TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Bacteriophages and their applications in the diagnosis and treatment of hepatitis B virus infection

Babak Bakhshinejad, Majid Sadeghizadeh

Babak Bakhshinejad, Majid Sadeghizadeh, Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran 14115-111, Iran

Author contributions: Bakhshinejad B performed literature mining and wrote the manuscript; Sadeghizadeh M critically revised the manuscript.

Correspondence to: **Majid Sadeghizadeh, Professor,** Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Al-e-Ahmad, Tehran 14115-111, Iran. sadeghma@modares.ac.ir

Telephone: +98-21-82884409 Fax: +98-21-82884484

Abstract

Hepatitis B virus (HBV) infection is a major global health challenge leading to serious disorders such as cirrhosis and hepatocellular carcinoma. Currently, there exist various diagnostic and therapeutic approaches for HBV infection. However, prevalence and hazardous effects of chronic viral infection heighten the need to develop novel methodologies for the detection and treatment of this infection. Bacteriophages, viruses that specifically infect bacterial cells, with a long-established tradition in molecular biology and biotechnology have recently been introduced as novel tools for the prevention, diagnosis and treatment of HBV infection. Bacteriophages, due to tremendous genetic flexibility, represent potential to undergo a huge variety of surface modifications. This property has been the rationale behind introduction of phage display concept. This powerful approach, together with combinatorial chemistry, has shaped the concept of phage display libraries with diverse applications for the detection and therapy of HBV infection. This review aims to offer an insightful overview of the potential of bacteriophages in the development of helpful prophylactic (vaccine design), diagnostic and therapeutic strategies for HBV infection thereby providing new perspectives to the growing field of bacteriophage researches directing towards HBV infection.

Key words: Bacteriophage; Hepatitis B virus; Phage display; Phage library; Biopanning; Diagnosis;Treatment; Vaccine development

Core tip: Gaining insight into the role played by bacteriophages, viruses with prokaryotic hosts, in the development of helpful diagnostic and therapeutic approaches for hepatitis B virus (HBV) infection is of paramount importance. Natural presence of bacteriophages in the human body, as a major constituent of the gut flora, creates new opportunities in directing the developed approaches towards HBV infection. Undoubtedly, exploitation of this hidden potential of bacteriophages paves the way for introduction of novel methodologies for the detection and therapy of prevalent infection of HBV.

Bakhshinejad B, Sadeghizadeh M. Bacteriophages and their applications in the diagnosis and treatment of hepatitis B virus infection. *World J Gastroenterol* 2014; 20(33): 11671-11683 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i33/11671.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i33.11671

INTRODUCTION

Hepatitis B virus (HBV) infection constitutes a significant health challenge worldwide and is considered as one of the most prevalent chronic viral infections in human. Chronic hepatitis can lead to a variety of liver disorders such as cirrhosis and hepatocellular carcinoma (HCC). HCC, one of the most common malignancies, is a leading cause of cancer-associated death in the world[1]. Human HBV belongs to the Hepadnaviridae family of viruses and human and higher primates are known as

Table 1 Names of the seven distinct proteins encoded by four overlapping genes contained in the genome of hepatitis B virus
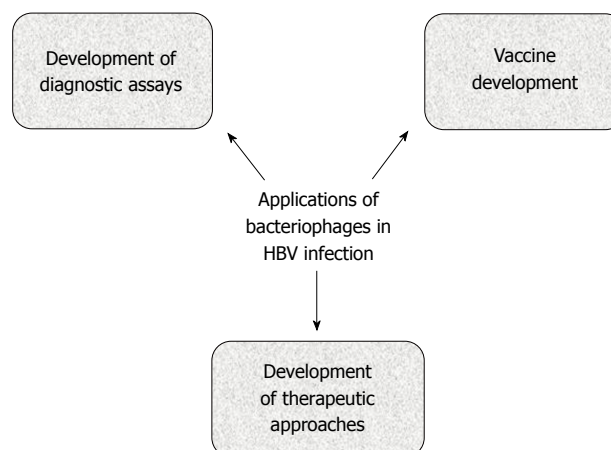
	List of HBV proteins
Large surface antigen	L-HBsAg
Medium surface antigen	M-HBsAg
Small surface antigen	S-HBsAg
Core antigen	HBcAg
Generated by proteolytic processing of the pre-core protein (non-structural protein)	HBeAg
Reverse transcriptase activity	DNA polymerase
Unknown function (non-structural protein)	HBx protein

HBV: Hepatitis B virus; HBsAg: Hepatitis B virus surface antigen; HBcAg: Hepatitis B virus core antigen; HBeAg: Hepatitis B e antigen.

the exclusive hosts for viral infection. The intact virion is composed of viral genome that is a 3.2 kb partially double-stranded circular DNA - enclosed in a nucleocapsid - and an outer layer. The nucleocapsid is made up of a large number of core antigen (HBcAg) molecules residing in the internal section of viral envelope. The outer layer contains surface antigen (HBsAg) molecules situated at the external portion of viral envelope. There are three different but related forms of HBsAg including large (L), middle (M) and small(S)-HBsAg. HBsAg harbors sets of epitopes known as immunodominant region. These highly conformational epitopes are positioned within a double-looped structure and trigger protective antibody responses in human. The antigenicity of immunodominant region can be used for detecting HBsAg. PreS1 (presurface 1) region - with 108 or 119 amino acids dependent on serotype - in the longest surface antigen (L-HBsAg) and on the outermost division of viral surface has been demonstrated to be implicated in the interaction of viral envelope with a specific receptor on the surface of hepatocytes. This HBV-hepatocyte attachment is known to be the initial step of viral infection. Furthermore, PreS1 region has been reported to play roles in the assembly and budding of virions[2-4]. Given the importance of PreS1 in viral assembly and infectivity, development of reagents with great affinity and specificity to this region of HBsAg is of particular importance for both diagnosis and treatment of HBV infection.

In general, HBV genomic DNA has four overlapping open reading frames that encode seven distinct proteins (Table 1). DNA polymerase, located within the nucleocapsid, is covalently attached to viral double-stranded DNA. Covalently closed circular DNA (cccDNA) which functions as the chief transcriptional template makes an essential contribution to the durability of infections caused by HBV and persists as an episome in HBV-infected hepatocytes. It can remain following antiviral therapy and even following apparent elimination of viral infection[5,6].

Currently there exist various diagnostic and therapeutic approaches for HBV infection. However, prevalence and also threatening consequences of HBV chronic infection emphasize the need to develop novel methodologies for the prevention, diagnosis and treatment of viral infection. Within the recent several years, numerous reports have revealed the potential of bacteriophages in this regard. These studies have shed light on the fact that one category of viruses called bacteriophage (with prokaryotic hosts) can serve as powerful tools to take prophylactic, diagnostic and therapeutic measures towards another category of viruses called hepatitis B virus (with eukaryotic hosts). The current review tells the tale of these two viruses. This review aims to provide opportunity for readers to gain a novel insight into the potential of bacteriophages in the emergence of new approaches for the prevention, detection and treatment of HBV infection. To this end, we discuss various applications of bacteriophages in three areas related to HBV infection. These areas include development of bacteriophage-based diagnostic tools, bacteriophage-based treatment modalities, and bacteriophage-based vaccines for HBV infection (Figure 1). In view of the paramount importance of phage display concept in presenting numerous bacteriophage-mediated diagnostic and therapeutic platforms for HBV, we address in detail phage display technology and phage display libraries and also provide description to various aspects of this powerful technology in order to formulate helpful diagnostics and therapeutics for HBV infection.

Figure 1 Various potentials of bacteriophages for hepatitis B virus infection.

BACTERIOPHAGES AS TOOLS FOR MEDICAL PURPOSES

Bacteriophages are naturally-occurring viruses that specifically infect bacterial cells. These prokaryotic viruses are the most abundant life forms in the biosphere. In comparison with bacterial cells, the number of phage virions is 10 fold higher and estimations of phage frequency approximate their number to 10^{30} particles. They can be tracked down in an innumerable variety of environments ranging from oceans' depths to hot springs. One of the most attractive habitats of phages is the body of human and animals particularly their gastrointestinal tract[7,8]. Di-

gestive system of human is home to a huge number of phage particles. These phages, along with their bacterial hosts, hold a major role in forming the gut flora. Interestingly, phages exhibit tremendous diversity in a manner that a large number of newly sequenced phage genes are lacking recognized homologous counterparts deposited in databases[9]. This is a reflection of the fact that the phage world is a terra incognita and many regions of this vast territory remain a mystery.

Structurally, bacteriophages harbor DNA or RNA as their genetic material which is surrounded by a protein coat or capsid. Being metabolically inert, bacteriophages take advantage of their host bacterial cells for growth and amplification. Based on the life cycle, they can be placed in two categories of lytic and lysogenic. Lytic or virulent phages multiply within the bacterial host, and subsequently release their assembled particles through lysis of the host cell. On the contrary, lysogenic or temperate phages integrate their genetic material into the host chromosome leading to their replication together with the host bacterium for generations[10]. In response to harsh conditions such as ultraviolet (UV) irradiation, the genome-residing prophage can undergo induction and change into lytic phage.

