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Phage-based vaccines

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ABSTRACT

Bacteriophages, or more colloquially as phages, are viruses that possess the ability to infect and replicate with bacterial cells. They are assembled from two major types of biomolecules, the nucleic acids and the proteins, with the latter forming a capsid and the former being encapsulated. In the eukaryotic hosts, phages are inert particulate antigens and cannot trigger pathogenesis. In recent years, many studies have been explored about using phages as nanomedicine platforms for developing vaccines due to their unique biological characteristics. The whole phage particles can be used for vaccine design in the form of phage-displayed vaccines or phage DNA vaccines. Phage-displayed vaccines are the phages with peptide or protein antigens genetically displayed on their surfaces as well as those with antigens chemically conjugated or biologically bound on their surfaces. The phages can then deliver the immunogenic peptides or proteins to the target cells or tissues. Phage DNA vaccines are the eukaryotic promoter-driven vaccine genes inserted in the phage genomes, which are carried by phages to the target cells to generate antigens. The antigens, either as the immunogenic peptides or proteins displayed on the phages, or as the products expressed from the vaccine genes, can serve as vaccines to elicit immune responses for disease prevention and treatment. Both phage-displayed vaccines and phage DNA vaccines promise a brilliant future for developing vaccines. This review presents the recent advancements in the field of phage-based vaccines and their applications in both the prevention and treatment of various diseases. It also discusses the challenges and perspectives in moving this field forwards.

Contents

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1. Introduction

The World Health Organization has recently classified human diseases into ten broad areas, including heart, lung and other organ diseases, blood and immune system diseases, cancer, injury, brain and nervous system diseases, endocrine system diseases, infectious and parasitic diseases, pregnancy and childbirth-related disease, inherited diseases, and environmentally-acquired diseases [1]. Some of these diseases can be prevented by vaccination, in particular, bacterial, viral [2,3] and fungal infections [4], parasitic illnesses [5], and cancer [6]. For example, cancer results from uncontrolled growth of abnormal cells, and has become a common cause of death worldwide. In 2017,the expected number of new cancer cases was about 1,688,780. Over the past few years, the increase in the cancer survival rate is eye-catching, owing to the development of effective prevention, early diagnosis and targeted therapy. The development of cancer vaccines can protect us from having cancer by indicting our immune system and producing specific antibodies against pathogens beforehand. Moreover, immunotherapy has been widely regarded as a promising solution to combat cancer by promoting anti-cancer immune responses [7,8]. Some immunotherapy agents such as antibodies for PD-1 and PD-L1 are now available for cancer therapy [9,10].

Conventional vaccines mainly originate from bacteria or viruses and have been commonly used in the disease prevention. According to the production methods or the mechanisms of actions, vaccines can be classified into live attenuated and inactivated vaccines. They are almost or completely devoid of pathogenicity via various ways but can trigger a protective immune response against specific antigens. Vaccines containing the infection agents derived from bacteria are called bacteria-based vaccines and those from viruses are termed virus-based vaccines. Live attenuate vaccines are obtained by altering diseases-causing bacteria or viruses. The bacteria or viruses of licensed live attenuate vaccines have the capability to replicate to form enough copies to induce protective immune responses, but the copy number is not large enough to cause diseases. Inactivated vaccines, are the easiest preparation to be used. They are prepared from microorganisms (e.g., viruses and bacteria) that have been killed through physical or chemical processes [11]. Due to their non-repeating characteristics, they need several doses over time to achieve the same level of protection as live vaccines.

Although the conventional vaccines have been commonly and successfully used for the prevention and control of most diseases, certain problems exist such as the difficulty in their transport or storage as well as the design of more effective vaccines. In addition, the complicated in vivo environment of large animals makes the targeted delivery of DNA vaccines a difficult problem to be solved [12]. Additionally, the safety and secondary effects of vaccines are also considerable problems.

Hybrid virus vaccines use viruses as the vehicle of vaccines. They are stable and cheap, and can be easily produced and purified. Currently, efforts are being made to explore the potency of viruses in the field of vaccine discovery and development. Bacteriophages, also known as phages, are unique viruses that can specifically infect bacteria. Through a long-term evolutionary process, phages have developed a friendly relationship with eukaryotes and are thus not infection agents for eukaryotes [13,14]. Therefore, phages provide a high safety profile and cannot induce pathogenesis in the human body. Additionally, the efficient immunostimulatory and potent adjuvant capacities of phages [15] make them capable of delivering vaccines with high immunogenicity [16,17]. Phages are chemically stable and easy to transport and store. They can also be produced in large scale and inexpensively by simply infecting bacteria [14]. Hence, phages as viral entities can solve the major problems of conventional vaccines such as the difficulty in transportation and storage as well as the targeted delivery. They have attracted growing interest as an optimal platform for designing vaccines. They can serve as a carrier to deliver both the peptides or proteins and the DNA, enabling the development of multifunctional vaccines.

2. History of phage-based vaccines

Phages are assembled from both genomic and proteomic materials. These prokaryotic viruses are considered the most abundant life forms on the Earth. They are more abundant and genetically more diverse than any other organisms. These viral entities occupy all habitats where bacteria reside (sea water, fresh water and soil etc.). It has been estimated that the number of phages in nature is over 10^{30} particles [13,18]. In the setting of constant emergence of novel phages without homologous equivalents, it is certain that our knowledge on the variety of phages and their characteristics represents only the tip of an iceberg.

In the fields of biotechnology and medicine, there is a lengthy controversy over the claim of the discovery of phages (Fig. 1). Actually, several bacteriologists or microbiologists had observed the existence of unidentified substance with a striking antibacterial activity, called bacteriophage phenomenon, such as British bacteriologist Ernest Hankin, Russian bacteriologist Gamaleya [19] and English bacteriologist Frederick Twort [20], before Felix d'Herelle “officially” discovered phages in 1917. However, except d'Herelle, all of them didn't continue to investigate phages further. Not long after the discovery of phages, many scientists including d'Herelle were interested in using the viral entities as antibacterial agents to cure pathogenic bacterial infections such as dysentery [21], suppurative conditions [22] and *Staphylococcus aureus* meningitis [23]. This therapeutic methodology, called phage therapy, dramatically decreased when penicillin was discovered by Alexander Fleming in 1928 (Fig. 1). Compared with the specific infections of phages, antibiotics are more likely to be regarded as a broader range of pathogenic bacteria. Therefore, the early clinical studies with phages were largely given up all over the world except the former Soviet [24–27] and Poland [28–33] and a few others.

Although the investigations about phage therapy expired during the antibiotic era, phages were still active in the biological realm as ideal experimental models and tools to explore the secrets of life. In 1977, Frederick Sanger et al. determined the complete sequence of the genome of phage ΦX174, which dramatically promoted researchers' understanding of phages [34]. Afterward, George Smith et al. invented the technology of filamentous phage display in 1985 [35] and proposed the idea of establishing a phage display library in 1988 [36]. Moreover, in 1989 [37] and 1990 [38], antibody fragments were first used as fusion peptides to be displayed on the surface of the phages by Lerner and Winter.

As the intractable problems of ever-increasing antibiotic resistance and the in-depth knowledge about phage biology, studies on phage therapy, especially phage vaccines, were restarted and drove a renaissance of interest among a wide range of scientists, clinicians and biotechnologists. In 1988, Vida et al. first used whole filamentous phages, displaying antigenic peptides, as a vaccine to trigger immune responses

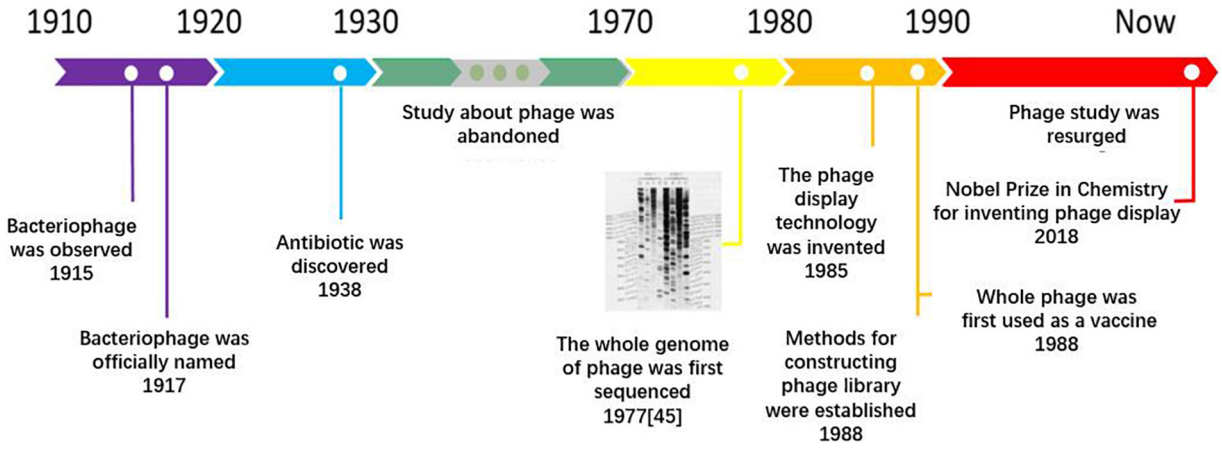

Fig. 1. History of bacteriophage development. The image in this figure was adapted from reference [45]. (Proc. Natl. Acad. Sci. USA).

