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Exploring phage engineering to advance nanobiotechnology

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abstract

Phage is a human-safe bacteria-infecting virus assembled from proteins and nucleic acids, enabling it to be genetically engineered to display functional peptides or proteins. It is also a biological nanomaterial that can be assembled into ordered nanostructured materials, laying the foundation for advancing nanobiotechnology. Phage, alone or in combination with non-biological nanomaterials, has been employed to develop new nanobiotechnologies for applications such as biosensing, drug and gene delivery, cancer therapy, vaccinations, tissue regeneration, antibacterial treatment, energy storage and generation, and higher-ordered materials assembly. This review summarizes the structure and biology of phage both as a biomacromolecule and as a nano-biomaterial. It also critically discusses the nanobiotechnological applications of phage and the challenges in this field. The current advancements of phage-based nanobiotechnology, as represented by those applications, demonstrate that phage can find unexplored applications in materials science, nanotechnology, energy, and medicine due to its unique properties such as genetic display of peptides or proteins and uniform size and shape.

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Introduction

In 2020, a sudden novel coronavirus epidemic swept the world. While countless anti-epidemic personnel are working hard, researchers in the biomedical field are also actively seeking effective means of detection, treatment and prevention. In the face of various drawbacks exposed by traditional medical materials, the development of new biological materials is urgent. With their excellent biological properties and modifiability, phages have shown great application potential in many medical fields, which are expected to further achieve clinical translation.

Phages have been used for the treatment of bacterial diseases since the beginning of their development and have a history of

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more than 100 years. In 1917, Felix d'Herelle announced that he had isolated a microbe that was resistant against Shigella. He gave the microbe a name of "bacteriophage" [1]. Subsequently, D'Herelle conducted extensive studies on phages and introduced the concept of "phage therapy" [2]. Since the discovery of penicillin by Alexander Fleming in 1928, the use of antibiotics has been dominant. Subsequently, the emergence of bacterial antibiotic resistance limited the use of antibiotics [3]. Phages had been used as an alternative to antibiotics since the late 20th century. Phage therapy was used against many multidrug-resistant bacteria [4]. In 1952, Alfred Hershey demonstrated that DNA, not protein, is the genetic material of life [5], for which he won the 1969 Nobel Prize in Physiology or Medicine. In 1985, George P. Smith successfully inserted the DNA of an exogenous peptide into the pIII capsid protein gene of f1 phage and made the peptide expressed on the shell of the phage, which marked the establishment of phage display technology [6]. In 1988, Stephen Parmley and George Smith described biopanning techniques for affinity selection and demonstrated that directed evolutionary selections could enrich clones present in one part per billion or less [7]. On this basis,

Gregory P. Winter established an antibody company in 1989 and used phage display technology to develop antibodies with the purpose of curing multiple sclerosis, cancer, and other diseases [8]. Phage display and biopanning techniques have become useful tools for drug discovery. Adalimumab (HUMIRA), the first antibody developed by the company, was authorized by the FDA in 2002 to treat psoriasis, ankylosing spondylitis, and rheumatoid arthritis [8]. In addition, phage technology has also been used to find new ligands for proteins such as enzymes and receptors [9], as well as for antigens for tumor diagnosis and targeted therapy [10]. In 2018, Smith and Winter, as two pioneers in phage display research, shared the Nobel Prize in Chemistry.

As a product of the cross-discipline of biology and materials science, phage display technology has opened up a new path for developing new nano-biomaterials and phage-based delivery systems came into being. The successful application of phage technology in important fields such as cancer therapy [11,12], scaffolds [13], antibacterial materials [14] and vaccines [15] is numerous. It is worth mentioning that the phage biopanning technology relies on the phage library (see Table 1), where a large number of random peptide sequences are presented by phages, making it possible to screen for specific target-affinity peptides. Targets can be mammalian cells [16,17], proteins [18] or organs [19,20]. They can also be nanoparticles [21], compounds [22,23], or semiconductor materials [24]. This has greatly expanded the applications of phage in other areas besides biomedical fields, such as battery materials [25], sensors for molecular detection [22,23], and highly-ordered assembled materials [26].