When tracing back to the era of emergence of molecular biology, it becomes clear that from the very beginning bacteriophages have played a leading role in the field. They have made an essential contribution to solving many fundamental questions of molecular biology and genetics. With the elapse of time, this role has expanded and triggered developments in a variety of biotechnological and medical areas. Some of these achievements have revolutionized the view of researchers towards biological issues. The ongoing advances in bacteriophage research have attracted tremendous attention to the potential applications of these bacterial viruses in the clinical context. These progresses have paved the way to use bacteriophages for the prevention, detection, and treatment of various pathological disorders.

PHAGE DISPLAY

Bacteriophages indicate a high level of genetic flexibility. This characteristic established ground work for the formulation of phage display methodology. The initial concept of phage display was presented in the pioneering work of George P Smith conducted at the University of Missouri in 1985[11]. In this molecular selection technique a foreign DNA fragment is inserted into the gene encoding one of the phage coat proteins. The DNA-encoded peptide/protein is then displayed as fusion to the coat protein on the surface of phage particle, where it is available to be involved in binding interactions with target molecules. In this manner, a physical linkage is formed between the surface-expressed peptide/protein and the DNA that encodes it. This genotype-phenotype connection is one of the most significant aspects of phage display[12,13]. This technique has served to display a variety

of molecules such as short peptides, individual protein domains, whole proteins, antibody fragments, receptors and enzymes on the surface of phage particles[14-16]. It is now well established that phage display as a robust and powerful technology represents huge potential to be used in a wide spectrum of biomedical and pharmaceutical areas including drug discovery, vaccine development, isolation of cell/tissue/disease-specific biomarkers (*e.g.,* a variety of cancers), protein-protein interactions, receptor-ligand characterization, gene/drug delivery and targeting, bioimaging and biosensing, neurobiology, proteomics, functional genomics, and antiviral research[17-25].

All five structural capsid proteins of M13 phage have the capacity to be exploited for expression of heterologous (poly)peptides. Each of the coat proteins has its own benefits and pitfalls. However, the most frequently used proteins for this purpose are minor coat protein (pⅢ) and major coat protein (pⅧ). pⅢ is a 400 amino acid-long protein implicated in binding of the phage particle to the host bacterium during infection. pⅧ with its 50 amino acids is recognized as the most abundant protein of M13 (about 2700 copies per phage virion) and plays a very critical role in shaping the long filamentous morphology of the phage. Generally, pⅧ is suitable for display of smaller proteins and pⅢ is proper for display of larger proteins. On the other hand, the abundance of pⅧ on the phage surface makes it possible for display of a higher number of protein of interest. But, pⅢ has fewer copies (3-5 copies) on the phage surface and is appropriate for display of a less number of protein of interest[26,27]. Other proteins of M13 are less matched for display purposes. This impaired capability for efficient display is due to the lack of full accessibility of these proteins in the context of intact phage particles. For example, some parts of pⅥ and pⅦ are not accessible in intact phage. Furthermore, the adverse effects of the displayed peptide/protein during phage assembly can, to some extent, result in low efficiency of display[28]. Choosing the coat protein (as fusion partner) on which the peptide or protein of interest will be displayed depends upon the type of the displayed ligand as well as the objective which is pursued.

Various bacteriophages such as filamentous phages, lambda, T4 and T7 have found application for surface display of foreign ligands. Platforms based on M13 (as a filamentous bacteriophage) constitute the most-widely used bacteriophage-based display tools. Filamentous bacteriophages (M13, f1 and fd) are rod-shaped bacterial viruses that are assembled at the membrane of the host bacterium where the phage genome is extruded through membrane pores. When assembled, the phage particle is composed of a single stranded, circular DNA enclosed in a proteinaceous coat with five different coat proteins[13,29]. Filamentous bacteriophages represent some advantages to be used as cloning vehicles and phage display technology platforms. Their genome can tolerate insertion of large DNA fragments in the nonessential regions without disrupting phage packaging. This high degree of adaptability is reflected by the fact that an increase in the

genome size is accompanied by a parallel increase in the length of the phage particle as well as the number of p Ⅷ molecules. Furthermore, M13 genome can be isolated as both single and double stranded forms. On the other hand, non-lytic multiplication of M13 phage leads to the accumulation of high concentrations of phage particles in the infected bacterial host[13,26].

PHAGE DISPLAY LIBRARIES

Combinatorial chemistry has proven to be a growingly potent and insightful methodology in modern drug development. This basic discovery technology can be used to generate combinatorial chemical libraries. In a general sense, combinatorial libraries are a diverse repertoire of molecules synthesized both chemically and biologically (genetically). This rich source of molecular collection can be screened for a function or affinity of interest. Genetically-encoded combinatorial libraries such as phage display libraries have taken a special place in the flourishing field of combinatorial technology particularly for the development of novel classes of tumor-avid molecules[30,31]. Genetic encoding makes it possible for molecules of a library with specific binding properties to be resynthesized and rescreened.

Construction of phage display random libraries has been one of the most fascinating and impressive developments in the area of phage display technology. In fact, phage display as a high throughput approach provides a robust basis for the production of massive libraries of molecules with different structural properties. Random peptide libraries are known to be one of the most frequent types of phage display libraries. These random libraries are made *via* cloning degenerate oligonucleotide sequences into a gene encoding one of the phage coat proteins. Each phage clone expresses a unique amino acid sequence on its surface, but the whole library may harbor billions of peptides[12,32,33]. Peptides in these random libraries have a length ranging from 5 to 20 amino acids. However, heptapeptide and dodecapeptide libraries - being commercially available - are the most broadly used peptide libraries.

These huge random libraries offer the advantage of using affinity selection to identify ligands with great specificity and affinity to any desired target. Interestingly, this procedure eliminates the need for any prior knowledge of characteristics of the target molecule. Multiple rounds of affinity selection are an extremely efficient and powerful strategy for the selection of very rare but highly specific binders from a huge pool of variants. To date, phage libraries have been applied to identify ligands against a wide variety of targets including purified proteins, antibodies, enzymes, cell surface receptors and in particular cancer-associated antigens[30,34,35]. Screening of phage libraries can be performed both in vitro against cultured cells and in vivo within the body of living animals through systemic circulation thereby leading to the isolation of cell-specific or organ-specific ligands, respectively[36-38].

Screening of a phage library over the desired target is carried out through an affinity selection-based process called biopanning. This process provides a means for selective enrichment of target-binding phages. In this approach, a library of phage-displayed peptides is passed over a plate coated with the target for which a binding ligand is sought. Bound phages are captured, while non-binders are eliminated by several washing steps. Due to the stability of phages, specifically bound phages can be eluted by acidic pH, denaturants (reducing agents) or ionic strength. Recovered phages are then amplified by infection of *E.coli* cells and undergo several - typically three to five - additional cycles of binding/amplification. This cyclic process of binding, washing, elution and amplification ultimately results in selective enrichment of high affinity binding sequences to the target. These enriched phage clones obtained from the last round are subjected to DNA sequencing in order to characterize and identify peptides with the highest affinity and specificity towards the target[39-41]. Because biopanning is a multistep selection procedure, very rare specific binders can be selected and amplified from a large background of phages with irrelevant binding property. One considerable benefit of phage display-based biopanning is that this screening modality provides various routes for the presentation of target to the library. This enables researchers to conveniently adapt screening protocol to the distinctive requirements of different targets. Target molecules can be immobilized onto a support such as coated tubes or plates, columns or magnetic beads and phage library is subsequently exposed to the immobilized target. Although being time-consuming, in this strategy only the target molecule is presented to the library. Targets can also be elements on the surface of cells either cultured in vitro or existent in different organs of the body of living animals. In this platform, target molecules are exposed in a (quasi)-natural position to the displayed library. But, the likelihood of binding of displayed peptides to many non-target molecules is an issue deserving further consideration[26,41].

Peptide ligands identified from phage libraries can be employed as leads in drug design and in vaccine development. Furthermore, some isolated peptides are bioactive and can inhibit the function of their target molecule (*e.g.,* enzyme inhibition). Therefore, these ligands represent potential to be used for various diagnostic and therapeutic applications. One of the most significant aspects of biopanning through phage libraries is searching for ligands - for example peptide ligands-specific to target molecules over-expressed on the surface of malignant cells. Peptides selected through phage libraries have been utilized for imaging, diagnosis, and treatment of a wide range of tumors such as glioblastoma, melanoma, leukemia, prostate and thyroid cancer[30,42-44]. These tumor-homing ligands are of great relevance for biomedical purposes and provide a means for selective delivery of various gene therapy vectors and chemotherapeutic drugs into neoplastic cells. Molecularly targeted delivery of anticancer agents enhances the therapeutic index and mini-

mizes the toxicity of these compounds.

BACTERIOPHAGE-BASED DIAGNOSTIC APPROACHES FOR HBV INFECTION

HBcAg, a major protein that forms the inner core of HBV virions, is regarded as one of most critical markers for detecting viral infection. The quantity of this protein demonstrates viral load as well as viral genomic DNA. HBcAg concentration in the serum correlates well with the amount of HBV genome implying its potential to be employed as a marker for viral load. Existence of anti-HBcAg antibodies in the serum can find utility as a specific serological marker for the diagnosis, monitoring of infected individuals, differentiation between acute and chronic forms of viral infection as well as epidemiological evaluations[45,46]. On the other hand, detection of antibodies generated against HBcAg is of particular importance in the identification of infected patients who are negative for HBsAg. Identification of this category of patients is crucial to impede HBV contamination of blood transfusion products[47].