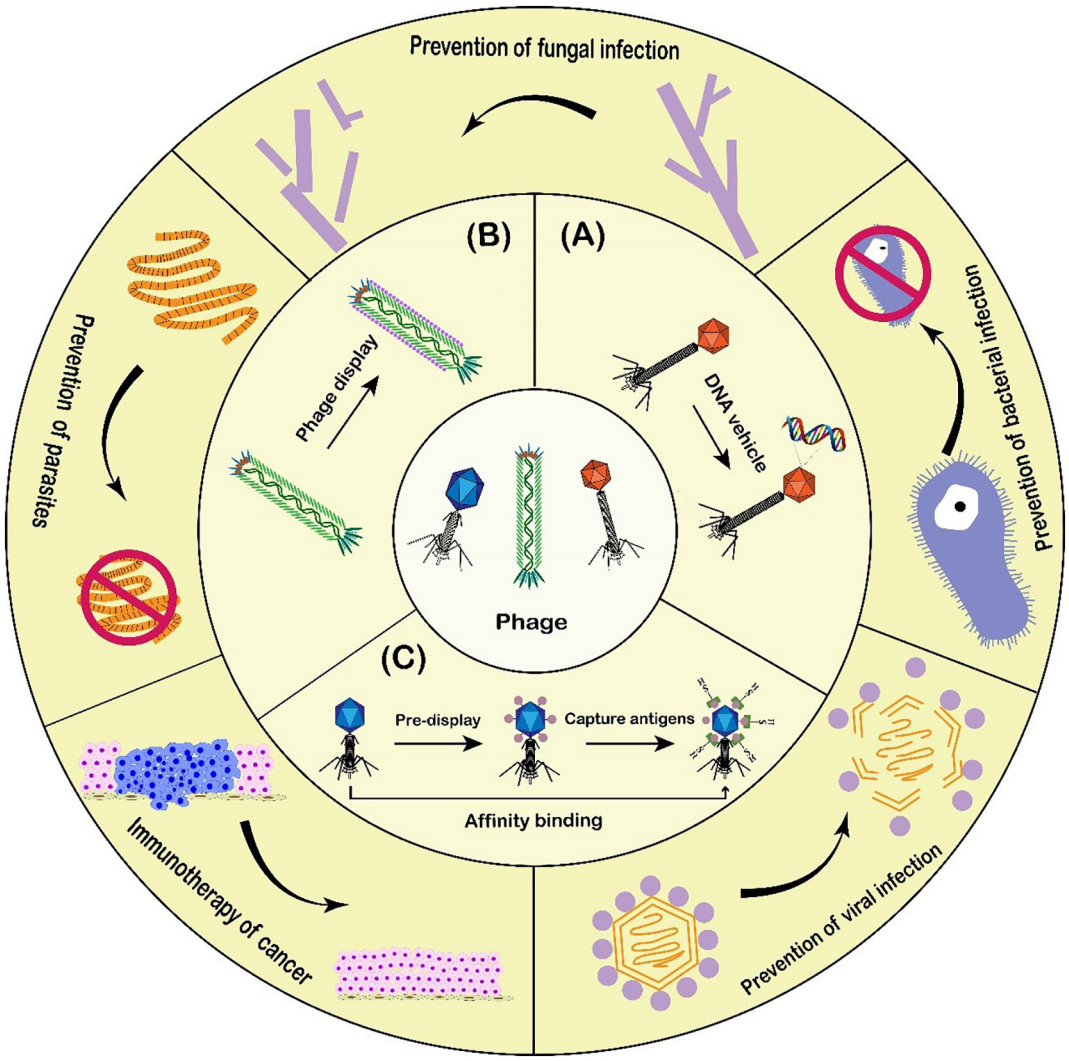

Fig. 2. Overview of the design and applications of phage-based vaccines. (A) DNA vaccines. The whole phage particles are employed as the vehicle of the gene encoding a protective antigenic peptide. In the body of host, the genetic material will be released to express an antigen to initiate immune responses. (B) Phage-displayed vaccines achieved via displaying peptides or proteins by genetic means. Through phage display, foreign peptides or proteins can be fused with the relevant coat protein efficiently and economically. Then the phage particles can be used as ideal vaccines to trigger the host immune system. (C) Phage-displayed vaccines achieved by allowing pre-displayed antigen-binding peptides to capture the antigens. Through an artificial linker, an antigen can be directly linked to the phage surface. Alternatively, an antigen-binding peptide can be identified by biopanning and displayed on the phage surface and then used to capture the antigen originally linked to a substrate through a cleavable linker. This strategy can improve the range of phage-displayed vaccines and ensure the correct special conformation of the foreign antigens. Phage-based vaccines can be designed through the above strategies and used to indict the host immune system and produce specific antibodies against various diseases such as viral infection, cancer, bacterial infection, fungal infection and parasites.

in rabbits model against *Plasmodium falciparum* [39]. Since then, many investigations about phage-based vaccines have been reported [16,40,41]. Consequently, phages, as a kind of viruses, are regarded as new candidate vaccines (Fig. 2).

Since Felix d'Herelle discovered the power of using phages to treat diseases, especially bacterial infections [42], and coined the name of bacteriophage about a century ago, phage study has received growing interest in various fields including protein-protein or protein-ligand interactions, disease diagnosis and therapy, gene/drug delivery vehicles and nanotechnology [43,44]. In the recent years, phage display and its exploitability as vaccine delivery platforms have been a rising star in exploiting the therapeutic use of phages. Phage display technique enables the more efficient and convenient design and production of phage-based vaccines. Thus this review is focused on the use of phages as a platform for vaccine design (Fig. 2).

3. The design of phage-based vaccines

As a platform for developing vaccines, phages have been exploited as vaccines in two formats: phage DNA vaccines and phage displayed vaccines (Fig. 2). Phage DNA vaccines are referred to as the use of phages to deliver DNA vaccines by incorporating the expression cassette of protective antigens or mimic epitopes into the phage genomes. With the protection from coat proteins, phage DNA vaccines are more stable for administration, storage and transport than the conventional DNA vaccines, which makes oral administration of phage DNA vaccines possible. However, compared with phage DNA vaccines, phage displayed vaccines are a more popular vaccine system and have received increasing attention. Phage displayed vaccines are the recombinant phages that display immunogenic peptides or proteins on their surface by transcriptional fusion or by using pre-displayed antigen-binding peptides to capture antigens. In this section, we'll describe these strategies and review their applications in the design of phage-based vaccines.

3.1. Phage display technology

Phage display technology is to display a unique sequence of peptides or proteins on the surface of the phages. It has played a vital role in the phage applications. Especially in developing vaccines, this technology provides an opportunity for identifying and selecting functional polypeptides or proteins with desired immunogenic characteristics [46,47]. Foreign peptides or proteins can be genetically fused to the relevant coat proteins, as the result of cloning antigen sequences into the phage coat protein genes. In an investigation conducted by Shi and coworkers, the peptide YGKDVKDLFDYAQE was displayed on the surface of filamentous phage [46]. This recombinant phage has shown protective effects against *C. albicans* infections.

The predominant phage display vector is filamentous phages of the Ff class (f1, fd and M13) for their specific morphological features. The filamentous phage is about 1~2 μm long (depending on the strain) and ~6 nm in diameter [48]. The surface of filamentous virion is made of one major coat protein (pVIII, a few thousand copies) and four minor coat proteins (pIII and pVI at one tip as well as pVII and pIX at the other distal tip, each with 5 copies) [49]. These coat proteins encapsulate the single-stranded (ss) circular viral DNA inside the lumen. Foreign ('guest') peptides and proteins can be easily displayed on the surface of the protein sheath by genetic fusion. As showed in Fig. 3, the genetic display involves two major steps: (1) insertion of a foreign DNA fragment into a specific site in the phage gene encoding one of the phage coat proteins; and (2) creation of a fusion coat protein with the exogenous sequence when phage particles express their genome during the process of infecting eukaryotic hosts. It is noteworthy that the foreign gene should be inserted into the midst between the coding sequences for the mature coat protein and the signal peptide to ensure the correct N-terminal display on these proteins. This technology correlates the phenotype of a peptide or protein and its related genotype. It has been widely used to construct a phage-displayed random peptide library and discover affinity ligands by a process called biopanning.

On the other hands, we can also harvest recombinant phages via using cleavable artificial linkers. In this method, the level of guest peptide or protein exposure is lower than genetic engineering, but the range of the sequences of the peptides or proteins is increased. For example, Kobayashi et al. introduced a new selection approach employing cleavable hapten–biotin conjugates to display hapten peptide on the phage surface [50]. In this approach, specific antigenic peptides (haptens) were conjugated with substrate-immobilized biotin through a cleavable linker including a disulfide bond, which can be easily cleaved via dithiothreitol (DTT) treatment. Following a biopanning procedure, specific phage particles displaying high-affinity anti-hapten peptides were isolated from a random phage library. These specific phages bound a hapten, while other non-binding phages were washed away. Subsequently, DTT was added to cleave the disulfide bond between the hapten and biotin, releasing phage-hapten conjugates from the substrate. By repeating this procedure, an increasing number of phages conjugated with multiple copies of hapten peptides per phage particle can be obtained. Hence, by this artificial linker-based approach, the

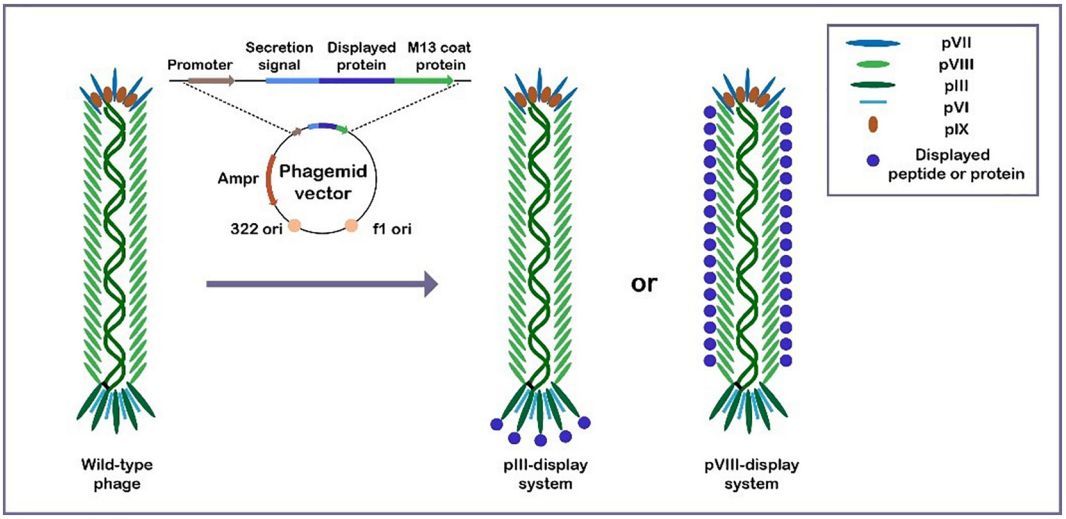

Fig. 3. Phage display of foreign peptides (or proteins) on the filamentous phage by genetic engineering. A unique and random set of peptides can be fused genetically to the pIII coat proteins to construct virus-peptide libraries. After a desired targeting peptide is selected by biopanning, that peptide can also be displayed on the major coat protein (pVIII) to improve its exposure level.

antigenic peptide can be displayed on the surface of phages in its immanent conformation, but the procedure of modification and amplification is very complicated and inefficient (Fig. 4).

3.2. Phage biopanning technology

The generation of a phage-displayed random peptide library allows rapid isolation of peptides that could bind to a given target molecule based on binding affinity through a strategy called biopanning. Earlier biopanning target molecules include purified biomolecules, such as antibodies [51–53], enzymes [54–57], and receptors [58–61]. Later biopanning targets have been extended to complex biological targets (like living animals) and non-biological elements (like nanoparticles) [62–64]. In general, this methodology can be divided into two groups, in vitro biopanning and in vivo biopanning. The in vitro biopanning is widely used to select a high-affinity peptide capable of binding biomolecules or inorganic materials whereas the in vivo biopanning is an effective method to obtain a cell/tissue-targeting peptide. In the vitro biopanning (Fig. 5), a phage library is allowed to interact with the target for allowing the phage-displayed peptides to bind the target during a specified period. After that, through a series of washing steps, the non- or weakly-bound phages are removed, and the specifically bound phages are captured. Then elution of bound phages is carried out by altering pH values or other strategies. The eluted phages are then propagated and exposed to the target for additional binding/amplification cycles. After multiple rounds, the phages that display peptides with a high affinity to the target are isolated, enriched and characterized by DNA sequencing.

The in vivo biopanning shares the similar principle with the in vitro biopanning. In this method, a phage-displayed random peptide library containing billions of phage clones is injected into the animals such as mice through femoral or caudal veins. The library can circulate in the body to incubate with target cells or tissues for a period of time. During this period, phages will migrate and bind to or internalize into their target cells/tissues. Subsequently, the unbound or weakly-bound phages are washed away. Then, an elution buffer can be used to elute the affinitive phages from the cells or tissues and a lysis buffer can be used to isolate the cell or tissue-internalized affinitive phages. The selected phages are propagated and exposed to the target again for additional binding/amplification cycles. After a few rounds, the phages displaying target-binding affinity peptides are purified, followed by DNA sequencing to determine the peptide sequences.

