

and LLGRMK at the C-terminus, in which LLGRMK served as a 'nano-glue' that directed and connected the CIP to the tip of the HBV capsid's spike (Figure 14). The glued CIP-capsid complex carrying fluorescein labelled oligonucleotides was then cross-linked chemically to strengthen the structure through formation of covalent bonds. This complex was then used to transfect HeLa cells, where fluorescence signals within the cells were detected, indicating successful delivery of the oligonucleotides (Figure 15). Therefore, a combination of phage display, virus-like particles and the nano-glue concept can create innovative applications, not only to combat HBV, but other diseases as well.

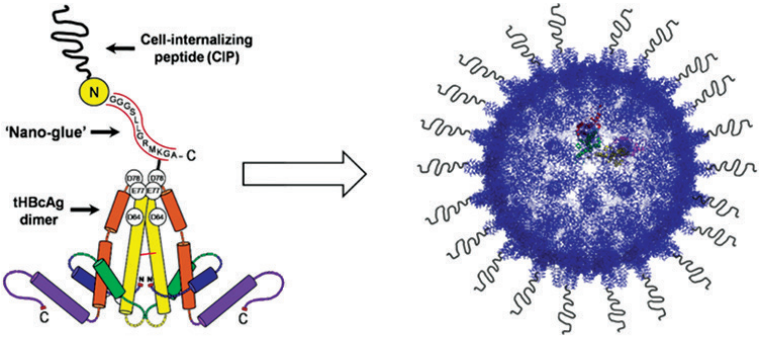

Figure 14 Display of cell-internalizing peptide (CIP) via the nano-glue. The nano-glue with the sequence LLGRMK is used to display cell-internalizing peptide (CIP) on the surface of the HBV capsid. The CIP is synthesized at the N-terminus of the nano-glue which interacts specifically with the HBcAg dimer. The Lys in the nano-glue is cross-linked to Asp or Glu, located at the tip of the HBcAg dimer.

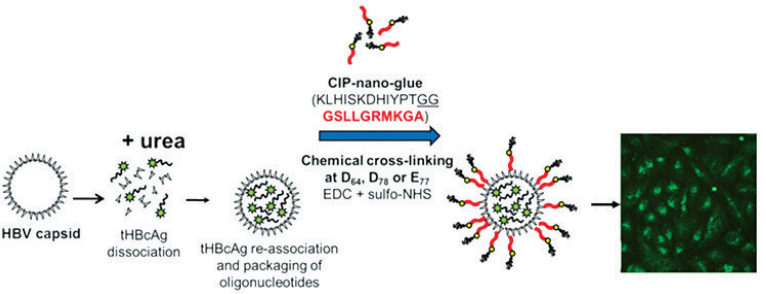

Figure 15 Delivery of fluorescent oligonucleotides into HeLa cells. HBV capsid is dissociated into dimer by using urea. Oligonucleotides labelled with fluorescence are added and the urea is removed by dialysis. The HBcAg self-assembles to package the oligonucleotides. CIP-nano-glue is cross-linked to the capsid packaged with the oligonucleotides and then added to HeLa cells.

DRUG AND GENE DELIVERY

Past & Present,	➢ Drugs are distributed throughout the body in traditional and conventional drug delivery systems.
Future,	➢ Specific drug delivery to the liver or specific organs by means of nanomedicine which employs nanoparticles. ➢ The loaded drugs can inhibit viral replication and kill cancer cells simultaneously.

CONCLUSION

Recombinant HBV vaccine produced in yeast has saved countless lives worldwide. However, the emergence of vaccine escape mutants and a dramatic increase in the population co-infected with HBV and HIV strongly justify the need for a better HBV vaccine. Virus-like particles are good options for scientists to invent a vaccine against HBV and HIV infections.

ELISA involving antibodies is widely used for the detection of HBV serological markers. However, production of antibodies with animals or hybridoma technology is laborious, time consuming and costly. These limitations can be overcome by displaying peptides and antibodies on phage particles as diagnostic reagents for detecting HBV serological markers. Our research group has demonstrated that phage-ELISA can be combined with qPCR for the detection of HBV antigens. In this digital age, point-of-care diagnostic methods can be linked to a mobile device for detecting HBV serological markers and the viral nucleic acids.

Currently, there is no effective cure for HBV infection. Interferon-α is commonly used to treat chronic hepatitis B patients but the success rate is less than 50%. Treatment of chronic hepatitis B carriers with nucleoside analogues has resulted in the selection of drug resistant mutants. Multiple drug therapy holds the best hope to clear the virus in chronic patients. In order to search for new therapeutics, phage displayed peptide libraries were used to select ligands that inhibit the intracellular and extracellular steps of the HBV life cycle. These ligands are lead compounds and will certainly occupy the experiments of many scientists who are developing potent anti-HBV drugs.