Phage display has been used for surface display of HBcAg as a novel approach for the production of anti-HBcAg monoclonal antibodies. In this framework, HBcAg-displaying phage serves as an immunogen for immunization purposes and potential development of HBcAg-specific monoclonal antibodies. This bacteriophage platform also represents promise for the production of anti-HBV vaccines. Bahadir *et al*[48] applied phage display to express full-length HBcAg protein on the surface of M13 phage as fusion to minor coat protein. This recombinant phage was found to be largely immunogenic in BALB/c mice with antibody responses comparable with that of commercial HBcAg. In this work, pⅢ protein - with only three to five copies on M13 surface - was chosen as the fusion partner of HBcAg. This is due to the fact that HBcAg is approximately too large for proper display in full-length form on pⅧ protein.

Also, phages displaying surface ligands (such as peptides or antibodies) with tight binding ability to HBcAg can be used as diagnostic tools for the detection of HBV presence in biological samples. M13 bacteriophage bearing a surface peptide sequence that interacts tightly and selectively with HBcAg has been demonstrated to have the capability of detecting this viral antigen using phage-ELISA, phage-dot blot and immunoprecipitation assays[49]. This peptide was isolated *via* biopanning of a phage display cyclic peptide library and further analysis revealed that it can specifically bind to HBcAg but not to HBsAg and HBeAg (Hepatitis B e Antigen). HBeAg is the extracellular form of HBcAg[50]. In addition, this bacteriophage system represented the ability to detect HBcAg released from virions in HBV-positive serum samples. In another report, the aforementioned fusion phage (displaying a selective peptide towards HBcAg) was used to develop a TaqMan based real-time method as a diagnostic assay for HBV detection in positive serum samples[51]. This special strategy was formulated based on the idea of phage display-mediated immune-polymerase chain reaction (PD-IPCR) originally introduced by Guo *et al*[52] PD-IPCR had previously been shown to be a highly sensitive method for the detection of viruses such as Hantaan virus nucleocapsid protein. Generally speaking, detection of HBcAg in blood samples can be performed by radioimmunoassay, ELISA and enzyme immunoassay[45,46,53]. The necessity of using monoclonal or polyclonal antibodies in these assays renders the procedure of HBV detection challenging, laborious and time-consuming. Development of a bacteriophage-based diagnostic method - with the capacity of rapid propagation in bacterial cells within several hours -immensely facilitates detection of HBV infection. PD-IPCR hugely increases detection sensitivity (100 to 10, 1000 fold) compared to conventional phage-ELISA. This enormous rise in sensitivity arises from remarkable amplification power of PCR[54]. The results of this study suggest that PD-IPCR can be used as an alternative to phage-ELISA for HBcAg diagnosis.

BACTERIOPHAGE-BASED THERAPEUTIC APPROACHES FOR HBV INFECTION

Initial efforts for bacteriophage-mediated transduction of mammalian cells date back to several decades ago. At the outset, chemical transfection (by using DEAE -diethylaminoethyl - dextran and lipopolyamine) was applied to transduce cultured mammalian cells with both filamentous (single stranded DNA) and lambda (double stranded DNA) phages[55-57]. Later on, Hart *et al*[58] demonstrated that filamentous bacteriophages displaying the RGD (arginine-glycine-aspartic acid) tri-peptide sequence can be selectively targeted to and subsequently internalized by mammalian cells. Although there was not any report on the capacity of bacteriophages for gene expression in eukaryotic cells, this study suggested cellular internalization of these viral agents *via* expressing surface ligands and led to the speculation that bacteriophages can be exploited as tools for the delivery of foreign genetic material into specific mammalian cells. Larocca *et al*[59] provided the first proof of principle of bacteriophages as a targeted gene delivery vehicle. They showed that M13 filamentous bacteriophages bearing a CMV (cytomegalovirus)-controlled green fluorescent protein (GFP) gene and genetically displaying fibroblast growth factor (FGF2) can target COS-1 cells in a FGF2 receptor-mediated manner triggering the expression of the phage encoded reporter gene in mammalian cells.

Targeting of various gene carriers towards specific cells is one of the most complicated issues in gene therapy that dramatically reduces toxicity concerns. To achieve successful outcomes in gene therapy, it is necessary for the therapeutic transgene of interest to be exclusively expressed in target cells sparing normal non-targeted tissues. Larocca's pioneering study laid the groundwork for potential application of bacteriophages as a unique novel
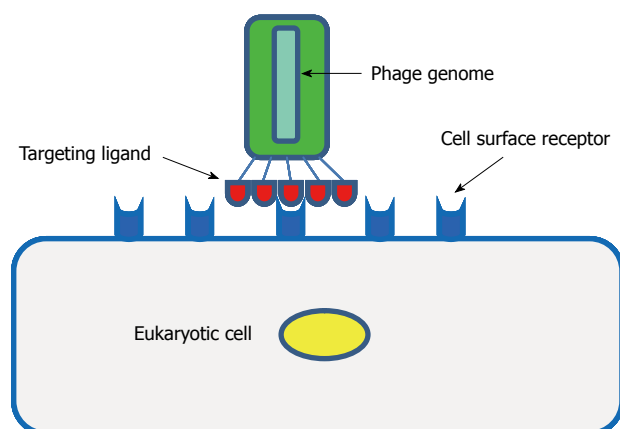
Figure 2 Cell-specific targeting of bacteriophages. Attachment of a ligand, with specific binding ability to a receptor on the surface of target cell, leads to targeted delivery of bacteriophage to the cell of interest. This ligand is fused to one of the phage coat proteins.

class of gene therapy vectors and provided strong support for the notion that bacteriophages, although having no tropism for mammalian cells, can be adapted for cell type-specific delivery of therapeutic genes into mammalian cells when properly targeted. This proper targeting can be made feasible through linking of a ligand -with binding capability to the desired cell surface receptor - to the surface of phage particles (Figure 2). Engagement of specialized cell surface receptors potentiates targeted bacteriophages to deliver their cargoes to cells through receptor-mediated endocytosis (RME)[60,61]. The use of RME potential presents a new perspective to the landscape of gene delivery. Within the past years, a variety of molecules including growth factors (basic fibroblast growth factor and epidermal growth factor), antibodies, and viral capsid proteins have been utilized for targeting bacteriophage vectors towards mammalian cells[62,63]. Phage display technology offers a powerful approach for targeted delivery of therapeutic cargoes. Bacteriophage-based surface display of peptides, poplypeptides or antibodies that show preferential binding to specific receptors on the surface of target cells, organs or tissues is a highly valuable modality in the discovery of new targeting methodologies. This strategy establishes new opportunities for the design of gene delivery vectors with improved targeting properties. Combination of disease-related receptors and their corresponding ligands forms a foundation based on which new systems for targeted delivery can be developed. In this regard, natural or artificial ligands with the capability of binding to receptors distinctively expressed on the surface of target cells might be used to direct therapeutics towards sites of interest.

Hepatitis B virus makes interactions with the surface of liver cells resulting in virus binding to the cell surface. This surface binding allows subsequent internalization of viral particles into hepatocytes. Hepatitis B virus uses some surface molecules- peptides or proteins- for interacting with hepatocytes. As previously noted, PreS1 is one of the most crucial elements underlying the attach-

ment of HBV particles to hepatocytes. One potential means for bacteriophage-based targeted delivery into liver cells - as the host cell of hepatitis B virus- is display of surface components of HBV that are necessary for binding to hepatocytes. This approach makes it possible to exploit phage display technique for the development of targeted platforms in order to treat HBV infection. Tang *et al*[64] took advantage of this strategy to develop a bacteriophage vehicle with potential application in human gene transfer and gene therapy. They fused several polypeptides of the PreS1 region of HBV in frame to the C-terminus of 10B capsid protein of phage T7. Their experiments demonstrated the efficacy of PreS1$_{60-108}$ polypeptide in transfection of HepG2 (Hepatoblastoma G2) cells. Also they found that higher phage concentrations and longer incubation periods trigger higher transfection efficiency highlighting the fact that phage internalization through displayed polypeptide of the PreS1 region occurs in a dose- and time-dependent manner. This study suggests that PreS1$_{60-108}$ region can be used as a targeting ligand to confer hepatocytic tropism to bacteriophages or any other gene and drug delivery vehicle. These targeted delivery vehicles offer a means for the delivery of therapeutic cargoes into liver cells thus providing a platform for the treatment of HBV infection, liver cancer and other hepatocyte-related disorders.