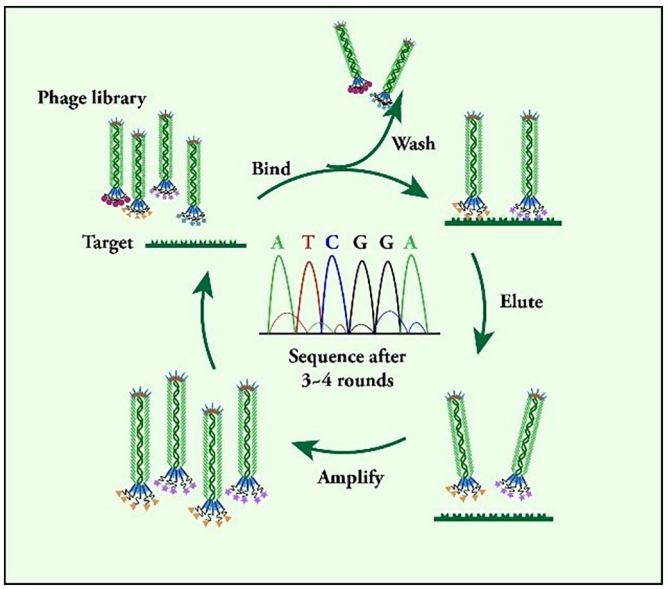

Fig. 5. In vitro phage biopanning. A phage library containing up to one billion phage clones is injected onto the surface of a target. After incubation, the unbound phages are washed, and the bound phages are collected by elution. The eluted phages are amplified and used as an input to interact with the target again by carrying out additional rounds of selection. After a few rounds, the affinitive phages are enriched and characterized by DNA sequencing to determine the sequences of target-binding peptides.

3.3. Phage-displayed vaccines

Phage display technology enables the phages to be engineered into ideal vaccines. In phage-displayed vaccines, as previously mentioned, there are two strategies for displaying foreign antigens on the phage surface: directly displaying antigens by constructing a transcriptional fusion with a coat protein and indirectly displaying antigens by using a pre-displayed antigen-binding peptide to capture the antigens [50]. Both methods have their pros and cons. Through the transcriptional

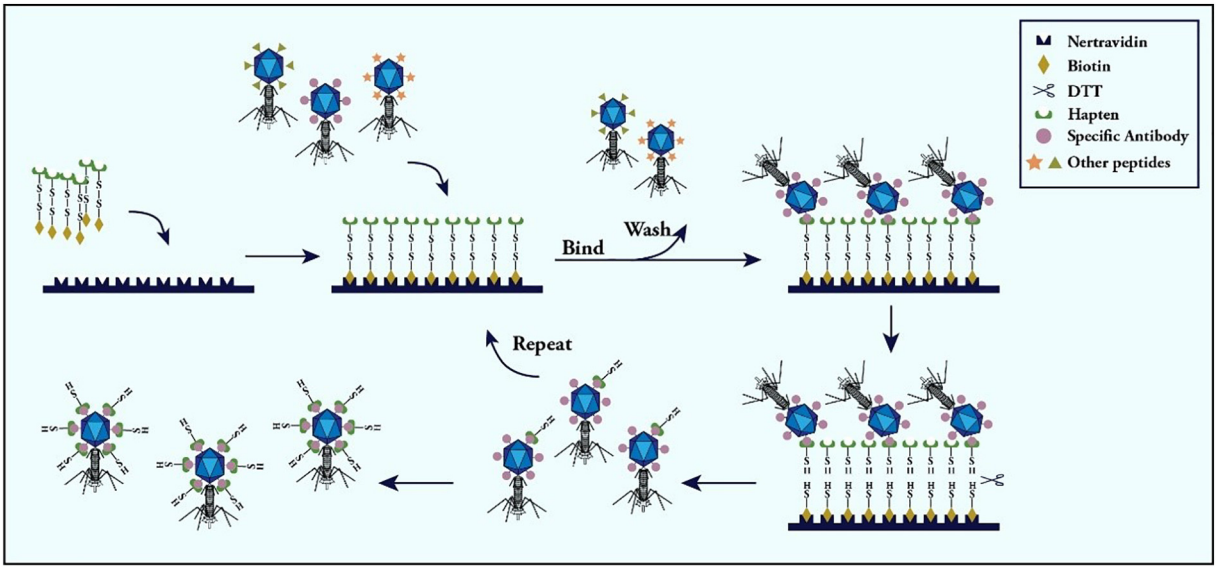

Fig. 4. Phage display of antigenic peptides by means of artificial linkers. At first, hapten peptides were conjugated with a disulfide-bearing linker anchored on a substrate. The linker connects the exposed hapten and the substrate (biotin). Then, a random phage-displayed peptide library was used to select phages displaying the peptide with a high affinity against the hapten. Subsequently, DTT was added to cleave the disulfide bond between the biotin and hapten. By repeating this procedure, an increasing number of phages conjugated with multiple copies of antigenic peptides can be obtained.

fusion, the production of vaccines is very efficient and economic. We can mass-produce the recombinant phages by simply using phages to infect bacteria. The recombinant phages can present a high copy number of displayed immunogens, which effectively improve the immunogenicity of vaccination. However, this method may limit the diversity and the ideal functional conformation of the displayed antigens. In the indirect display strategy, the diversity of displayed antigens can be widened but the procedure of vaccine manufacture is truly troublesome for repetitive conjugation or binding of the antigens to the phage surface (Fig. 4). Hence, the direct display by genetic means is a more common method in obtaining phage-displayed vaccines. Nowadays, phage display technology has offered a larger contribution for vaccine design and delivery. There are several phage display systems that are used to develop phage-displayed vaccines, including filamentous phages, T4 phage, T7 phage, phage lambda and even some RNA phage display systems.

3.3.1. Filamentous phage display systems

Filamentous phages mainly refer to M13, fd, and f1 phages, which possess the capability to infect gram-negative bacteria with F pilus. These phages are biological nanofibers and regarded as an ideal platform for the production of phage-displayed vaccines. Their structures are all similar and can be pictured as nanofibers. Namely, they are assembled from coat proteins and ssDNA genome. The coat proteins include a few thousand copies of the major coat protein (pVIII) on the side wall and five copies of each of the minor coat proteins (pIII, pVI, pVII, and pIX) at the tips of the nanofibers [65]. The immunogenicity of filamentous phage-displayed vaccines largely depends on the category of the displayed peptides and display methods. Currently, peptide display is achieved by fusion to either the pIII [35] or pVIII [66]. But pIII or pVIII display has specific pros and cons. pVIII has a high copy number (a few thousand molecules per phage nanofiber) and can provide an elevated level of immunogens exposure to elicit effective immune responses. But pVIII only can be employed for the display of short peptides (usually less than 20 amino acids). In contrast, pIII can display large proteins of about 100 kDa, but forms a lower level of immunogens exposure (5 molecules per phage nanofiber), reducing the immunogenicity of vaccines.

M13 phage is a common and useful display system. But there is a key drawback. Due to the nonlytic proliferation mechanism of this phage, all components of the phages must be secreted through the host cell inner membrane before they are assembled into phage nanofibers. Therefore, the displayed proteins must be capable of going through such a process. Consequently, the length, sequence, and folding characteristics of the displayed proteins are undoubtedly restricted. In principle, using lytic phage display systems can skillfully avoid this limitation, because phage assembly would take place entirely in the cytoplasm of the host cells.

3.3.2. T4 phage display systems

T4 phage is a lytic bacteriophage that infects *Escherichia coli* bacteria. It harbors double-stranded (ds) DNA genome encased in an elongated icosahedral capsid. Compared with filamentous phages, T4 phage has a structure that is relatively complicated and consists of a head, a tail and twelve tail fibers. The head of a mature phage is a prolate icosahedral capsid (850 Å-wide and 1150 Å-long), which encapsidates the dsDNA genome (168 kbp). At one end of the head, there is a contractile tail (925 Å-long and 240 Å-wide) attracted to a special portal vertex of the head. At the distal end of the contractile tail, there is a hexagonal baseplate (270 Å in height and 520 Å in diameter). Six long tail fibers (LTFs) and six short tail fibers (STFs) are connected to the hexagonal base and folded beneath the baseplate respectively [67]. The head of T4 phage is covered by 9–19 categories of proteins. Among these proteins, two are highly antigenic, including the highly outer capsid protein (HOC) and the small outer capsid protein (SOC). Therefore, these two are commonly used to display foreign immunogens. Each capsid includes decorated 155 copies of HOC protein (39 kDa) and 810 copies of SOC protein (10 kDa), both of which are non-essential coat proteins. The dominating superior feature of T4 display systems is that T4 phage not only can display foreign antigens on the single HOC or SOC site, but also can display dual foreign antigens on both SOC and HOC sites [68]. With regard to some enveloped viruses, such as rhabdoviruses, antibodies should bind a large number of the available surface determinants to wipe out the infection [69]. Thus T4 phage as a dual display system can induce effective immune responses with a high level of immunogens exposure.

The portal of T4 phage head is assembled by twelve copies of gp20. The large motor protein (gp17; 70KDa) is one of the components of a terminal complex. With the expense of ATP hydrolysis, linear (or circular) DNA can be encapsidated through motor gp17 and portal gp20. Under the internal pressure of a tightly packed genome, the head expands in size by about 15% along both long and short dimensions. When the motor complex is dissociated from the head, the gene packing is terminated. Kondabagil and co-workers reconstituted the DNA packing process in vitro via mixing the purified prohead, gp17 and ATPase [70]. This packing system can package 171 kb T4 DNA or other double-stranded DNA. A similar result was observed in a study in which multiple oligonucleotides as short as 30 bp was packaged inside the same head [71]. This finding suggests that T4 phage can be employed to deliver multiple pathogen genes encoding various vaccine antigens. An increasing number of reports have revealed the potential use of T4 phage using as a vaccine vector. Some representative investigations are summarized in Table 1.

3.3.3. T7 phage display systems

T7 phage has an icosahedral capsid encapsulating a dsDNA. The capsid is composed of 415 copies of the capsid proteins. In addition, these proteins are arranged as 11 pentamers at the vertices and 60 hexamers on the faces of the capsid shell. A head-tail connector, a short conical tail and six tail fibers are hooked to one end of the capsid shell (the head). Like T4 phage, T7 phage is also lytic. Phage assembly occurs inside the host cell and the mature particles are released by bacterial lysis.

Table 1
The important studies about phage-based vaccines in preventing diseases.