In conventional drug delivery systems, anti-viral drugs are distributed throughout the body which causes side effects in normal cells. Thus, there is an urgent need to develop a specific drug delivery system to target anti-HBV drugs to the liver. The delivery vehicle must be stable under physiological conditions and the cargo released when the vehicle is close to the liver cells or taken up by the cells. In this light, HBcAg VLP is an excellent packaging material for therapeutic drugs. These drugs can be targeted to a specific organ or a tissue by displaying a CIP on the VLP via the nano-glue concept. However, the stability and pharmacological

activity of the nano-vehicle and its therapeutic drugs must first be studied thoroughly.

The entire world is now facing scary HBV threats with the emergence of vaccine escape and drug resistant mutants, as well as co-infection with HIV. The weapons currently being used to fight this deadly virus are losing their efficiency. Thus, they have to be sharpened immediately. New heroes and innovative ideas are desperately needed to continue this incomplete battle.

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BIOGRAPHY

Wen Siang was born in Perlis, Malaysia, in 1968. He obtained a BSc (Hons) degree in Biochemistry and Microbiology in 1993, from Universiti Pertanian Malaysia (UPM). Upon graduating, he continued his MSc study at UPM with Dr. Khatijah Yusoff (now Prof. Datin Paduka) as his supervisor. In 1994, he was awarded a UPM scholarship to pursue a PhD degree at the University of Edinburgh under the supervision of Prof. Sir Kenneth Murray (Figure 16), one of the most eminent and highly respected molecular biologists in the world, who developed the first effective recombinant vaccine against the hepatitis B virus (HBV) and saved countless lives worldwide. Wen Siang and Prof. Murray developed methods to study HBV proteins and improved the yield of the HBV core antigen produced in bacteria. Wen Siang then returned to Malaysia in late 1997 and managed to set up a laboratory equipped with some basic instruments to study molecular biology of viruses. He was promoted to an Associate Professor in 2002 and a full Professor in 2008.

Wen Siang is an enthusiast of phage display and has successfully applied this technology in affinity selection of ligands, epitope mapping, identification of inhibitors and in the design of diagnostic reagents for HBV, Newcastle disease virus (NDV) and the Nipah virus (NiV). His studies on phage display and molecular biology of HBV, NDV and NiV have paved the way for the development of drugs and vaccines against these viruses. He is also passionate about designing and producing virus-like particles (VLPs) as well as in their practical uses in the development of multi-component vaccines and immunological reagents. Along with colleagues, he has developed many new methods to separate and purify VLPs from HBV, NDV and NiV. This has led to the production of cheaper vaccines and diagnostic reagents. In 2005, he was awarded the

Norken Stiftung Research Fellowship to study structural biology in the laboratory of Prof. Malcolm Walkinshaw, Chair of Structural Biology, Edinburgh. He succeeded in crystallizing and solving the structure of the HBV capsid which provides an insight into the use of the capsid as a vaccine carrier and also as a gene delivery vehicle. With the structural information, he developed methods to package drugs in the capsid and invented chimeric VLPs that served as nano-vehicles to target cancer cells. Recently, he introduced an innovative 'nano-glue' concept for the display of ligands on the surface of viral capsids and specific delivery of drugs and nucleic acids into cancer cells.

Wen Siang loves to share his findings and inventions with scientific communities whereby so far he has published over 130 papers in leading scientific journals (total impact factor over 350, citation over 1700 times, and *h*-index=23), more than 260 papers in scientific proceedings and one book. He is one of the inventors for 17 patents, 6 of which have been granted by the US, Singapore and Malaysian Patent Agencies. In addition, 3 trademarks and 1 trade secret have been registered under his name.

Wen Siang has completed the supervision of 22 PhD and 33 MSc students and is currently supervising another 9 PhD and 5 MSc students. Many of his students have followed his footsteps to become academicians or researchers in Malaysia, Indonesia, Singapore, Japan, South Korea, Canada, Australia, Iran, India, Thailand, United Kingdom, Germany, Taiwan, New Zealand and the United States. More than 20 associations and institutions have invited him to deliver lectures at conferences and workshops, including plenary lectures. He has also represented his university in national and international competitions, and won many prizes and awards, including the Best Invention in Biotechnology, presented by RamRais and Partners. In 2012, he was a recipient of the Top

Research Scientists of Malaysia award by the Academy of Sciences Malaysia.