Another framework represented by phage display for the treatment of HBV infection is the utilization of random phage display libraries - both peptide and antibody libraries - in order to obtain ligands with selective binding to surface molecules of hepatitis B virus. These HBV-binding ligands might prove useful in targeted delivery of various carriers into HBV-infected haptocytes and also as antiviral drugs against HBV infection. Wang *et al*[65] screened a commercial phage display dodecapeptide library against purified PreS1 protein of HBV. This library contained peptide variants as fusion to minor coat protein (pⅢ) of M13 phage. They identified several distinct PreS1 binding peptides among which one peptide was the most enriched with the tightest ability for binding to the target protein. Further analysis revealed that this peptide strongly decreased the attachment of HBV virions to the PreS1 antibody in a dose-dependent manner exhibiting its capability in effective blocking of the relevant epitope of PreS1 protein. Peptide ligands or motifs obtained from phage display libraries with the merit of recognizing and blocking the surface molecules of HBV offer promise to be a candidate for the treatment of HBV infection. As a result, they can be utilized for the design of novel drugs against HBV. In another study, Deng *et al*[66] reported screening of a random peptide library against the PreS region of HBV as target. In contrast to the previous case, in this work peptides were displayed as fusion to major coat protein (pⅧ) of M13 phage. On the other hand, peptides of this library were structurally constrained in which random peptides were enclosed in a loop flanked by a pair of cysteine residues. Five rounds of biopanning gave rise to the enrichment and selection of PreS-binding

constrained peptide ligands.

Antibodies with the ability of being internalized into HBV-infected cells hold enormous potential for the development of treatment methods against hepatitis B infection. Park *et al*[67] constructed a large nonimmunized/naive human antibody phage library in scFv (single-chain variable fragment) format. Peripheral blood mononuclear cells (PBMC) of nonimmunized healthy donors were used as a source of lymphoid tissue. Subsequent to screening of this phage library, two functional anti-PreS1 scFvs were obtained. Survey of biological activity of the isolated scFvs established that they can recognize PreS1 and neutralize the binding of PreS1 protein and HBV virions to a human hepatoma cell line. As interaction between PreS1 (on the surface of HBV) and hepatocyte is vital for HBV infection, inhibition of this interaction *via* the identified scFvs may be promising in immunoprophylaxis and immunotherapy against HBV infection. Furthermore, this type of phage library can be an advantageous and applicable source of antibodies to any viral or malignant cell target. Wen *et al*[68] exploited screening of a natural immune antigen binding fragment (Fab) antibody phage library against HBsAg. This library had been constructed from the lymphocytes of a volunteer immunized with HBsAg. A number of Fab fragments with considerable ability for binding to the target antigen were identified following several rounds of biopanning. Subsequently, these Fab fragments were reconstructed into scFvs for further analysis. This reconstruction of display format of the isolated antibodies was due to the fact that scFvs represent interesting features including low molecular weight and convenient permeability while retaining binding capacity. All scFvs maintained a high affinity against HBsAg on the membrane of HBV-infected cells. Further analysis revealed that one of scFvs is heavily internalized into HBsAg-positive HepG2.2.15 cells. On the other hand, as transferrin is a marker for clathrin-mediated endocytosis, colocalization of this scFv with tranferrin suggests the possible role of clathrin-mediated endocytic pathway in the trafficking of scFv into infected cells.

It is interesting to note that phage display technology presents the possibility for affinity maturation of HBV-binding ligands. In this context, phage display can find utility as a tool for the production of a mutant antibody library with the intention of augmenting affinity of an antibody. Yang *et al*[69] made use of phage display library to enhance the affinity of an anti-PreS1 antibody with HBV-neutralizing activity in chimpanzees but inadequate affinity for clinical application in human. They generated HCDR3 (heavy chain complementarity determining region)-randomized library in scFv-phage display format. Biopanning of phage-displayed library against the PreS1 antigen resulted in selecting several affinity-matured scFv variants of the original anti-PreS1 antibody. These antibody mutants, with higher affinity towards target antigen, present improved potency in neutralizing HBV infection in comparison with wild type antibody thereby providing more efficacious tools for anti-HBV immunotherapy.

BACTERIOPHAGE-BASED VACCINE DEVELOPMENT FOR HBV

Many vaccines are designed based on recombinant viral and bacterial carriers. One major complication of these vaccines in translation to the clinic is their ability to generate strong immune responses that result from previous exposure of human cells to these bacterial and viral agents[70]. The use of bacteriophages as viruses whose naturally occurring hosts are non-human prokaryotic cells can bring new opportunities to the field of immunotherapy and vaccine development. Bacteriophages have recently been introduced as efficient vehicles for vaccine delivery. Bacteriophage-carried antigens have the capacity to trigger humoral and cell-mediated immune responses. Various bacteriophages including filamentous phage, lambda phage, T4 and T7 can be exploited for vaccination purposes[71].

Bacteriophages represent some advantages for the development of vaccine delivery systems. Bacteriophage-based antigen delivery platforms are low-cost and stable under relatively extreme environmental conditions. They can be readily and simply produced in large quantities because of their exponential multiplication on a simple bacterial host. These vaccine vehicles offer an approximately large cloning capacity; for example up to 20 kb for lambda-phage-based vaccines[71-73]. This provides potential for the delivery of multiple different constructs (different vaccines or multiple copies of the same vaccine) by a single phage particle, inclusion of adjuvant (cytokine and chemokine) genes on the phage vaccine, and also cloning of large intron-harboring genes of eukaryotes. The latter property is of considerable significance to tailor bacteriophage vaccines for eukaryotic parasites such as *Plasmodium falciparum* (causal pathogen of malaria) or *Trypanosoma brucei* (causal pathogen of trypanosomiasis); disease-causing agents with complicated life cycles or antigen switching activity[73]. In contrast to vaccines based on eukaryotic viruses, bacteriophages are lacking ability to propagate in eukaryotic cells that excludes the possibility of vaccine replication in human cells. With the birth of phage therapy concept in the initial decades of the 20th century, there have been a multitude of studies - particularly in Eastern Europe countries - in which these prokaryotic viruses were safely utilized for the treatment of bacterial infections[74,75]. This implies the fact that there is a long tradition of safe application of bacteriophages in human for clinical purposes.

There are two main strategies for the exploitation of bacteriophages as vaccine delivery platforms: phage DNA vaccines and phage display vaccines[76]. In phage DNA vaccines, the sequences necessary for the synthesis of vaccine antigen under the control of an appropriate eukaryotic expression cassette are inserted into the phage genome. Following DNA packaging in vitro and propagation of phage virions on the bacterial cells, the

whole recombinant phage particles are used as a delivery carrier for DNA vaccination and immunization of the host. When introduced into APCs (antigen presenting cells) of the host immune system, the phage protein coat is removed and the vaccine-encoding DNA is expressed. Compared with standard plasmid DNA vaccination, phage DNA vaccines have been indicated to mount enhanced antibody responses in mice and rabbits[10]. March *et al*[72] used lambda-phage DNA vaccine containing HBsAg (λ-HBsAg) for immunization of rabbits and mice. Compared with those vaccinated with equivalent plasmid construct encoding HBsAg, λ-HBsAg-immunized animals presented a higher titer of anti-HBsAg antibodies and this antibody response did not show any sign of reduction more than six months post-immunization. Clark *et al*[77] incorporated an expression cassette containing HBsAg of hepatitis B virus into the genome of lambda phage and used this phage-based vaccine for vaccination against HBV in rabbits. Furthermore, they compared the immunization capacity of λHBsAg with a commercially available protein vaccine, EngerixB, that is made up of HBsAg recombinant protein. They demonstrated that the phage construct in comparison with Engerix B protein vaccine is able to produce significantly higher antibody responses. This difference can be ascribed in part to the adjuvant effects of lambda phage particles. Immunostimulatory effects of bacteriophage particles have been previously shown in several studies[74,78]. In another study, Clark and March intramuscularly injected mice with λ-gt11 phages harboring HBsAg of hepatitis B virus[71]. Mice groups vaccinated with λ-HBsAg were found to yield elevated anti-HBsAg responses in comparison with unmodified λ-gt11 phage or HBsAg-containing plasmid (naked DNA). Additionally, a potent antibody response was observed against the coat protein of the carrier phage lambda. This phenomenon leads to the formation of immune complexes that play a critical role in effective targeting of antigen presenting cells thereby boosting efficiency of the delivery system. Although this finding reflects momentousness of initial priming of the immune system against the phage carrier, investigation of the impact of anti-phage response on the efficacy of the delivery system remains to be elucidated.

In phage display vaccines, bacteriophages are engineered in order to display a specific peptide or protein with antigenic property on their surface. A number of studies have revealed the potential of phage displayed antigens for the development of vaccines against pathogens such as hepatitis C virus[79], HIV-1 (Human Immunodeficiency Virus-1)[80], *Plasmodium falciparum*[81], *Neisseria meningitidis*[82], as well as diseases including Alzheimer[83] and cancer[84] in animal models. These reports have successfully exhibited the capability of these vaccines in the activation of immune responses. In this regard, a vaccine against *Taenia solium* pig cysticercosis is known as the first vaccine developed through phage display for a large animal like pig[85]. Tan *et al*[86] displayed major antigenic region of HBV, the immunodominant region (amino acids 111-156) of S-HBsAg, on the surface of T7 phage as fusion to the C-terminal end of 10B capsid protein. This region has proven to be capable of eliciting protective antibodies. T7 phage-HBsAg construct was indicated to generate desirable antigenic responses with the guinea pig anti-HBsAg antibody and human anti-HBsAg serum in ELISA tests. This observation implies the merit of this phage construct for the detection of antibodies produced against HBsAg in infected individuals. Moreover, the sera purified from rabbits immunized with T7 phage-HBsAg exhibited high levels of anti-HBsAg antibodies suggesting the potential of these phage particles as candidates for vaccine production. The results were in support of antigenic and immunogenic characteristics of the immunodominant region of HBsAg displayed on T7 phage. This antigenicity and immunogenicity represent the potential of T7 engineered phage particles to be employed as immunological reagents in detection assays for HBV infection also as immunogens for the development of economical vaccine platforms.