Pathogen types	Infectious agents	Phage types	Phage protein used for displaying foreign peptides	References
Virus	*HIV*	T4	Hoc, Soc	[113,145]
	Influenza A virus	T7	10B	[146]
		f88	pVIII	[147]
	Hepatitis B	M13	pIII	[117]
			pVIII	[118]
	Papillomavirus	M13	pVIII	[119]
	White spot syndrome virus	M13	pVIII	[120]
	porcine Circovirus 2	lamdba	LDP-D-CAP	[148]
	foot-and-mouth disease virus	T7	10B	[115].
Bacterium	*Yersinia pestis*	T4	Soc	[125]
	Mycoplasma hyopneumoniae	M13	pIII	[127]
Fungus	*Sporotrichosis*	M13	pIII coat protein	[130]
	Candida albicans (CA)	fd88	pVIII	[131,149]
		fd8SHS	pVIII	[150]
Cancer	*Lewis lung carcinoma*	M13	pVIII	[40]
		T4	Soc	[141]
		T7	10B	[142]
	4 T1 breast tumor	T7	10B	[143]
	Melanoma	fd	pVIII	[144]
	MAGE-A^{3+}tumor	fd	pVIII	[140]
Parasites	*Rhipicephalus microplus*	M13	pIII	[132]
	Taenia solium	M13	pVIII	[133]
			pIII and pVIII	[134]
	Malaria	M13	Qβ	[136]

Hence, the displayed peptides or proteins are not required to be translocated across the inner membrane of host bacteria when T7 phage is being assembled.

In the T7 phage display system, the capsid proteins used to display peptides or proteins usually include two forms, 10A and 10B. 10A and 10B are composed of 344 and 397 amino acids (aa) with the latter formed by a translational frameshift at amino acid 341 of the former. The ratios of the two proteins can be varied to construct functional capsids. Therefore, the T7 phage display system could accommodate a variation in peptide or protein sequences and thus display peptides or proteins in high copy number although the copy number tends to be decreased when the sequence size is increased.

Moreover, T7 phage is extremely robust and its proliferation is more rapid than other phages such as filamentous phages and phage lambda. These additional advantages make T7 phage an attractive economical display vector and widen its applications in various platforms [72–76]. In an investigation constructed by Xu and co-workers, they found that T7 phage could tolerate the insertion of foreign genes of 2 kb without damage to its structural integrity [77]. This study revealed the potential use of T7 phage in DNA vaccine delivery. The delivery efficiency can be improved via the display of Tat protein on the surface of T7 phage [78,79].

3.3.4. Phage lambda display systems

Phage lambda has an isometric head (~50 nm in diameter) and a flexible tail (~150 nm in length). Each phage particle is composed of 405 copies of a capsid protein, D head protein, arranged in an icosahedral shell. Its tail involves 32 disks, each of which is made of six copies of a major tail protein termed pV. The pV possesses two folding domains. The C terminus of the pV is not necessary and can be displaced by foreign peptides or proteins without obviously influencing phage proliferation. Therefore, both the D head protein [80–85] and pV tail protein [86,87] can be employed to fuse foreign peptides or proteins. However, compared with pV protein, the N-or C-terminal of D head protein is more common as a display site because it has a larger copy number and its display does not interfere with phage's multiplication. Although each phage particle contains six copies of pV, usually less than one molecule can be fused to pV for each particle.

The genome packaging of phage lambda is also catalyzed by terminase enzymes. Fueled by ATP hydrolysis, lambda terminase can package DNA to near-crystalline density and generate an internal capsid pressure. The D head protein is essential to stabilize the capsid carrying large genes, including a 48.5-kb phage lambda genome [88]. The large genomes of phage lambda enable it to bear an excellent ability to package foreign genes encoding various vaccine antigens. Additionally, phage lambda has been largely employed as the vector of DNA vaccine, which is summarized in Table 2.

Compared with filamentous phages, phage lambda can display multiple copies of the same complicated large proteins on the D head protein, making it potentially an effective vaccine. For example, recently, a study has demonstrated that the display level of fusion peptides on the phage lambda surface was much more than that on the M13 fusion construct, which suggested the great potential of phage lambda being a vaccine delivery vehicle [85]. Additionally, some kinds of peptides that are difficult to secret through the membrane in filamentous phage display systems can also be displayed on the surface of phage lambda. On the other hand, there are still some problems limiting the wide applications of phage lambda. This virus has a temperate lifestyle with the possibility of both lysogeny and lysis and this complex biology has made phage lambda present lower titers than filamentous phages. Moreover, phage lambda has a larger genome, making genetic manipulations more complicated.

Table 2
The important studies about phage DNA vaccine in preventing diseases.

Pathogen types	Infectious agents	Phage types	Genotype	References
Virus	HSV-1	M13KO7	ssDNA	[121]
	hepatitis B	Lambda phage	dsDNA	[97,122,123,151]
	hepatitis C	Lambda phage	dsDNA	[124]
Cancer	Lewis lung cancer	T7	dsDNA	[152]
Chlamydia	Chlamydia abortus	Lambda phage	dsDNA	[41]

3.4. Phage DNA vaccines

Although phage-displayed vaccines provide large promise, in some cases, epitopes displayed on the phage surface are difficult to maintain their primary active conformations and lack eukaryotic post-translational glycosylation signals for the proliferation of phages. Hence, phage-displayed vaccines cannot induce effective immune responses for certain diseases. As an alternative, phage DNA vaccines can be used for special pathogens and present multiple benefits in comparison to phage or conventional DNA vaccines.

A standard DNA vaccination is referred to as the injection of the whole plasmid, which consists of the gene coding for specific proteins (antigens) from a pathogen, and a eukaryotic expression cassette. After intracellular expression of the antigens, greater cytotoxic T-lymphocyte (CTL) responses could be effectively induced. Due to the poor results of naked DNA vaccination in large animals, many methods have been established to protect and deliver the plasmids, such as gene gun [89–91], electroporation [92], and using nano−/micro-particles such as liposomes [93,94] and magnetic silica nanoclusters [95]. But all these technologies have increased the complication and cost of vaccine production. As an alternative vehicle, phages are a good vehicle for the delivery of DNA vaccines because they can carry genes in the form as inserts in their DNA and then release them in the target cells for further expression. In this strategy of designing phage-based vaccines, the whole phage particles are employed as the vehicle for the genes encoding protective antigenic peptides. Because phages should be propagated in prokaryotes, the vaccine gene must function under the guidance of a eukaryotic expression cassette to ensure the proper expression of the vaccine genes and proper folding of their products. An integrated eukaryotic expression comprises an open reading frame, a promotor, and a 3′ untranslated region [96]. Through in vitro packaging of the expression cassette into phage particles, followed by their proliferation and purification, the whole phage particles as a DNA vaccine are injected into the host. As an exogenous antigen, phage particles will be uptaken by the antigen presenting cells (APCs) and other cell types of the host immune system. Following the lysing of phage vehicles, genetic material will be released and expressed into antigens to initiate immune responses (Fig. 6). However, the mechanisms of phage lysis and vaccine gene expression by APCs or other cell types are unclear at present and need to be further studied. Compared with phage displayed vaccine, phage DNA vaccine can ensure the correct presentation of conformationally active epitopes due to the intracellular expression of proteins. Compared with other DNA vaccines, phage DNA vaccine can be easily propagated through the amplification of phages by infecting host bacterial cells, resulting in the decrease in the cost of vaccines.

Nowadays, the details about the in vivo fate of phages are not very clear. Some scientists have made an attempt to explore the exact mechanism of this kind of DNA vaccines. For instance, Clark et al. used phage lambda as the vehicle for delivering the gene of the hepatitis B surface antigen (HBsAg), which can effectively initiate anti-HBsAg immune responses. Additionally, they also constructed recombinational phages with the gene of green fluorescent protein (GFP) inserted in the phage genome. After 8 h, GFP-tagged antigen was detected on the surface of APCs via immunohistological staining, suggesting that the phages could be rapidly taken up and the cloned inserts in the phage DNA was then expressed by APCs in vitro [97].

In conclusion, phages as a vehicle can improve the stability of genetic materials and prevent them from being degraded in the in vivo

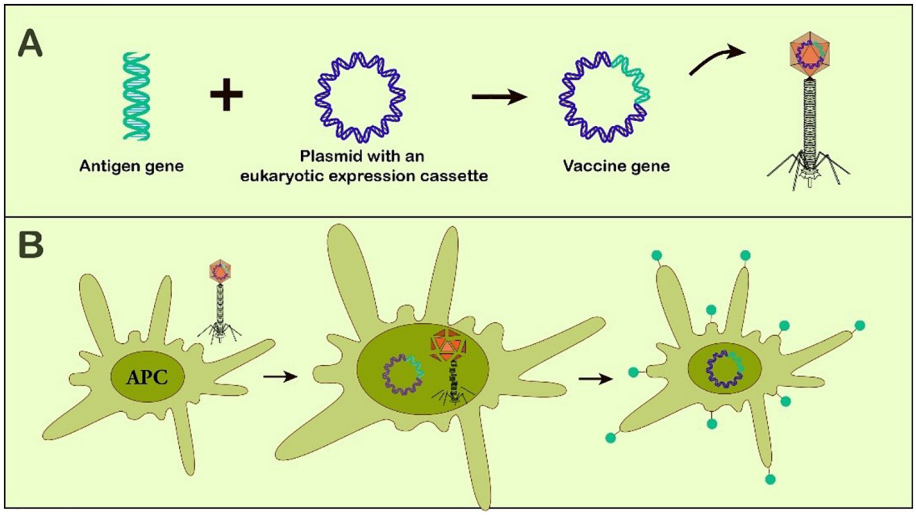

Fig. 6. The construction and working mechanism of phage DNA vaccines. (A) An antigen gene is spliced into a eukaryotic expression cassette, which is in vitro packed into recombinant phage particles. (B) Following the in vivo administration of phages, the recombinant phages are taken up by APCs, resulting in the expression of antigens on the APC surface to induce immune responses.

environment. Additionally, because phages could be genetically modified to bear antigen genes and deliver the genes to APCs, resulting in the expression of antigens in situ, phage DNA vaccines can largely improve the level of immune responses in host animals. Furthermore, phage DNA vaccines can be produced effectively and economically by means of phage amplification through infecting bacteria. Compared with filamentous phages, phage lambda provides a large gene capacity and is more useful in the field of phage DNA vaccines.

4. Immunogenic properties of phages

The interaction of phages with immune cells is a vital index to evaluate the application value of phage-based vaccines. This interaction mainly includes two aspects: the phage immunogenicity and the phage immunomodulatory activity. The phage immunogenicity is the natural capability of phages to trigger specific immune responses, including the production of antibodies against phage antigens. The phage immunomodulatory activity is the nonspecific effects of phages on different populations of immunes cells involved in both innate and adaptive immune responses [98]. Knowledge about the two aspects is essential for the rational design and applications of phage-based vaccines.

4.1. The phage immunogenicity

The phage immunogenicity is referred to as the ability of phages in eliciting specific immune responses, which include two main aspects: anti-phage humoral responses and anti-phage cellular responses. One of the main components of the immunity system is humoral response with the production of a population of antiviral antibodies. As for phages, studies have suggested that phages can trigger the generation of specific antiviral antibodies in vivo [99–102]. The vast majority of these antiviral antibodies are neutralizing antibodies, which can bind the tails of phages and affect their antibacterial activity. In this context, the generation of anti-phage antibodies and the phage-antibody interactions can lead to the inactivation of phages and limit the effective immunogen of phage-based vaccines, which are large challenges related to the phage-based vaccines. An investigation have demonstrated that the anti-phage antibodies may be present in the sera of non-immunized individuals, due to the omnipresence of phages [102]. In addition, after the systemic administration, the intensity of anti-phage antibodies may depend on the phage category, administration paths and the patients' status [103].