Apart from research and teaching, Wen Siang is also heavily involved in administrative duties at the national and international levels. He was a lead auditor for the Quality Management System in UPM and an auditor for the Malaysian Quality Assurance (MQA). He was the Head of the Safety and Health Committee in his faculty. He was also the advisor for the Biochemistry and Microbiology Postgraduate Club. He has served as a member in various committees, including the Adjunct Professor Selection Committee (UPM), workshops and seminars. Further, he was an advisor for the International Conference on Beneficial Microbes, 2014.

Wen Siang also serves the scientific and non-scientific communities in several ways. He is an editorial board member for the Pertanika Journal of Tropical Agricultural Science and he was the chief editor for the Research@BioTech, a panel member of the National Science Fellowship (NSF), MOSTI Science Fund Grant, Monash University's BSc programmes, an external examiner for the International Medical University's (IMU) Biotechnology Programme, an assessor for the appointment of Associate Professor and Professor at Universiti Kebangsaan Malaysia (UKM), University of Nottingham, Universiti Tunku Abdul Rahman and UCSI University. Wen Siang has reviewed over 80 scientific articles for leading scientific journals and been appointed examiner for more than 90 MSc and PhD theses. He is an advisor for the Education Chamber of Selangor Citizens, and is also an active member of several professional bodies and scientific societies. He is happily married to Bee Kee whom he first met in a laboratory and the couple is blessed with two children.

Figure 16 Wen Siang and Ken Murray.

The photo was taken in December 2005, when Wen Siang was in Edinburgh learning crystallography and NMR spectroscopy.

ACKNOWLEDGEMENTS

First, I wish to express my most sincere gratitude to my PhD supervisor, Prof. Sir Kenneth Murray (1930-2013) for his invaluable guidance in my career development. His passion for experiments, science, commercialization and charity continues to inspire creativity and innovation in every aspect of my life. On behalf of my family, once again I would like to thank him for his care, concern and love.

I am also greatly indebted to Prof. Datin Paduka Dr. Khatijah Yusoff for introducing me to the first virus (Newcastle disease virus) in my career. I had a good start in science because I did my undergraduate and MSc projects under her supervision. Further, I can hardly express enough gratitude to Prof. Dr. Tey Beng Ti for his encouragement, advice and jokes whenever I faced difficulties in research. Interestingly, he has always been able to come up with innovative ideas to solve our research problems.

Special thanks go to the following postgraduate students for sharing their discoveries and inventions with me and the entire world: Dr. Amir Rabu, Dr. Azira Mohamad, Dr. Chew Few Ne, Dr. Chong Fui Chin, Dr. Eddie Chia Suet Lin, Dr. Goh Zee Hong, Dr. Ho Kok Lian, Dr. Jane C.J. Liew, Dr. Jee Jap Meng, Dr. Kho Chiew Ling, Dr. Koh Cha San, Dr. Kok Wai Ling, Dr. Lee Khai Wooi, Dr. Lee Thong Chuan, Dr. Michele Ng Yee Tan, Dr. Micky Lee Fu Xiang, Dr. Nacisse Mary Joseph, Dr. Ong Hooi Tin, Dr. Ong Swee Tin, Dr. Ong Yien Yien, Dr. Priadarishni Ramanujum, Dr. Raha Ahmad Raus, Dr. Raksha Sunhare, Dr. Rattana Wongchuphan, Dr. Shamala A/P Salvamani, Dr. Sharifah Mariam Sayed Hitam, Dr. Sieo Chin Chin, Dr. Siti Salwah Hasmoni, Dr. Tan Geok Hun, Dr. Tan Sheau Wei, Dr. Tang Swee Seong, Dr. Wong Sin King, Dr. Yap Wei Boon, Dr. Yong Chean Yeah, Dr. Zulkefley Othman, Ms. Beh Poay Ling, Ms. Chia Jyh Yea, Ms. Eni Kusumaninglyas,

Mrs. Fazia Mohd. Sinang, Ms. Gan Bee Koon, Ms. Kueh Chare Li, Ms. Hagilaa Ganesan, Mr. Ho Chin Woi, Ms. Lalita Ambigai A/P Sivasamugham, Ms. Lau Gee Leng, Ms. Lim Swee Lu, Mr. Made Angga Akwiditya, Ms. Masniza Mohamed @ Mahnood, Ms. Mayamarni Shamsu Zaman, Mrs. Noor Suhana Adzahar, Ms. Nur Asilah Ibrahim, Mrs. Nur Azmina Syakirin Mohd, Mrs. Nursyaza Zulkifli, Ms. Nurwatti Md Aris, Mr. Ong Hui Kian, Ms. Rafidah Saadun, Ms. Razieh Monjezi, Mrs. Roya Biabani Khankahdani, Ms. Seyedehsara Masoomi Dezfooli, Ms. Siti Sarah Mohd. Noor, Ms. Tan Yean Peng, Mr. Tang Kah Fai, Mrs. Tang Kie Hie, Ms. Tayebeh Azam Saedi Yadollah, Ms. Thong Qiu Xian, Mrs. Ummi Fairuz Hanapi, Ms. Wong Chuan Loo, Mr. Yaw Chong Wey, Mrs. Yoon Kam Yee and Mrs. Zeinabsadat Mohammadi.