In the aforementioned reports, the capacity of bacteriophage-based vaccines in inducing humoral immunity was brought into focus. However, specific cellular immune responses also play substantial roles in generating anti-virus immunity thereby taking a special place in the establishment of bacteriophage-based vaccines against HBV infection. Keeping this in mind, it is of particular importance to explore whether vaccines developed based on bacteriophage particles can stimulate T cell responses especially antigen-specific cytotoxic T cell (CTL) responses. Wan *et al*[87] displayed a MHC (major histocompatibility complex) class I molecule (H-2d)-binding peptide called HBs28-39, an epitope generated by exogenous processing of hepatitis B virus surface anigen, on the surface of filamentous phage particles (as fusion to the pVIII coat protein) and injected the constructed phage into BALB/c mice without adjuvants. Several days following injection of low doses of phage particles into mice, a specific CTL response for MHC class I-restricted HBs28-39 epitope was found. This study suggested that filamentous phage particles can be processed by macrophages for presentation by MHC-I molecules. In another report, Manoutcharian *et al*[88] grafted a T-cell epitope that was predicted through analysis of the *Taenia crassiceps* proline-rich protective antigen KETc7 into immunoglobulin heavy-chain CDRs and displayed it on the surface of filamentous bacteriophage particles. The CD4+ and CD8+ T cells isolated from mice immunized with this phage display vaccine stimulated the generation of interferon gamma (INF-γ). The results indicated that phage particles can be efficiently processed and presented by MHC class I molecules eliciting strong CTL responses that yield ultimate resistance to challenge by infection. In the study conducted by Hashemi *et al*[89] recombinant filamentous phage particles containing the expression cassette of HSV-1 (Herpes Simplex Virus 1) glycoprotein D were used for vaccination of mice. Glycoprotein D is necessary for viral entry, mediates fusion of membranes following viral attachment

and is considered as an important target for the immune system[90]. After inoculation of mice with different titers of phage particles, both humoral and cell-mediated immune responses were measured in the immunized mice. In this work, antigen specific cellular response was evaluated through ELISA measurement of secreted granzyme B in the supernatant of effector-target cells co-culture. Granzyme B, a serine protease, is regarded as one of the most significant cytotoxic mediators exocytosed by CTLs upon specific recognition of antigens presented by self cell surface MHC class I molecules and triggers granule-mediated apoptosis through cleavage of different cellular substrates. The results of the study exhibited that there is a dose-response relationship in both antiviral neutralizing antibody and specific cellular immune responses. Therefore, phage virions displaying a fusion antigenic peptide can be taken up and subsequently processed by both MHC class I and class II in antigen presenting cells and in this way stimulate the immune response to the phage-displayed epitope. In another work, it was revealed that fd virions that displayed peptide RT2 (corresponding to residues 309 to 317 of the reverse transcriptase of HIV-1) harbor the capability of priming a CTL response specific to RT2 epitope in human cell lines[91]. A T-helper epitope (pep23 corresponding to residues 249 to 263 of the reverse transcriptase of HIV-1) is required for successful priming of specific CTL response against RT2 epitope. This results in the activation of antigen specific CD4^{+} T cells. Furthermore, HLA-A2 transgenic mice that were immunized with bacteriophages displaying RT2 peptide mounted an efficient and specific anti-HIV-RT2 CTL response.

These studies reflect the fact that bacteriophages offer potential to elicit T cell-mediated immunity in particular antigen specific cytotoxic T cell responses. The ability of bacteriophages for inducing cellular immunity, together with humoral immune response, can have significant implications for the development of bacteriophage-based vaccine platforms.

In addition to phage DNA and phage display vaccines previously mentioned, phage display methodology can also be used in another manner for vaccine development. In this approach, a specific antiserum or the serum of convalescent individuals is employed as a means for screening phage peptide libraries to recognize new protective antigens or mimotopes - peptides mimicking secondary structure and antigenic characteristics of a protective protein, carbohydrate or lipid, although bearing a different primary structure - as novel potential vaccines against a certain disease[92-94].

Another practical framework for the use of bacteriophages in vaccine design, inspired by combining phage DNA and phage display vaccines, is development of hybrid phage vaccines. This more recently established methodology, as its name represents, takes advantage of both previously mentioned approaches of phage DNA and phage display vaccines. In this strategy of vaccine development, a DNA vaccine is inserted into the phage genome, while a phage display variant of the antigen encoded by the incorporated DNA vaccine is expressed on the phage surface. This vaccine type exhibits a more considerable efficacy in targeting of both humoral and cellular components of the immune system[10,76].

Targeting of bacteriophages towards APCs is another important issue which is worthy of consideration in the development of bacteriophage-based vaccines. Surface modification of bacteriophage vaccines through specific molecules can be used as a means for preferential targeting of these vaccines towards certain types of immune cells such as APCs. With regard to targeting of bacteriophage vaccines towards cells of the immune system, dendritic cells (DCs) as the most important category of professional APCs have received considerable attention. DCs make essential contribution to the initiation of the immune response. These specialized immune cells are able to sample antigens from their surrounding environment, process the antigens that have entered the endocytic pathway, and ultimately present the processed antigens as immunostimulatory peptides to T lymphocytes in the context of MHC antigens. DCs, in addition to playing roles in priming naive T cells, are also involved in the growth and secretion of immunoglobulin molecules by B lymphocytes. As a consequence of these diverse functions, DCs support a major role in battle with viral infections and other pathogenic conditions through inducing both humoral and cellular immunity[95]. Previous studies have demonstrated the potential of DCs as effective vaccines in human clinical trials[96].

Currently, the lack of DC-specific molecules has made the design of DC-targeting strategies challenging. Great efforts have been made to develop optimal means for the delivery of immunogenic antigens to DCs. Targeting of molecules to DCs is considered a potentially helpful vaccination strategy in eliciting anti-virus immune responses. Undoubtedly, the efficacy of bacteriophage-based antigen delivery systems can be improved through targeting of bacteriophage particles to DCs. It has been demonstrated that phage peptide libraries can be exploited to identify ligands with specific binding ability to DCs. These peptides make it possible to specifically guide immunogenic antigens to DCs through molecules expressed exclusively on the surface of these immune cells. In line with this, Curiel *et al*[97] screened a PhD.12-mer peptide phage display library to obtain human DC-homing peptides. The isolated DC-binding peptides were subsequently fused to the NS3 (nonstructural protein3) of hepatitis C virus - as a model immunogen and as an antigen proposed for HCV vaccines - to facilitate DC capture and presentation. This genetic fusion also preserved the selectivity of DC targeting and antigen immunogenicity. The NS3-DC-peptide fusion was efficiently presented to CD4^{+} and CD8^{+} T cells obtained from HCV-positive blood cells and induced antigen specific activation and proliferation of T lymphocytes. In chimeric NOD-SCID (Nonobese Diabetic/Severe Combined Immunodeficiency) mice transplanted with human cells, NS3-DC-peptide fusion

was able to prime naive CD4$^+$ and CD8$^+$ T cells for potent antigen specific proliferation and cytokine secretion. The capacity of DC-targeting peptides isolated from phage display libraries to direct immunogenic antigens to dendritic cells may present a novel platform for vaccine development. In another report, selective targeting ability of the above mentioned DC-targeting peptide was used to deliver a therapeutic siRNA into DCs. In this study, DC-specific 12-mer peptide was fused to nona-D-arginines (9dR) to selectively direct siRNA - specific to an envelope sequence of dengue virus - to DCs[98]. Dendritic cells and macrophages are the major in vivo targets of dengue virus being regarded as the predominant infected cell types. The results of the study indicated the potential of this DC-targeting approach for targeted delivery of specific therapeutic siRNA to dendritic cells. This effectively suppressed dengue virus replication leading to simultaneous decrease of viral load and aberrant cytokine responses in DCs. In another study, a semi-synthetic scFv antibody phage display library in combination with fluorescence-activated cell sorting was employed to isolate antibodies that bind to subpopulations of DCs present in human peripheral blood[99]. These DC-targeting scFv antibody fragments can be genetically fused to antigens or chemically coupled to nucleic acids thereby providing an immunotherapeutic means for targeted delivery of vaccines to DCs.