In cellular responses, both $CD8^+$ and $CD4^+$ are engaged in the potent CTL response, which is playing a pivotal role in the inhibition of viral infection and the clearance of tumor cells. Conventional vaccines lack the capability to induce cellular responses for the existing non-living components. In this context, phage-based vaccines stand a good chance of overcoming this limitation in vaccine design. Studies have showed that the administration of phages [104] or phage-displayed peptides [105] can induce potent cellular responses. For example, Langbeheim et al. explored the cellular responses induced by whole phage particles and synthetic conjugates from phage capsid proteins [104]. Through measuring the local induration of mice after the infection of pathogens and the proliferative responses, they evaluated the cellular responses in vitro and in vivo. They eventually discovered that the administration of the whole phages could induce stronger cellular responses than that of the synthetic conjugates both in vitro and in vivo.

4.2. The phage immunomodulatory activity

The phagocytosis effect of phagocytic cells plays a decisive role in the elimination of invaded bacteria. Investigations about the influence of phages on the phagocytic cells will help us have a better understanding about the mechanism of phage vaccines in preventing and treating diseases. However, several studies revealed absolutely different effects of phages on the phagocytic cells [106–110]. These studies suggest that the interactions between phages and phagocytic cells are intricate and depend on various parameters, such as the category of phages and pathogens, incubation time, concentrations of coincubated bacteria, phages, and leukocytes, the activity of phages, and the manner of phage inactivity. The first report relevant to this was conducted by Felix d'Herelle [108]. In his studies, Shigella phages could dramatically increase the effects of the phagocytosis of Shigella in the model of guinea pig. However, Kantoch et al. investigated the effect of T5 and T2 phage on the phagocytosis of *E. coli* in the model of guinea pig and horse, respectively [109,110]. They found the different results that the existence of T5 phage did not affect the phagocytosis of *E. coli*, whereas the T2 phage could reduce the phagocytosis of leukocytes. Furthermore, the phenomenon of phagocytosis inhibition was also revealed in other kinds of phages and appeared to be time- and concentration-dependent [106,107].

Lymphocytes are the essential part of immune systems, including T and B cells. Many studies have demonstrated the immunosuppressive effects of phages on the function of lymphocytes. This phenomenon can be referred to as two main aspects: the secretion of specific complex

and the adhesion of phages to lymphocytes. Gorski et al. showed that through the CD3-TCR complex, T4 phage could suppress the proliferation and activity of human immune cells [111]. Additionally, Kniotek et al. demonstrated that the adhesive interaction between immune cells and phages could obstruct the contact between immune cells and their ligands, which undoubtedly inhibited the function of lymphocytes [112]. Moreover, in the experiment conducted by Gorski and coworkers, phages inhibited the activation of a key transcription factor (NF-κB), which regulated the expression of many genes [111]. They proved the immunosuppressive effects of phages in vivo. In their study, phages could extend the survival of allogeneic skin transplants in a mice model.

So far, no data are available in the literature to give a definite answer to the question of how phage-based vaccines affect and modify immune functions in human bodies. We believe that many factors affecting the success of treatment should be considered together in the study for answering this question.

5. The application of phage-based vaccines

Phages have been mainly used as vaccines for preventing diseases. Table 1 and Table 2 list some important studies about the use of phage-displayed vaccines and phage DNA vaccines in preventing diseases, respectively. In what follows, different applications were discussed in terms of preventing or treating different diseases including viral, bacterial, fungal infection, parasitic diseases, and cancer.

5.1. Prevention of viral infection

Viruses can cause a large number of illnesses in animals such as influenza and acquired immunodeficiency syndrome (AIDS). Both antiviral drugs and vaccines can be employed to treat viral infection. However, some viruses harbor the mutated ability to resist antiviral drugs or avoid the immune responses such as human immunodeficiency virus (HIV).

As phage-displayed vaccines, reconstructed phages displaying single or multiple antigens can be used to induce effective immune responses and provide protection in the viral infection. Sathaliyawala et al. fused single antigen and multiple antigens with Hoc capsid protein of T4 phage (Fig. 7). Through the phage display technology, HIV antigens were exposed as many copies on the T4 phage surface, eliciting strong and broad neutralizing antibodies without external adjuvant. Therefore, the engineered antigen-displaying T4 phage can be used as an efficacious HIV vaccine [113].

The T7 display system is also used to design vaccines for the prevention of viruses. Influenza A virus is a member of influenza viruses that can cause annual epidemics worldwide. Influenza A virus has a tetrameric type III membrane protein, termed matrix protein 2 (M2). The extracellular domain (M2e) and Hepatitis B virus core subunit (HBc) of the M2 are often used as components for conventional influenza A vaccines [114]. Similarly, the recombinant T7 phage was also used as a food-and-mouth disease virus (FMDV) vaccine candidate. Xu et al. demonstrated that the major antigenic site of FMDV is the G-H loop of FMDV VP1 structure protein. Through inserting the gene coding the antigenic sites into the T7 genome, they obtained the recombinant T7-GH phage that displayed the antigen (G-H loop peptide). In a pig model, the phage displayed vaccine was found to trigger more effective immune responses than commercially available FMDV vaccines (e.g., InactVac and PepVac) [115].

Additionally, filamentous phages are a common platform for developing phage-displayed vaccines. Deng et al. used phage f88 to display amino acids 2–16 of M2e (termed M2e2–16 with a sequence of SLLTEVETPIRNEWG) on the major coat surface and explored its potential to act as an influenza A vaccine [116]. The resultant phage was termed f88M2e2–16. Additionally, they also constructed a recombinant phage (f88ctr) displaying a peptide (VHEPHEFRHVALNPV) on the N-terminus of the pVIII of f88 (Fig. 8-A, B). This peptide is influenza-irrelevant but can induce the production of anti-aggregating β-amyloid antibodies with the EFRH epitope of human β-amyloid peptide. They confirmed the display of the two peptides (M2e2–16 and VHEPHEFRHVALNPV) by western blot and injected the two recombinant phages into mice models for immunization. Then they extracted the serum from the immunized mice and determined the ability of serum to bind the infected Madin-Darby canine kidney (MDCK) cells. They also immunized mice with M2eHepatitis B core particles (M2eHBc) as a control group. The serum of immunized mice with f88M2e2–16, f88ctr and M2eHBc were collected and used to immunostain the fixed MDCK cells following the infection with influenza A virus. As shown in Fig. 8C, the serum collected from the mice of f88M2e2–16 group could specifically bind to the infected MDCK cells. In the influenza A virus challenge, the mice vaccinated by f88M2e2–16 could survive and the hematoxylin and eosin (H&E) staining of lung samples showed little immune cell infiltration in the lungs of f88M2e2–16 group (Fig. 8-D). These results show that the recombinant phage (f88M2e2–16) can be employed as a substitute vaccine to elicit effective immune responses against influenza A virus.

Recently, two kinds of antigens of Hepatitis B virus were fused to the surface of M13 phage, respectively. Bahadir and colleagues inserted the hepatitis B core antigen (HBcAg) gene into the pIII of M13 phage, resulting in the fusion of HBcAg to the pIII at the tips of the phages. Then the whole HBcAg-displaying recombinant phages were injected into the BALB/c mice through the tail vein to test their antigenicity and immunogenicity, which confirmed the successful immunization [117]. Wan et al. used the pVIII of M13 phage to display the Hepatitis

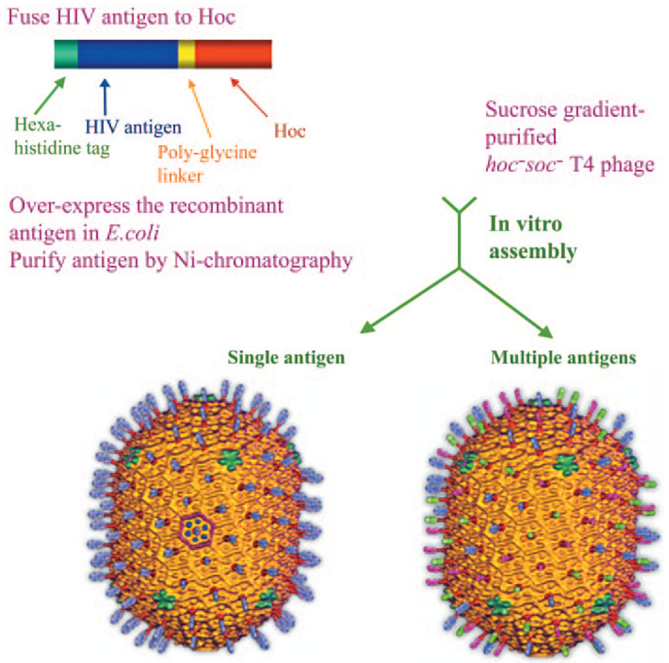

Fig. 7. The design strategy of T4 reconstructed phage-based vaccine with the immunogenicity against HIV. By splicing the gene of HIV and Hoc gene, the reconstructed phage DNA vaccine can be obtained. The left structure model represents the single antigen display system in which the blue spikes represent the single antigen (p24-gag). The right structure model represents the multiple antigens display system in which the blue, green, and pink spikes represent the display of p24-gag, Nef, and gp41 C-trimer antigens, respectively. The dark red spikes represent Hoc proteins; the gold domains represent the hexameric gp23* protrusions, and the Soc subunits bridging the gp23* subunits form the capsid shell; in one of the hexagons of the icosahedral face (left reconstruction), the gp23* and Soc subunits are shown in blue and purple, respectively. The vertex at the base of the capsid represents the unique portal vertex to which the neck and tail attach (not shown). (Reprinted with permission from ref. [113], copyright 2006, J Virol).