Many thanks to all my collaborators for sharing their ideas, knowledge and facilities in the advancement of science, education, engineering and technology: Prof. Dr. Abdul Rahman Omar (UPM), Prof. Datin Paduka Dr. Aini Ideris (UPM), Dr. Ahmad Tarmizi Hashim (MPOB), Assoc. Prof. Dr. Alan Ong Han Kiat (UTAR), Dr. Alvin Hee (UPM), Dr. Amir Syahir Amir Hamzah (UPM), Dr. Andrew Cronshaw (University of Edinburgh), Assoc. Prof. Dr. Bimo A. Tejo (UCSI), Assoc. Prof. Dr. Chan Eng Seng (Monash University), Dr. Chee Hui Yee (UPM), Prof. Lee Hsuan Shu (NTU, Taiwan), Assoc. Prof. Dr. Crystale Lim Siew Ying (UCSI), Dr. David Bhella (University of Glasgow), Dr. Dusan Uhrin (University of Edinburgh), Dr. Edward Ooi Chien Wei (Monash University), Dr. Ho Kok Lian (UPM), Prof. Dr. Ho Yin Wan (UPM), Assoc. Prof. Dr. Janna Ong Abdullah (UPM), Prof. Datin Paduka Dr. Khatijah Yusoff (UPM), Assoc. Prof. Dr. Kok Wai Ling (University of Nottingham), Prof. Dr. Ling Tau Chuan (UM), Dr. Majid Eshaghi (Biopolis, Singapore), Prof. Malcolm Walkinshaw (University of Edinburgh), Dr. Mariatulqabtiah Abdul Razak (UPM), Dr. Michael

Dyson (Iontas Ltd, UK), Prof. Dr. Mohd Basyaruddin Abdul Rahman (UPM), Prof. Dr. Mohd. Hair Bejo (UPM), Assoc. Prof. Dr. Mohd. Puad Abdullah (UPM), Assoc. Prof. Dr. Muhajir Hamid (UPM), Assoc. Prof. Dr. Noorjahan Banu Alitheen (UPM), Assoc. Prof. Dr. Norazizah Shafee (UPM), Prof. Dr. Norhani Abdullah (UPM), Prof. Dr. Seow Heng Fong (UPM), Assoc. Prof. Dr. Sieo Chin Chin (UPM), Assoc. Prof. Dr. Sharifah Syed Hassan (Monash University), Prof. Dr. Sheila Nathan (UKM), Prof. Dr. Shin-ichiro Suye (Fukui University), Assoc. Prof. Dr. Subha Bhassu (UM), Dr. Syahida Ahmad (UPM), Assoc. Prof. Dr. Takenori Satomura (Fukui University), Dr. Tan Chong Seng (MARDI), Dr. Tan Sheau Wei (UPM), Prof. Dr. Tan Soon Guan (UPM), Assoc. Prof. Dr. Tan Wee Tee (UPM), Dr. Tang Swee Seong (UM), Prof. Dr. Tey Beng Ti (Monash University), Prof. Dr. Thong Kwai Lin (UM) and Dr. Yeap Swee Keong (UPM).

I would like to thank Universiti Putra Malaysia for providing a conducive environment for my career development. I am also fortunate that the following organizations provided me with research and travel grants: the Ministry of Science, Technology and Innovation of Malaysia (MOSTI), Ministry of Higher Education, Malaysia (MOHE), Ministry of Agriculture & Agro-Based Industry, Malaysia (MOA), European Synchrotron Radiation Facilities (ESRF), Third World Academy of Sciences (TWAS), Japan Aerospace Exploration Agency (JAXA), Japan Student Services Organization (JASSO), Roseanne Campbell Trust for Hepatitis Research, Die Norken Stiftung, the Royal Society Short Visit Scholarship and the Boehringer Ingelheim Fonds.

Finally, special thanks to my wife, Lim Bee Kee, for her sacrifices, patience, motivation and love. I owe her more than she knows. I extend my thanks to our parents, children (Tan Jean Yin and Tan Yee Herng), brothers and sisters for their endless love and support.

Wen Siang Tan

LIST OF INAUGURAL LECTURES

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