REFERENCES

1   **Wright TL**. Introduction to chronic hepatitis B infection. *Am J Gastroenterol* 2006; **101** Suppl 1: S1-S6 [PMID: 16448446 DOI: 10.1111/j.1572-0241.2006.00469.x]

2   **Petersen J**, Dandri M, Mier W, Lütgehetmann M, Volz T, von Weizsäcker F, Haberkorn U, Fischer L, Pollok JM, Erbes B, Seitz S, Urban S. Prevention of hepatitis B virus infection in vivo by entry inhibitors derived from the large envelope protein. *Nat Biotechnol* 2008; **26**: 335-341 [PMID: 18297057 DOI: 10.1038/nbt1389]

3   **Tan WS**, Tan GH, Yusoff K, Seow HF. A phage-displayed cyclic peptide that interacts tightly with the immunodominant region of hepatitis B surface antigen. *J Clin Virol* 2005; **34**: 35-41 [PMID: 16087122 DOI: 10.1016/j.jcv.2005.01.007]

4   **Xie Y**, Zhai J, Deng Q, Tiollais P, Wang Y, Zhao M. Entry of hepatitis B virus: mechanism and new therapeutic target. *Pathol Biol* (Paris) 2010; **58**: 301-307 [PMID: 20570056 DOI: 10.1016/j.patbio.2010.04.001]

5   **Shepard CW**, Simard EP, Finelli L, Fiore AE, Bell BP. Hepatitis B virus infection: epidemiology and vaccination. *Epidemiol Rev* 2006; **28**: 112-125 [PMID: 16754644 DOI: 10.1093/epirev/mxj009]

6   **Ganem D**. Assembly of hepadnaviral virions and subviral particles. *Curr Top Microbiol Immunol* 1991; **168**: 61-83 [PMID: 1893779 DOI: 10.1007/978-3-642-76015-0_4]

7   **Dabrowska K**, Switała-Jelen K, Opolski A, Weber-Dabrowska B, Gorski A. Bacteriophage penetration in vertebrates. *J Appl Microbiol* 2005; **98**: 7-13 [PMID: 15610412 DOI: 10.1111/j.1365-2672.2004.02422.x]

8   **Solomon B**. Filamentous bacteriophage as a novel therapeutic tool for Alzheimer's disease treatment. *J Alzheimers Dis* 2008; **15**: 193-198 [PMID: 18953108]

9   **Hambly E**, Suttle CA. The viriosphere, diversity, and genetic exchange within phage communities. *Curr Opin Microbiol* 2005; **8**: 444-450 [PMID: 15979387 DOI: 10.1016/j.mib.2005.06.005]

10 **Clark JR**, March JB. Bacteriophages and biotechnology: vaccines, gene therapy and antibacterials. *Trends Biotechnol* 2006; **24**: 212-218 [PMID: 16567009 DOI: 10.1016/j.tibtech.2006.03.003]

11 **Smith GP**. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* 1985; **228**: 1315-1317 [PMID: 4001944 DOI: 10.1126/science.4001944]

12 **Paschke M**. Phage display systems and their applications. *Appl Microbiol Biotechnol* 2006; **70**: 2-11 [PMID: 16365766 DOI: 10.1007/s00253-005-0270-9]

13 **Pande J**, Szewczyk MM, Grover AK. Phage display: concept, innovations, applications and future. *Biotechnol Adv* 2010; **28**: 849-858 [PMID: 20659548 DOI: 10.1016/j.biotechadv.2010.07.004]

14 **Barbas CF**, Kang AS, Lerner RA, Benkovic SJ. Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc Natl Acad Sci USA* 1991; **88**: 7978-7982 [PMID: 1896445 DOI: 10.1073/pnas.88.18.7978]

15 **Burritt JB**, Bond CW, Doss KW, Jesaitis AJ. Filamentous phage display of oligopeptide libraries. *Anal Biochem* 1996; **238**: 1-13 [PMID: 8660577 DOI: 10.1006/abio.1996.0241]

16 **Winter G**, Griffiths AD, Hawkins RE, Hoogenboom HR. Making antibodies by phage display technology. *Annu Rev Immunol* 1994; **12**: 433-455 [PMID: 8011287 DOI: 10.1146/annurev.iy.12.040194.002245]

17 **Tohidkia MR**, Barar J, Asadi F, Omidi Y. Molecular considerations for development of phage antibody libraries. *J Drug Target* 2012; **20**: 195-208 [PMID: 21950316 DOI: 10.3109/1061186X.2011.611517]

18 **Krumpe LR**, Mori T. The Use of Phage-Displayed Peptide Libraries to Develop Tumor-Targeting Drugs. *Int J Pept Res Ther* 2006; **12**: 79-91 [PMID: 19444323 DOI: 10.1007/s10989-005-9002-3]

19 **Hertveldt K**, Beliën T, Volckaert G. General M13 phage display: M13 phage display in identification and characterization of protein-protein interactions. *Methods Mol Biol* 2009; **502**: 321-339 [PMID: 19082565 DOI: 10.1007/978-1-60327-565-1_19]

20 **Manoutcharian K**, Gevorkian G, Cano A, Almagro JC. Phage displayed biomolecules as preventive and therapeutic agents. *Curr Pharm Biotechnol* 2001; **2**: 217-223 [PMID: 11530876 DOI: 10.2174/1389201013378671]

21 **Sergeeva A**, Kolonin MG, Molldrem JJ, Pasqualini R, Arap W. Display technologies: application for the discovery of drug and gene delivery agents. *Adv Drug Deliv Rev* 2006; **58**: 1622-1654 [PMID: 17123658 DOI: 10.1016/j.addr.2006.09.018]

22 **Mao C**, Liu A, Cao B. Virus-based chemical and biological sensing. *Angew Chem Int Ed Engl* 2009; **48**: 6790-6810 [PMID: 19662666 DOI: 10.1002/anie.200900231]

23 **Ahmadvand D**, Rahbarizadeh F, Moghimi SM. Biological targeting and innovative therapeutic interventions with phage-displayed peptides and structured nucleic acids (aptamers). *Curr Opin Biotechnol* 2011; **22**: 832-838 [PMID: 21420292 DOI: 10.1016/j.copbio.2011.02.012]

24 **Li K**, Nguyen HG, Lu X, Wang Q. Viruses and their potential in bioimaging and biosensing applications. *Analyst* 2010; **135**: 21-27 [PMID: 20024176 DOI: 10.1039/b911883g]

25 **Castel G**, Chtéoui M, Heyd B, Tordo N. Phage display of combinatorial peptide libraries: application to antiviral research. *Molecules* 2011; **16**: 3499-3518 [PMID: 21522083 DOI: 10.3390/molecules16053499]

26 **Willats WG**. Phage display: practicalities and prospects. *Plant Mol Biol* 2002; **50**: 837-854 [PMID: 12516857]

27 **Prisco A**, De Berardinis P. Filamentous bacteriophage fd as an antigen delivery system in vaccination. *Int J Mol Sci* 2012; **13**: 5179-5194 [PMID: 22606037 DOI: 10.3390/ijms13045179]

28 **Hamzeh-Mivehroud M**, Alizadeh AA, Morris MB, Church WB, Dastmalchi S. Phage display as a technology deliver-

ing on the promise of peptide drug discovery. *Drug Discov Today* 2013; **18**: 1144-1157 [PMID: 24051398 DOI: 10.1016/j.drudis.2013.09.001]

29   **Kehoe JW**, Kay BK. Filamentous phage display in the new millennium. *Chem Rev* 2005; **105**: 4056-4072 [PMID: 16277371 DOI: 10.1021/cr000261r]

30   **Landon LA**, Deutscher SL. Combinatorial discovery of tumor targeting peptides using phage display. *J Cell Biochem* 2003; **90**: 509-517 [PMID: 14523985 DOI: 10.1002/jcb.10634]

31   **Houghten RA**, Pinilla C, Blondelle SE, Appel JR, Dooley CT, Cuervo JH. Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery. *Nature* 1991; **354**: 84-86 [PMID: 1719428 DOI: 10.1038/354084a0]

32   **Laakkonen P**, Vuorinen K. Homing peptides as targeted delivery vehicles. *Integr Biol* (Camb) 2010; **2**: 326-337 [PMID: 20657951]

33   **Christianson DR**, Ozawa MG, Pasqualini R, Arap W. Techniques to decipher molecular diversity by phage display. *Methods Mol Biol* 2007; **357**: 385-406 [PMID: 17172704]

34   **Shukla GS**, Krag DN. Phage display selection for cell-specific ligands: development of a screening procedure suitable for small tumor specimens. *J Drug Target* 2005; **13**: 7-18 [PMID: 15848950 DOI: 10.1080/10611860400020464]

35   **Staquicini FI**, Sidman RL, Arap W, Pasqualini R. Phage display technology for stem cell delivery and systemic therapy. *Adv Drug Deliv Rev* 2010; **62**: 1213-1216 [PMID: 20932865 DOI: 10.1016/j.addr.2010.09.014]

36   **Barry MA**, Dower WJ, Johnston SA. Toward cell-targeting gene therapy vectors: selection of cell-binding peptides from random peptide-presenting phage libraries. *Nat Med* 1996; **2**: 299-305 [PMID: 8612228 DOI: 10.1038/nm0396-299]

37   **Kolonin MG**, Sun J, Do KA, Vidal CI, Ji Y, Baggerly KA, Pasqualini R, Arap W. Synchronous selection of homing peptides for multiple tissues by in vivo phage display. *FASEB J* 2006; **20**: 979-981 [PMID: 16581960 DOI: 10.1096/fj.05-5186fje]

38   **Cortese R**, Monaci P, Luzzago A, Santini C, Bartoli F, Cortese I, Fortugno P, Galfrè G, Nicosia A, Felici F. Selection of biologically active peptides by phage display of random peptide libraries. *Curr Opin Biotechnol* 1996; **7**: 616-621 [PMID: 8939640 DOI: 10.1016/S0958-1669(96)80072-3]

39   **Zhao S**, Zhao W, Ma L. Novel peptide ligands that bind specifically to mouse embryonic stem cells. *Peptides* 2010; **31**: 2027-2034 [PMID: 20713104 DOI: 10.1016/j.peptides.2010.08.004]