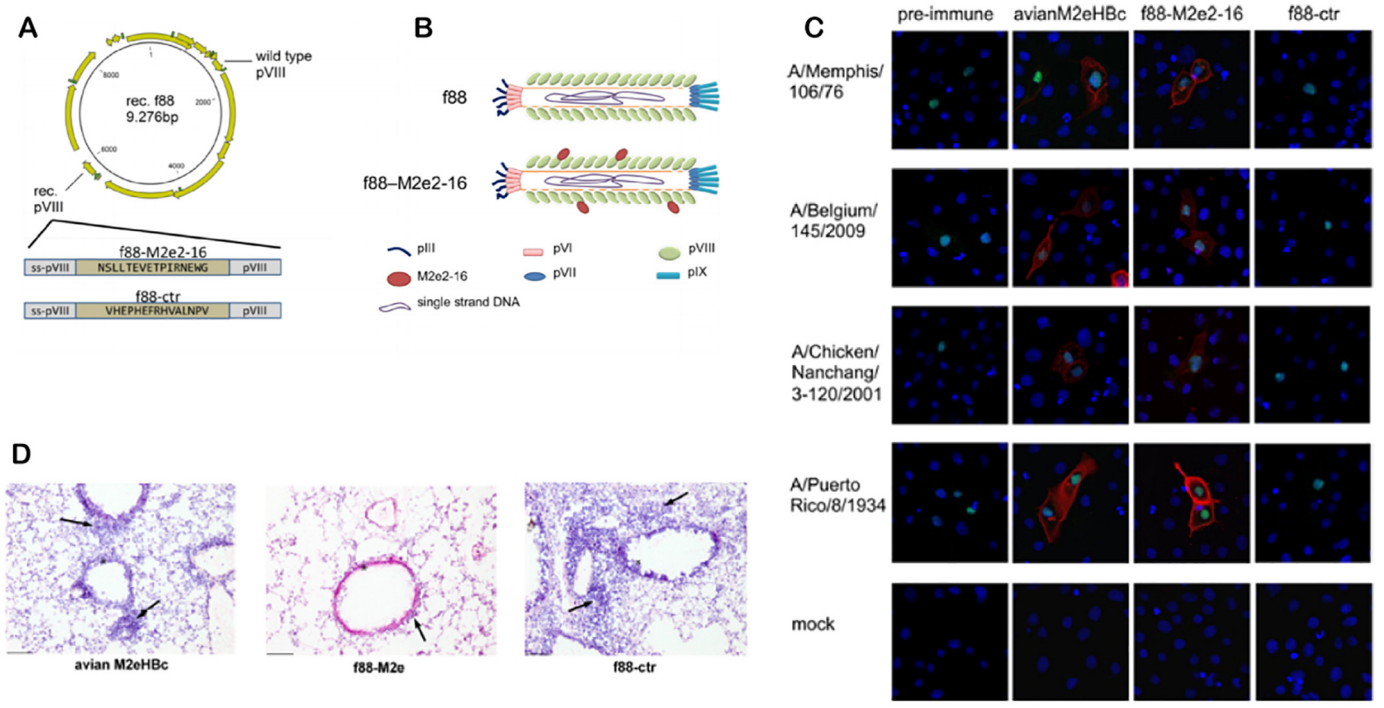

Fig. 8. The design strategy of f88 reconstructed phage-based vaccines with the immunogenicity against influenza A. (A) The plasmid of the reconstructed phage. (B) The structure model of wild and reconstructed f88 phage. (c) The image of immune stain of pre-immune and immune sera showing that immunization of mice with f88M2e2–16 phage induced anti-M2e specific serum IgG that bound to human and avian influenza A virus infected mammalian cells. (d) The lung section and H&E stain of vaccinated mice. With incomplete Freund's adjuvant, 10^{10} f88M2e2–16 phage particles, avian M2eHBc or f88ctr are injected into eight-weeks-old female BALB/c mice (six per group) through the intraperitoneal route for 3 times at an interval of 3 weeks. The mice were sacrificed on day 10 after influenza A virus challenge. (Reprinted with permission from ref. [116], copyright 2015, Applied Biochemistry & Biotechnology).

B virus epitope S_{28-39}. In the BALB/c mice model, the reconstructed phages could also trigger effective hepatitis B virus-specific CTL responses in vivo [118]. M13 phage was also employed to construct the phage-displayed vaccines for other viral diseases, such as papillomavirus [119] and white spot syndrome virus [120]. For example, a protein (termed E7) in an oncogenic human papillomavirus type 16 (HPV16) was used as a model antigen to evaluate the site-specific immunization (SSI) method [119]. In this work, a fragment of the HPV16 E7 was fused with the pVIII of M13 phage. The phage-displayed vaccine was found to effectively trigger immune responses due to the generation of monoclonal antibodies (MABs) against the HPV16 E7 protein in mice. They also used biopanning to determine the mimotopes of the MABs and demonstrated that M13 phage could also be used for epitope mapping.

Besides human vaccines, the phage-based vaccines can also be employed in the animal vaccination. White spot syndrome virus (WSSV) can cause the huge economy loss for the global shrimp industry. Lucero and colleagues constructed a phage-displayed vaccine by expressing the full-length VP28 protein of WSSV on the surface of M13 phage. In vivo challenge study showed that the vaccinated groups presented a much lower mortality than the untreated group after challenging [120]. Additionally, f88 phage, another member of filamentous phage family, can also be used for the design of phage-displayed vaccines. For example, the extracellular domain of M2e of influenza A virus was genetically fused with N-terminal of the pVIII of f88 phage. The challenging study in BALB/c mice showed that the recombinant f88-M2e phage could induce robust M2e-specific serum IgG for preventing viral infection [116,119].

As a phage DNA vaccine, recombinant filamentous phages can also be used as DNA vaccine delivery vehicles. Recently, Hashemi and colleague constructed a recombinant filamentous phage by inserting the expression cassette of Herpes simplex virus 1 (HSV-1) glycoprotein D into the M13 phage genome. The glycoprotein D is very important for the attachment and entry of HSV-1. After genetic immunization by the whole recombinant phages, they evaluated both the humoral and cell-mediated immune responses in the BALB/c mice. The results suggested that the filamentous phages could be considered as optimal vehicles of DNA vaccines, due to the cost-effective production and purification [121].

Besides filamentous phages, phage lambda is widely used in the design of phage DNA vaccines. Clark et al. used phage lambda to construct the phage DNA vaccines by expressing the small surface antigen (HBsAg) of hepatitis B. After three vaccinations in a rabbit model, the phage DNA vaccines could trigger a much higher antibody response against the HBsAg antigen than the commercial vaccines [122]. Similarly, March et al. designed the phage lambda DNA vaccine inserted with the gene encoding for the hepatitis B surface antigen (λ-HBsAg) and confirmed its strong immunogenicity in both rabbit and mice models [123]. The phage lambda system was also successfully applied to the design of phage DNA vaccines for hepatitis C virus [124].

To explore the phage lambda nanoparticle uptake and the gene expression by APCs in vitro, Clark et al. added a reporter gene (GFP) to the vaccine gene under the control of the cytomegalovirus promoter (P_{CMV}) to activate mouse peritoneal macrophages. They found that the phage lambda nanoparticles could be rapidly uptaken by the APCs and then clone sites were expressed on the surface of APCs for eliciting immune responses for viral protection [124]. All of the aforementioned studies show that phage-based vaccines are promising in preventing viral infection.

5.2. Prevention of bacterial infection

Bacteria are single-celled prokaryotic microorganisms and thrive in most of habitats on the Earth. Some bacteria are harmless to human, but some can cause serious illnesses. With the excessive use of antibiotics, an increasing number of bacteria present antibiotic resistance. To overcome this issue, many scientists propose to employ phages to induce immune responses against bacterial infection.

Pneumonic plague is one of the deadliest virulent infection known to humans, caused by the bacterium *Yersinia pestis* (*Y. pestis*). Two components of *Y. pestis*, the capsular protein (F1) and the low calcium response V antigen (V), have been employed in the design of vaccines against *Y. pestis*. In a recent study, Tao et al. investigated novel plague vaccines by two basic methods. One is the design of structure-based immunogens and another is the delivery of T4 phage. By designing the structure of F1 protein and fusing the mutated F1 with the V antigen, the resultant F1mut-V protein became a soluble monomer. Then the F1mut-V protein antigen was fused with Soc on the T4 phage (Fig. 9). The resultant capsid protein of T4 phage showed a potent immunogenicity. Both the F1mut-V protein and the recombinant T4 phage can provide a full protection against the challenge of *Y. pestis* in two models (rodent and cynomolgus macaque). Therefore, these novel vaccine systems offer a new direction for plague vaccine development [125].

Pneumococcal, a Gram-positive bacterium, could cause pneumonia and meningitis in human, especially in both the children and the elderly. Most pneumonia vaccines are based on the bacterial polysaccharide (PS) capsule and can trigger the production of specific antibodies against pneumococcal. Buchwald and coworkers used a M13 phage displayed random peptide library to select the high-affinity phages [126]. During the biopanning, a human monoclonal immunoglobulin A (IgA) antibody (NAD) specific to type 8 Streptococcus pneumoniae capsular polysaccharide (type 8 PS) was used as the target. After three rounds of screening, the specific phages displaying the peptide FHLPYNHNWFAL were selected. They conjugated this peptide with a protein carrier (tetanus toxoid) and evaluated its immunogenicity in BALB/c mice. Their results showed that a type 8 PS-specific antibody response was induced, which suggests that the selected phage displaying this peptide is a mimotope of type 8 PS and can be used as a candidate vaccine against pneumococcal.

Similarly, a phage displayed random peptide library is also largely used in the epitope mapping of various bacterial infection, such as Mycoplasma hyopneumoniae (*M. hyopneumoniae*) [127], *Leptospira* [128] and *Avibacterium paragallinarum* (*A. paragallinarum*) [129]. *M. hyopneumoniae* can cause porcine enzootic pneumonia among pigs of all ages. Yang and colleagues explored the use of M13 phage displayed random peptide library to map the epitope of *M. hyopneumoniae* and used the screened phages as the vaccines to immunize the mice directly to evaluate their immunogenicity [127]. In their research, the protein A-purified IgG of the rabbit anti-*M. hyopneumoniae* hyperimmune serum was used as the target during the biopanning. The selected phages were employed to immunize the mice directly. The generation of IgA coproantibody, IgA in bronchoalveolar lavage fluid, and serum IgG successfully suggested that these selected phages could be used as the candidate vaccines against *M. hyopneumoniae*. Additionally, Wang et al. also employed the M13 phage library to select the immunodominant mimotopes of a serovar A strain of *A. paragallinarum* [129]. They used polyclonal antibody directed against *A. paragallinarum* strain 0083 (serovar A) as the target during biopanning and screened the specific phages displaying the peptide YGLLAVDPLFKP. To evaluate the immunogenicity of the phage-derived peptide, they constructed a recombinant *E. coli* GI826 displaying this peptide on the surface. In a chicken model, this recombinant *E. coli*