40   **Derda R**, Tang SK, Li SC, Ng S, Matochko W, Jafari MR. Diversity of phage-displayed libraries of peptides during panning and amplification. *Molecules* 2011; **16**: 1776-1803 [PMID: 21339712 DOI: 10.3390/molecules16021776]

41   **Marr A**, Markert A, Altmann A, Askoxylakis V, Haberkorn U. Biotechnology techniques for the development of new tumor specific peptides. *Methods* 2011; **55**: 215-222 [PMID: 21640826 DOI: 10.1016/j.ymeth.2011.05.002]

42   **Newton J**, Deutscher SL. Phage peptide display. *Handb Exp Pharmacol* 2008; **(185 Pt 2)**: 145-163 [PMID: 18626602 DOI: 10.1007/978-3-540-77496-9_7]

43   **Sidhu SS**. Phage display in pharmaceutical biotechnology. *Curr Opin Biotechnol* 2000; **11**: 610-616 [PMID: 11102798 DOI: 10.1016/S0958-1669(00)00152-X]

44   **Deutscher SL**. Phage display in molecular imaging and diagnosis of cancer. *Chem Rev* 2010; **110**: 3196-3211 [PMID: 20170129 DOI: 10.1021/cr900317f]

45   **Kimura T**, Rokuhara A, Matsumoto A, Yagi S, Tanaka E, Kiyosawa K, Maki N. New enzyme immunoassay for detection of hepatitis B virus core antigen (HBcAg) and relation between levels of HBcAg and HBV DNA. *J Clin Microbiol* 2003; **41**: 1901-1906 [PMID: 12734224 DOI: 10.1128/JCM.41.5.1901-1906.2003]

46   **Bredehorst R**, von Wulffen H, Granato C. Quantitation of hepatitis B virus (HBV) core antigen in serum in the presence of antibodies to HBV core antigen: comparison with assays

of serum HBV DNA, DNA polymerase, and HBV e antigen. *J Clin Microbiol* 1985; **21**: 593-598 [PMID: 3988901]

47   **Katchaki JN**, Siem TH, Brouwer R, Brandt KH, van der Waart M. Detection and significance of anti-HBc in the blood bank; preliminary results of a controlled prospective study. *J Virol Methods* 1980; **2**: 119-125 [PMID: 7228974 DOI: 10.1016/0166-0934(80)90045-2]

48   **Bahadir AO**, Balcioglu BK, Uzyol KS, Hatipoglu I, Sogut I, Basalp A, Erdag B. Phage displayed HBV core antigen with immunogenic activity. *Appl Biochem Biotechnol* 2011; **165**: 1437-1447 [PMID: 21915589 DOI: 10.1007/s12010-011-9365-1]

49   **Hasmoni SS**, Yusoff K, Tan WS. Detection and precipitation of hepatitis B core antigen using a fusion bacteriophage. *J Gen Appl Microbiol* 2005; **51**: 125-131 [PMID: 15942873 DOI: 10.2323/jgam.51.125]]

50   **Ho KL**, Yusoff K, Seow HF, Tan WS. Selection of high affinity ligands to hepatitis B core antigen from a phage-displayed cyclic peptide library. *J Med Virol* 2003; **69**: 27-32 [PMID: 12436474 DOI: 10.1002/jmv.10266]

51   **Monjezi R**, Tan SW, Tey BT, Sieo CC, Tan WS. Detection of hepatitis B virus core antigen by phage display mediated TaqMan real-time immuno-PCR. *J Virol Methods* 2013; **187**: 121-126 [PMID: 23022731 DOI: 10.1016/j.jviromet.2012.09.017]

52   **Guo YC**, Zhou YF, Zhang XE, Zhang ZP, Qiao YM, Bi LJ, Wen JK, Liang MF, Zhang JB. Phage display mediated immuno-PCR. *Nucleic Acids Res* 2006; **34**: e62 [PMID: 16682441 DOI: 10.1093/nar/gkl260]

53   **Usuda S**, Okamoto H, Tsuda F, Tanaka T, Miyakawa Y, Mayumi M. An enzyme-linked immunosorbent assay with monoclonal antibodies for the determination of phosphorylated hepatitis B core protein (p21c) in serum. *J Virol Methods* 1998; **72**: 95-103 [PMID: 9672136 DOI: 10.1016/S0166-0934(98)00019-6]

54   **Niemeyer CM**, Adler M, Wacker R. Immuno-PCR: high sensitivity detection of proteins by nucleic acid amplification. *Trends Biotechnol* 2005; **23**: 208-216 [PMID: 15780713 DOI: 10.1016/j.tibtech.2005.02.006]

55   **Yokoyama-Kobayashi M**, Kato S. Recombinant f1 phage-mediated transfection of mammalian cells using lipopolyamine technique. *Anal Biochem* 1994; **223**: 130-134 [PMID: 7695088 DOI: 10.1006/abio.1994.1557]

56   **Yokoyama-Kobayashi M**, Kato S. Recombinant f1 phage particles can transfect monkey COS-7 cells by DEAE dextran method. *Biochem Biophys Res Commun* 1993; **192**: 935-939 [PMID: 8484795 DOI: 10.1006/bbrc.1993.1505]

57   **Okayama H**, Berg P. Bacteriophage lambda vector for transducing a cDNA clone library into mammalian cells. *Mol Cell Biol* 1985; **5**: 1136-1142 [PMID: 3158804]

58   **Hart SL**, Knight AM, Harbottle RP, Mistry A, Hunger HD, Cutler DF, Williamson R, Coutelle C. Cell binding and internalization by filamentous phage displaying a cyclic Arg-Gly-Asp-containing peptide. *J Biol Chem* 1994; **269**: 12468-12474 [PMID: 8175653]

59   **Larocca D**, Kassner PD, Witte A, Ladner RC, Pierce GF, Baird A. Gene transfer to mammalian cells using genetically targeted filamentous bacteriophage. *FASEB J* 1999; **13**: 727-734 [PMID: 10094933]

60   **Larocca D**, Burg MA, Jensen-Pergakes K, Ravey EP, Gonzalez AM, Baird A. Evolving phage vectors for cell targeted gene delivery. *Curr Pharm Biotechnol* 2002; **3**: 45-57 [PMID: 11883506]

61   **Larocca D**, Baird A. Receptor-mediated gene transfer by phage-display vectors: applications in functional genomics and gene therapy. *Drug Discov Today* 2001; **6**: 793-801 [PMID: 11470588 DOI: 10.1016/S1359-6446(01)01837-2]

62   **Di Giovine M**, Salone B, Martina Y, Amati V, Zambruno G, Cundari E, Failla CM, Saggio I. Binding properties, cell delivery, and gene transfer of adenoviral penton base displaying bacteriophage. *Virology* 2001; **282**: 102-112 [PMID:

11259194 DOI: 10.1006/viro.2000.0809]

63   **Poul MA**, Marks JD. Targeted gene delivery to mammalian cells by filamentous bacteriophage. *J Mol Biol* 1999; **288**: 203-211 [PMID: 10329137 DOI: 10.1006/jmbi.1999.2678]

64   **Tang KH**, Yusoff K, Tan WS. Display of hepatitis B virus PreS1 peptide on bacteriophage T7 and its potential in gene delivery into HepG2 cells. *J Virol Methods* 2009; **159**: 194-199 [PMID: 19490973 DOI: 10.1016/j.jviromet.2009.03.015]

65   **Wang W**, Liu Y, Zu X, Jin R, Xiao G. Blocking peptides against HBV: preS1 protein selected from a phage display library. *Biochem Biophys Res Commun* 2011; **412**: 633-637 [PMID: 21856287 DOI: 10.1016/j.bbrc.2011.08.014]

66   **Deng Q**, Zhuang M, Kong YY, Xie YH, Wang Y. Screening for PreS specific binding ligands with a phage displayed peptides library. *World J Gastroenterol* 2005; **11**: 4018-4023 [PMID: 15996026]

67   **Park SG**, Jeong YJ, Lee YY, Kim IJ, Seo SK, Kim EJ, Jung HC, Pan JG, Park SJ, Lee YJ, Kim IS, Choi IH. Hepatitis B virus-neutralizing anti-pre-S1 human antibody fragments from large naïve antibody phage library. *Antiviral Res* 2005; **68**: 109-115 [PMID: 16290278 DOI: 10.1016/j.antiviral.2005.06.012]

68   **Wen WH**, Qin WJ, Gao H, Zhao J, Jia LT, Liao QH, Meng YL, Jin BQ, Yao LB, Chen SY, Yang AG. An hepatitis B virus surface antigen specific single chain of variable fragment derived from a natural immune antigen binding fragment phage display library is specifically internalized by HepG2.2.15 cells. *J Viral Hepat* 2007; **14**: 512-519 [PMID: 17576393 DOI: 10.1111/j.1365-2893.2007.00843.x]

69   **Yang GH**, Yoon SO, Jang MH, Hong HJ. Affinity maturation of an anti-hepatitis B virus PreS1 humanized antibody by phage display. *J Microbiol* 2007; **45**: 528-533 [PMID: 18176536]

70   **Kaplan JM**. New cancer vaccine approaches. *Drugs Today* (Barc) 2004; **40**: 913-929 [PMID: 15645004 DOI: 10.1358/dot.2004.40.11.872580]

71   **Clark JR**, March JB. Bacteriophage-mediated nucleic acid immunisation. *FEMS Immunol Med Microbiol* 2004; **40**: 21-26 [PMID: 14734182 DOI: 10.1016/S0928-8244(03)00344-4]

72   **March JB**, Clark JR, Jepson CD. Genetic immunisation against hepatitis B using whole bacteriophage lambda particles. *Vaccine* 2004; **22**: 1666-1671 [PMID: 15068849 DOI: 10.1016/j.vaccine.2003.10.047]

73   **Jepson CD**, March JB. Bacteriophage lambda is a highly stable DNA vaccine delivery vehicle. *Vaccine* 2004; **22**: 2413-2419 [PMID: 15193403 DOI: 10.1016/j.vaccine.2003.11.065]

74   **Gorski A**, Dabrowska K, Switala-Jeleń K, Nowaczyk M, Weber-Dabrowska B, Boratynski J, Wietrzyk J, Opolski A. New insights into the possible role of bacteriophages in host defense and disease. *Med Immunol* 2003; **2**: 2 [PMID: 12625836 DOI: 10.1186/1476-9433-2-2]

75   **Hanlon GW**. Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *Int J Antimicrob Agents* 2007; **30**: 118-128 [PMID: 17566713 DOI: 10.1016/j.ijantimicag.2007.04.006]

76   **Clark JR**, March JB. Bacterial viruses as human vaccines? *Expert Rev Vaccines* 2004; **3**: 463-476 [PMID: 15270651 DOI: 10.1586/14760584.3.4.463]

77   **Clark JR**, Bartley K, Jepson CD, Craik V, March JB. Comparison of a bacteriophage-delivered DNA vaccine and a commercially available recombinant protein vaccine against hepatitis B. *FEMS Immunol Med Microbiol* 2011; **61**: 197-204 [PMID: 21204995 DOI: 10.1111/j.1574-695X.2010.00763.x]

78   **Miedzybrodzki R**, Fortuna W, Weber-Dabrowska B, Gorski A. Bacterial viruses against viruses pathogenic for man? *Virus Res* 2005; **110**: 1-8 [PMID: 15845250 DOI: 10.1016/j.virusres.2005.01.009]

79   **Puntoriero G**, Meola A, Lahm A, Zucchelli S, Ercole BB, Tafi R, Pezzanera M, Mondelli MU, Cortese R, Tramontano A, Galfre' G, Nicosia A. Towards a solution for hepatitis C virus hypervariability: mimotopes of the hypervariable region 1 can induce antibodies cross-reacting with a large number of viral variants. *EMBO J* 1998; **17**: 3521-3533 [PMID: 9649423 DOI: 10.1093/emboj/17.13.3521]

80   **Scala G**, Chen X, Liu W, Telles JN, Cohen OJ, Vaccarezza M, Igarashi T, Fauci AS. Selection of HIV-specific immunogenic epitopes by screening random peptide libraries with HIV-1-positive sera. *J Immunol* 1999; **162**: 6155-6161 [PMID: 10229859]

81   **de la Cruz VF**, Lal AA, McCutchan TF. Immunogenicity and epitope mapping of foreign sequences via genetically engineered filamentous phage. *J Biol Chem* 1988; **263**: 4318-4322 [PMID: 2450091]

82   **Menéndez T**, De Haz I, Delgado M, Garay H, Martín A, Vispo NS. Immunisation with phage-displayed variable region 2 from meningococcal PorA outer membrane protein induces bactericidal antibodies against Neisseria meningitidis. *Immunol Lett* 2001; **78**: 143-148 [PMID: 11578688 DOI: 10.1016/S0165-2478(01)00245-0]

83   **Frenkel D**, Katz O, Solomon B. Immunization against Alzheimer's beta -amyloid plaques via EFRH phage administration. *Proc Natl Acad Sci USA* 2000; **97**: 11455-11459 [PMID: 11027345 DOI: 10.1073/pnas.97.21.11455]

84   **Fang J**, Wang G, Yang Q, Song J, Wang Y, Wang L. The potential of phage display virions expressing malignant tumor specific antigen MAGE-A1 epitope in murine model. *Vaccine* 2005; **23**: 4860-4866 [PMID: 16029917 DOI: 10.1016/j.vaccine.2005.05.024]

85   **Manoutcharian K**, Díaz-Orea A, Gevorkian G, Fragoso G, Acero G, González E, De Aluja A, Villalobos N, Gómez-Conde E, Sciutto E. Recombinant bacteriophage-based multiepitope vaccine against Taenia solium pig cysticercosis. *Vet Immunol Immunopathol* 2004; **99**: 11-24 [PMID: 15113650 DOI: 10.1016/j.vetimm.2003.12.009]

86   **Tan GH**, Yusoff K, Seow HF, Tan WS. Antigenicity and immunogenicity of the immunodominant region of hepatitis B surface antigen displayed on bacteriophage T7. *J Med Virol* 2005; **77**: 475-480 [PMID: 16254965 DOI: 10.1002/jmv.20479]

87   **Wan Y**, Wu Y, Bian J, Wang XZ, Zhou W, Jia ZC, Tan Y, Zhou L. Induction of hepatitis B virus-specific cytotoxic T lymphocytes response in vivo by filamentous phage display vaccine. *Vaccine* 2001; **19**: 2918-2923 [PMID: 11282203 DOI: 10.1016/S0264-410X(00)00561-2]

88   **Manoutcharian K**, Terrazas LI, Gevorkian G, Acero G, Petrossian P, Rodriguez M, Govezensky T. Phage-displayed T-cell epitope grafted into immunoglobulin heavy-chain complementarity-determining regions: an effective vaccine design tested in murine cysticercosis. *Infect Immun* 1999; **67**: 4764-4770 [PMID: 10456929]

89   **Hashemi H**, Bamdad T, Jamali A, Pouyanfard S, Mohammadi MG. Evaluation of humoral and cellular immune responses against HSV-1 using genetic immunization by filamentous phage particles: a comparative approach to conventional DNA vaccine. *J Virol Methods* 2010; **163**: 440-444 [PMID: 19903497 DOI: 10.1016/j.jviromet.2009.11.008]

90   **Spear PG**, Manoj S, Yoon M, Jogger CR, Zago A, Myscofski D. Different receptors binding to distinct interfaces on herpes simplex virus gD can trigger events leading to cell fusion and viral entry. *Virology* 2006; **344**: 17-24 [PMID: 16364731 DOI: 10.1016/j.virol.2005.09.016]

91   **De Berardinis P**, Sartorius R, Fanutti C, Perham RN, Del Pozzo G, Guardiola J. Phage display of peptide epitopes from HIV-1 elicits strong cytolytic responses. *Nat Biotechnol* 2000; **18**: 873-876 [PMID: 10932158 DOI: 10.1038/78490]

92   **Folgori A**, Tafi R, Meola A, Felici F, Galfré G, Cortese R, Monaci P, Nicosia A. A general strategy to identify mimotopes of pathological antigens using only random peptide libraries and human sera. *EMBO J* 1994; **13**: 2236-2243 [PMID: 7514533]

93   **Phalipon A**, Folgori A, Arondel J, Sgaramella G, Fortugno P, Cortese R, Sansonetti PJ, Felici F. Induction of anti-carbo-

hydrate antibodies by phage library-selected peptide mimics. *Eur J Immunol* 1997; **27**: 2620-2625 [PMID: 9368618 DOI: 10.1002/eji.1830271022]

94   **Meola A**, Delmastro P, Monaci P, Luzzago A, Nicosia A, Felici F, Cortese R, Galfrè G. Derivation of vaccines from mimotopes. Immunologic properties of human hepatitis B virus surface antigen mimotopes displayed on filamentous phage. *J Immunol* 1995; **154**: 3162-3172 [PMID: 7534789]

95   **Banchereau J**, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; **392**: 245-252 [PMID: 9521319 DOI: 10.1038/32588]

96   **Banchereau J**, Schuler-Thurner B, Palucka AK, Schuler G. Dendritic cells as vectors for therapy. *Cell* 2001; **106**: 271-274 [PMID: 11509176 DOI: 10.1016/S0092-8674(01)00448-2]

97   **Curiel TJ**, Morris C, Brumlik M, Landry SJ, Finstad K, Nelson A, Joshi V, Hawkins C, Alarez X, Lackner A, Mohamadzadeh M. Peptides identified through phage display direct immunogenic antigen to dendritic cells. *J Immunol* 2004; **172**: 7425-7431 [PMID: 15187120]

98   **Subramanya S**, Kim SS, Abraham S, Yao J, Kumar M, Kumar P, Haridas V, Lee SK, Shultz LD, Greiner D, N M, Shankar P. Targeted delivery of small interfering RNA to human dendritic cells to suppress dengue virus infection and associated proinflammatory cytokine production. *J Virol* 2010; **84**: 2490-2501 [PMID: 20015996 DOI: 10.1128/JVI.02105-08]

99   **Lekkerkerker A**, Logtenberg T. Phage antibodies against human dendritic cell subpopulations obtained by flow cytometry-based selection on freshly isolated cells. *J Immunol Methods* 1999; **231**: 53-63 [PMID: 10648927 DOI: 10.1016/S0022-1759(99)00140-4]

Baishideng®