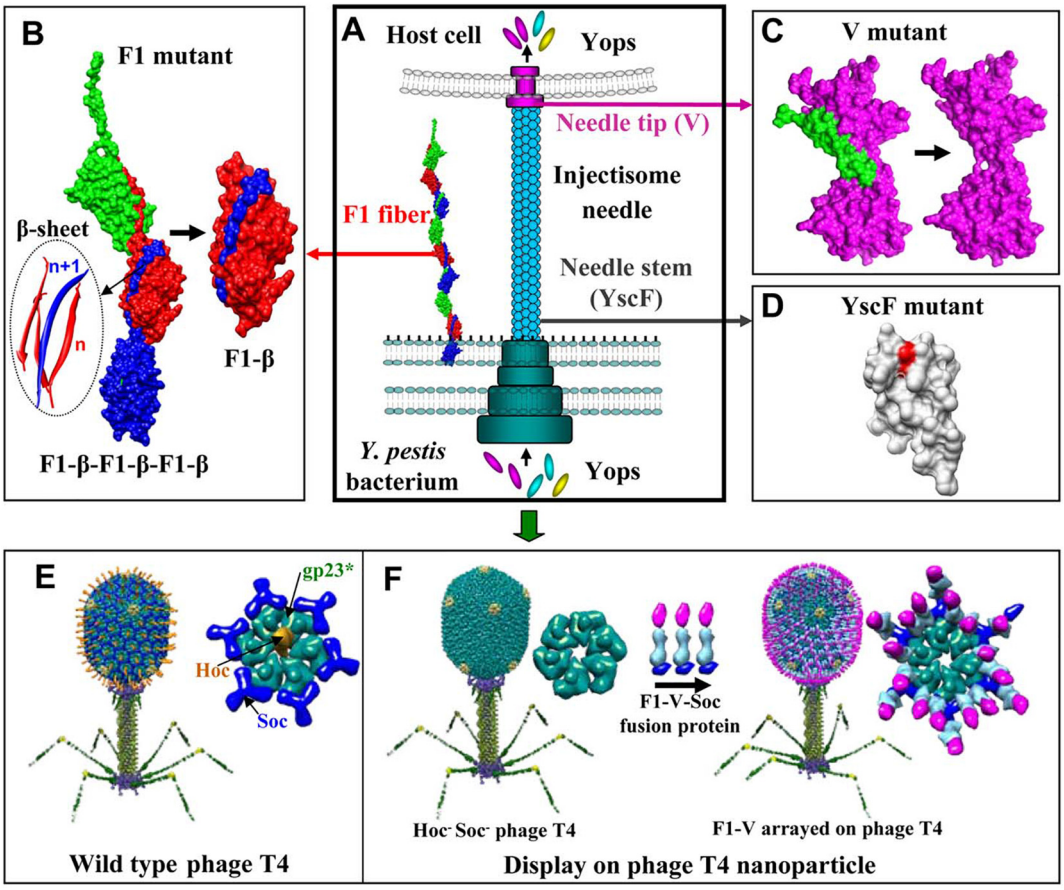

Fig. 9. The design strategy of reconstructed T4 phage-based vaccines with the immunogenicity against pneumonic plague. (A) Schematic of the *Y. pestis* surface structure and components. F1 refers to the structural unit of the capsular layer. Yops can translocate into the host cell through a pore at the tip of the inject-some needle formed by antigen V. YscF refers to the structural unit of the injectisome needle. (B) Schematic of the monomeric F1. "n" subunits of F1 are showed as red strands and "n + 1" subunits of F1 are showed as blue strands. (C) The putative immunomodulatory of V antigen are deleted. (D) Schematic of the YscF mutant with mutagenesis of Asn35 and Ile67. (E) The structure and morphology of wild type phage T4. (F) F1mut-V-Soc was fused to the phage surface to form a phage-based vaccine. (Reprinted with permission from ref. [125] copyright 2013, Plos Pathogens).

can induce immune responses and provide protection in the challenge with *A. paragallinarum*, which demonstrated the potential of phage libraries in the area of epitope mapping.

Another phage, T7 phage, can also be exploited to establish a phage-displayed peptide library and such library can be used to identify the epitope of an antibody involved in vaccine development. For example, a T7 phage displayed random peptide library was successfully used to identify epitopes of monoclonal antibodies against *Leptospira*. The specific affinity of individual clones was estimated through enzyme-linked immunosorbent assay (ELISA). In the ELISA, 90% selected phage clones gave a positive signal. The peptides displayed on the surface of selected phages were identified via DNA sequencing and were compared with the protein database from the gene bank. By this method, several consensus sequences from the displayed peptides (-SSKSYR-, -PKKS-, -KSGRC- and -TNSKRK-) were found to match well with some domains of the proteins from *Leptospira*, indicating that these peptides may be potential immunogens. This study suggested that the T7 phage library is also an optimal platform for epitope mapping in vaccine design [128].

5.3. Prevention and treatment of fungal infection

A fungus is kind of eukaryotic organisms, such as yeasts, molds and mushrooms. There are a variety of pathogenic plant fungi in the fungus kingdom, which may cause serious diseases in humans, animals and plants.

Sporothrix globose (*S. globose*) is a kind of fungus that can cause a subcutaneous mycosis called Sporotrichosis. A 70-kDa glycoprotein (Gp70) of *Sporothrix*, a major adhesion factor, was found to confer notable virulence attributes. Therefore, Chen et al. fused an epitope peptide of Gp70 with the coat protein (pIII) of M13 phage and investigated the immune responses elicited by the recombinant phage in comparison with heat-killed *S. globosa*. As a result, both humoral and cellular immune responses were potently induced by the recombinant phages without the need for an adjuvant. After exposure to and infection with viable yeast cells, recombinant-phage-immunized mice showed an effectively improved survival rate without apparent adverse effects in the hematological or biochemical parameters. Hence, the recombinant phage holds great promise for novel vaccine candidates against *S. globose* [130].

C. albicans (CA), a kind of opportunistic fungus, can cause high morbidity and mortality in immunocompromised groups, such as HIV, diabetes and cancer patients. Pointing to this problem, our group employed fd phage to display the immunodominant epitope (termed EPS) of Sap2 (the major virulence factors of CA) on the surface of fd phage and constructed the fusion phage (termed EPSP). Through intraperitoneally injecting EPSP into the mice for three times, we obtained EPSP-immunized mice. Subsequently, we carried out the CA infection challenge in the EPSP-immunized mice and measured fungus loading in kidney, visceral lesion and survival rates to evaluate the immune efficiency of the phage vaccines (Fig. 10). We found that the EPSP-immunized mice could not be infected by the fungi even when the mice were challenged with CA infection. On the other hand, we collected the serum of EPSP-immunized mice and extracted the IgG antibody to cure the CA infected mice. Both of these tests received positive results. Then we evaluated the antibody response against recombinant Sap2 (rSap2) and EPSP through Western Blot and immunofluorescence. As a result, antibodies exacted from the EPSP-immunized mice could effectively bind rSap2 and EPSP [131].

5.4. Prevention of parasitic diseases

Parasites are the predators that eat prey in units of less than one, which include protozoa, animals, plants and fungi. Conventional methods to achieve the prevention of parasitic diseases in cultivation industry are expensive and labor-intensive. Therefore, the development and applications of the phage-based vaccines for preventing parasitic diseases might promise a huge benefit on the economy and society.

Prudencio and colleagues have explored the immunogenicity of recombinant filamentous phage vaccines against the *Rhipicephalus microplus* (*R. microplus*) ticks in mice and cattle models. In their study, they used a M13 phage library to select specific phages with high-affinity to chicken polyclonal IgG antibodies raised against larval proteins of *R. microplus*. Through biopanning, they selected nine phage clones that could specifically recognize the polyclonal IgG via dot blot and ELISA. These nine phage clones displaying nine different amino acid sequences were further tested to assess the primary immunogenicity in the mice model. The results showed that each of these phage clones could induce immune responses in mice immunization, which suggested that phage-displayed vaccines could act as an effective method for ectoparasite control [132].

Taenia solium cysticercosis is a common parasitic disease affecting the health of human and rustic pigs. One of the antigens of cysticercosis is S3Pvac which is composed of several protective peptides. Morales and colleagues displayed S3Pvac on the surface of M13 filamentous phage [133]. The whole phage was found to induce effective immune responses against cysticercosis in the pig models. Manoutchariana and colleagues constructed similar recombinant M13 phage displaying three antigens (KETc1, KETc2, GK1) and a recombinant antigen (KETc7) of porcine cysticercosis and explored their immunogenicity through oral vaccination [134]. Their results showed that antigen-specific cellular responses were induced in the pig models, and demonstrated that phage vaccines were relatively stable and could be used through oral administration for protecting parasitic diseases.

Malaria, caused by *Plasmodium falciparum*, is a mosquito-borne infectious disease. As one of the world's greatest public health problems, it would be of immense importance to develop effective malaria vaccines. Over the past few decades, various antigens and immunodominant mimotopes of malaria were identified, such as apical membrane antigen-1 (AMA1) [135] and circumsporozoite protein (CSP) [136]. Phage display-based biopanning approach provides an optimal platform for antigen identification and vaccine discovery. Recently, Crossey and coworkers identified the conserved epitopes on the *Plasmodium falciparum* blood stage antigen AMA1 [137]. In their study, an RNA phage (MS2) library was used to select peptides with a high affinity against 4G2. 4G2 has been identified as one of the most broadly inhibitory anti-AMA1 mAb, which could specifically recognize AMA1 [138]. After 3 rounds of affinity selection, eight different phage particles were selected. In the further test, one selected phage displaying a specific peptide (VTHDAWRPD) on its surface could induce AMA1 cross-reactive IgG responses in the mice models. Similarly, Casey and colleagues explored filamentous phages to identify a peptide mimic of the 4G2 epitope [139]. From these studies, we can see that the phage display technology has been successfully applied to identify malaria antigens and discover malaria vaccines.

In addition to antigen display on the phages by genetic engineering, antigens can also be displayed on the phage surface by chemical conjugation to develop vaccines against malaria (Fig. 11). For example, Khan and coworkers used a multi-step chemical coupling of circumsporozoite protein (CSP) to the surface of Qβ phages [136]. First, purified CSP diluted in PBS was treated with SATA (N-Succinimidyl S-Acetylthioacetate) and other reagents to generate the free-SH groups (cysteine-CSP). Then the purified Qβ virus-like-particles (VLP) were mixed with SMPH (Succinimidyl-1-6-[(β-maleimidopropionamido) hexanoate]) to generate free maleimide groups on the Qβ VLPs (Qβ-Malemide). Subsequently, the cysteine-CSP and Qβ-Malemide were mixed and incubated at room temperature to conjugate CSP with Qβ, leading to the production of the CSP-Qβ vaccines. With an adjuvant like Alum, the CSP-Qβ vaccines could induce a prominent level of immune responses in the C57Bl/6 mouse model. Compared with genetic engineering, this chemical conjugation makes the full-length display of larger proteins become possible.

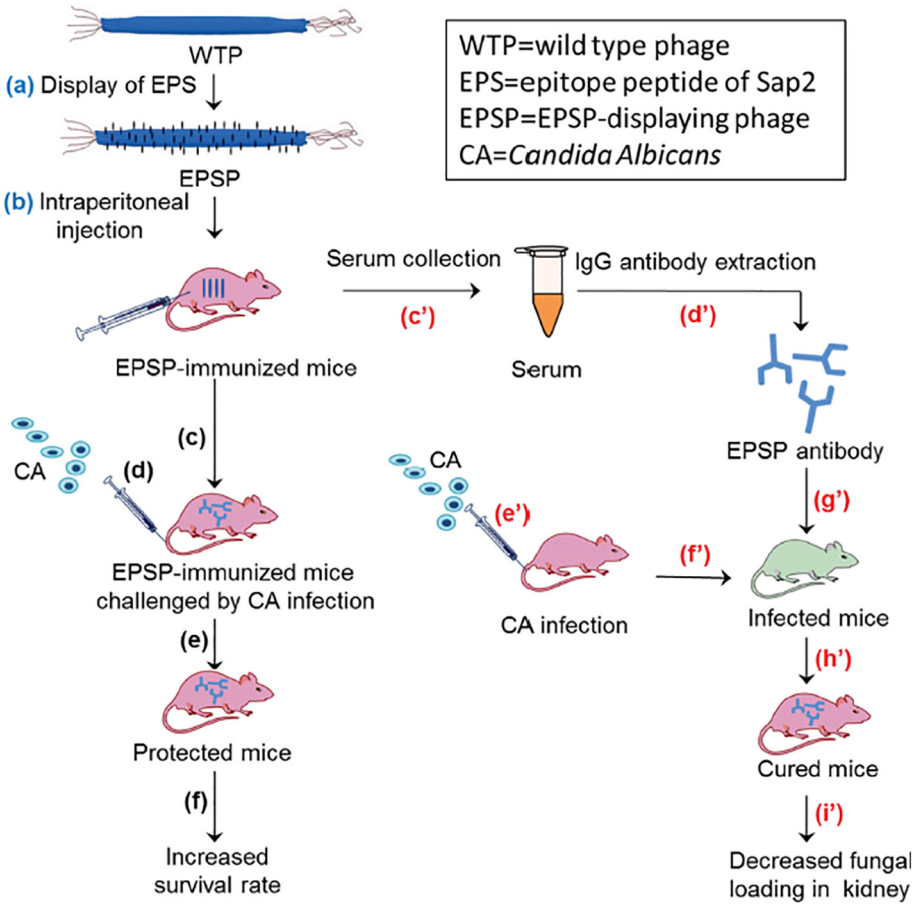

Fig. 10. The design strategy of ESEP reconstructed phage-based vaccines for preventing and curing CA infection. a) At first, the phage-based vaccine was constructed by expressing EPS on the wildtype phage (WTP) surface. b) The EPSP-immunized mice were obtained through intraperitoneally injecting ESEP phage into the mice for three times. Then, two strategies were adopted to evaluate the immunogenicity of the phage vaccine. In the first strategy (c-f), EPSP-immunized mice were challenged by injecting 2×10^6 CA cells via tail vein. The results showed EPSP phage could protect mice from fungal infection effectively, as evidenced by the increased survival rate. In the second strategy (c′-i′), the serum of EPSP-immunized mice was collected and EPSP antibodies were obtained through the extraction of IgG. Subsequently the mice were infected by CA cells via tail vein injection. Then the EPSP antibodies were injected intravenously into the infected mice, which led to the curing of CA infection as evidenced by the significantly reduced fungal loading in kidneys. (Reprinted with permission from ref. [131], copyright 2016, Advanced Healthcare Materials).

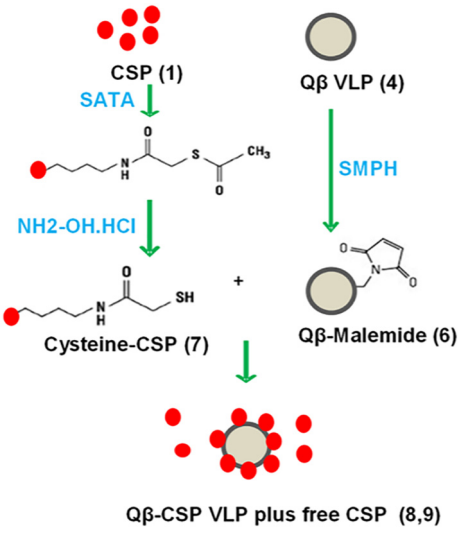

Fig. 11. Outline of the chemical conjugation of antigens onto the phage particles. First, CSP was treated with SATA, NH_3-OH, HCL and other reagents to generate the free-SH groups, leading to the formation of cysteine-CSP. Qβ particles were treated with SMPH to generate the free maleimide groups, resulting in the production of Qβ-Malemide. Then, cysteine-CSP and Qβ-Malemide were mixed to produce Qβ-CSP vaccines. (Reprinted from ref. [136], copyright 2016, PloS One).

5.5. Prevention and treatment of cancer

Recently, cancer immunotherapy, regarded as a promising method to treat cancer, has received increasing attention among clinical oncologists. Tumor cells can circumvent host immune surveillance by forming an immunosuppressive tumor microenvironment through several mechanisms. To solve this problem, recombinant phages that display antigen mimotopes can be employed as a candidate vaccine to improve immune responses and achieve the prophylactic and therapeutic effects.

In the recent years, many human tumor-associated Antigens (Ags), which can be recognized by CTLs, have been identified and characterized. This success largely pumps the investigations in the field of cancer immunotherapy. Cancer/testis (C/T) Ags are the most rapidly expanding group of tumor-associated Ags, which are expressed in various kinds of tumors but not in normal tissues. Therefore, C/T Ags are optimal candidates for cancer vaccines due to their rigid tumor-specific characteristics. Sartorius and colleagues used filamentous fd phage to co-express T helper (Th) cell epitopes and C/T Ags for inducing Th-dependent CTL responses [140]. The recombinant phage could induce potent specific CTLs responses both in vitro (using a human cell system) and in vivo (using a humanized murine model). The application of filamentous phages effectively improved the immunogenicity of tumor-associated Ags and hampered tumor growth. In the control group, the wild-type filamentous phage mixed with the same concentration of Th peptides

and C/T Ags could induce cytotoxic activities both in vitro and in vivo, which demonstrated that phages could be used as a powerful platform for the cancer immunotherapy [140]. Similarly, Asadi-Ghalehni et al. investigated the use of recombinant M13 phage, displaying epidermal growth factor receptor (EGFR) mimotope on the pVIII coat protein, as a Lewis lung carcinoma vaccine [40]. They showed that the recombinant phage vaccines could largely induce immune responses and elicit specific antibodies targeting cancer cells.

Non-filamentous phages such as T4 and T7 have been employed as vaccines to enhance cancer therapy after proper peptides are displayed on them. For example, Rena et al. used T4 phage to display vascular endothelial growth factor (VEGF) and then employed it as a vaccine against Lewis lung carcinoma in a mice model. The resultant T4-mVEGFR2 phage could effectively suppress the angiogenesis and possessed significant antitumor activity [141]. In addition, T7 phage is also used to display the five fragments of the EGFR mimotope. Through genetical engineering, the EGFR was fused with the 10B coat protein of T7 phage. The results showed that the EGFR was successfully expressed on the surface of T7 phage and the recombinant phage could effectively inhibit the growth of the tumor in the model of BALB/c mice [142].

Identification of tumor-specific antigens could enable the successful design of phage displayed vaccines for cancer prevention. For example, Shadidi et al. employed a proteomics-based method to identify the tumor antigens of breast cancer [143]. They designed vaccines by displaying the identified tumor antigens on the T7 phage and test the level of immune responses in mice after oral administration. Their data indicate that the surface display of tumor antigens on the phage can effectively trigger immune responses and the resultant engineered phage can be viewed as a promising mucosal cancer vaccine. Similarly, in a fd phage display system, the tumor specific antigen epitope, melanoma antigen $A1_{161-169}$, was fused with pVIII coat protein of fd phage. The resultant hybrid phage was found to effectively inhibit tumor growth, as confirmed by an in vivo tumor protection assay [144]. All of these studies clearly demonstrate that the phages could be engineered to become a vaccine to enhance cancer therapy.

6. Perspectives and current challenges

Although phage-based vaccines have achieved some successes, there are still issues that need to be solved to move this field one step further. First, there is a huge gap between the published phage-based vaccines and those available to the average consumers at local clinics. Although phages do not infect eukaryotes and cannot trigger pathogenesis in the human body, a full study on the biology of phages and the clinical trials to test the effectiveness of phage-based vaccines in human disease prevention are needed before the phage-based vaccines could get government approval. Second, phage-based vaccines have not been developed to protect superbugs. Currently, there are 700,000 deaths each year caused by the drug resistant viruses, bacteria, fungi and parasites [153]. Antimicrobial resistance (AMR) is becoming a big killer. We believe that phage-based vaccines could be a key approach to tackling such crisis. Recently, there is a special case that a patient who was infected by a superbug was cured by the therapeutic use of phages [154]. In this case, phages were injected into the patient's body to cure the infection by a deadly superbug (*Acinetobacter baumannii*). This success shows that phages could be injected into human bodies for treating superbugs. We believe that phage vaccination could be used in combination with antibacterial phage therapy in preventing and treating superbugs once a superbug-specific phage is identified for a specific superbug. Third, currently there have been no truly effective vaccines for preventing cancer and thus phage-based vaccines need to be further developed to more efficiently prevent cancer. Now there are a variety of categories of cancer treatment, such as chemotherapy, radiation therapy, surgery, and immunotherapy. Phage-based vaccines could be integrated into some of these therapy strategies such as immunotherapy to advance the field of cancer prevention and therapy. Fourth, the mechanism by which a phage displayed vaccine or phage DNA vaccine elicits desired immune protection needs to be further studied. Many questions remain unanswered. For example, how phages carrying a DNA vaccine are lysed to release the DNA vaccine at the target mammalian cell is still a puzzle [97,155]. Moreover, the phages could be engineered to target a particular cell or tissue by displaying a cell- or tissue-targeting peptide discovered by in vitro or in vivo biopanning [43]. Exploring the combination of such targeting properties and immunogenicity of phages can be a future direction for improving the efficiency in delivering the phage-displayed vaccines or phage DNA vaccines to target cells or tissues.

Phages are not only a nanoscale carrier that can be mass-produced cost-efficiently, but also genetically modifiable to bear targeting motifs as well as antigens or vaccine genes. Hence, phage display is still a powerful methodology in vaccine development although it will not resolve all barriers we may face in the field of vaccine production. More recently, an obvious step in the development of phage-based vaccines is to produce a 'hybrid phage vaccine' with the combination of phage-displayed and phage DNA vaccines in a single phage particle [156]. A hybrid phage vaccine can be designed by achieving two goals in the same phage particles, namely, displaying peptide or protein antigens on their surface and inserting a eukaryotic promoter-driven gene encoding the same (or different) antigens into their genome. Thus, the resultant hybrid phages could potentially target both major histocompatibility complex (MHC) class I and class II arms of the immune systems. So far, this strategy is still hypothetical, but we believe that hybrid phage vaccines could serve as a promising platform for vaccine design following a better understanding of phage-mediated immunization. We also believe that phages or phage-based vaccines can be integrated with other biomolecules to form a novel type of vaccines that can be more efficient and home to target cells or tissues. For instance, the engineered phages displaying galactose could effectively target galactose-recognizing hepatic receptors and stimulate the uptake of the phages [157]. It was also found that the recombinant phages with the fusion of cocaine sequestering antibodies could home into the central nervous system [158]. Therefore, it is crystal-clear that phage-based vaccines will continue to develop and offer a bright future to the prevention and treatment of many diseases such as cancer and neurodegenerative disorders through immunotherapy.

7. Concluding remarks

Since bacteriophages were discovered, scientists and clinicians have committed themselves to the investigation of phage therapy. Soon after the early exploration, scientists almost gave up this manner due to the emerging of antibiotics and the shortage of biological knowledge about phages. However, witnessing the severe situation of the antibiotic-resistance, scientists have to revisit the value of bacteriophages. Nowadays, phage-based vaccines have revealed an enormous potential in preventing and treating diseases. Through random genome or peptide-based phage libraries, immunogenic peptides can be easily identified. Subsequently, we can obtain novel phage-displayed vaccines by fusing these immunodominant epitopes to protein domains on the phage surface through phage display technology. Moreover, antigen genes can be inserted into phage genomes and carried by phages to target cells. The product of the antigen genes can also serve as antigens for eliciting immune responses for disease prevention. Moreover, phages can effectively induce immune responses to serve as protective antibodies without an adjuvant. Importantly, phage-based vaccines can be mass-produced easily and economically, through infecting bacterial culture supernatants. Furthermore, the transport and storage cost of phage particles are much lower than that of the traditional vaccines which rely on a cold chain for storage. All these points make phages an ideal source for the design, development, delivery and storage of vaccines. Although there are still many unexplored aspects of phage-based vaccines, they

are providing new strategies for the prevention and treatment of diseases.

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