This review summarizes the nanobiotechnology related to phages in detail (Fig. 1), which is the principal basis for its further applications and research. Phages have shown great application potential in many fields. Although several reviews on phages have been published, this review is novel in many respects by contrast. Published reviews pay more attention to research progress in the field of biomedicine, while this review looks at phages from the perspective of materials, not only summarizing the applications of phages in biomedicine, but also including studies on energy and ordered structure materials. In addition, it provides the latest research advances in phage-based nanobiotechnology, such as phage-based vaccine development against the recent infectious disease COVID-19. Although the basic and applied research on phages is increasing, this review also puts forward some problems that need to be solved urgently. While affirming the advantages and development potential of phages, it also proposes prospects and challenges of this challenging field.

Phage as a biomacromolecule and nanobiomaterial

Phage

Phage, bacteriophage, is a nano-scale microorganism that can only rely on certain bacteria to multiply. The structure of the phage is simple. Phage is composed of capsid proteins encapsulating a core containing nucleic acids (DNA or RNA). DNA and RNA molecules are linear or circular and can be single-stranded or double-stranded. Currently known genome structures include linear dsDNA (e.g., λ , T7, T3, T4, P22 phage, etc.), circular dsDNA (e.g., PM2 phage, etc.), circular ssDNA (e.g., M13, fd phage, etc.), linear ssRNA (e.g., MS2 phage, etc.), segmented dsRNA (e.g., 46 phage, etc.) and so on.

The International Committee on Taxonomy of Viruses (ICTV) classified phages based on morphology and nucleic acid [27]. At present, phages are divided into 26 families, among which the long-tailed phage family (e.g., λ phage, etc.), the short-tailed phage family (e.g., T7, T3, P22 phage, etc.) and the filamentous phage (e.g., M13, fd, f1 phage, etc.) have been studied most deeply. Both the long-tailed bacteriophage family and the short-tailed bacteriophage family consist of an icosahedral head, a neck, and multiple tails.

According to the relationship between phage and host cells, the phage can be divided into lytic phage (e.g., T7, T3, P22 phage, etc.) and lysogenic phage (e.g., λ , M13, fd, f1 phage, etc.). The former is to inject its genome into the host bacteria to replicate nucleic acids, encode proteins, assemble into progeny phage and then lyse the host bacteria to release the progeny phage. However, the

Table 1
Phage libraries.

Fig. 1. Overview of phage-based nanobiotechnology. Phage-based nanobiotechnology has been applied to many material fields, including cancer therapy, scaffold development, biosensors, antibacterial materials, energy storage, and higher-ordered structures.

lysogenic phage could be secreted from the host cells but not lyse the cells.

T7 phage

T7 phage belongs to the *Podoviridae* family of the order *Caudo-virales*. It has an icosahedral head composed of a linear double-stranded circular DNA genome of 39,937 bp and inner core proteins (gp8, gp14, gp15, gp6) encapsulated by capsid proteins (gp10 A and gp10 B) connected with tail proteins (gp6, gp7, gp11,

gp12, gp17). Compared with filamentous phage, T7 phage has a shorter life cycle, about 17 min at 37 C from the infection to the lysis. Therefore, the T7 phage could be used in rapid detection and biopanning and can kill host bacteria (Fig. 2A) [28]. The T7 phage is a nanoscale particle with an icosahedron 55 nm in diameter and six tails 19 nm in length. Like the other *Caudovirales*, the T7 phage could be displayed with hundreds of copies of large molecular proteins (up to 1200 amino acids) on the coat protein and six copies of the peptide on the gp17 tail fiber proteins. The life cycle of the T7

Fig. 2. (A) Schematic of the T7 phage structure. (B) Schematic of the filamentous phage.

phage starts as the tail fiber recognizes the receptor of the host cell, followed by the viral genome being injected into the cytoplasm [29]. Then class I and class II are transcribed by host RNA polymerase and T7 RNA polymerase, respectively. After that, the host cell genome is degraded, and the T7 DNA polymerase replicates the T7 genome to form the viral genome concatemers. Then, the class III genes are transcribed by T7 RNA polymerase, and the resultant proteins are assembled into the empty procapsids and packaged with the T7 DNA. Finally, the mature phage lyses the host cells and is released, generating many copies of progeny phage particles.

M13 and fd phage

The filamentous phage, including M13, fd, and f1 phage, belongs to the *inoviridae* family of the order *Tubulavirales*. They have a nanoscale tubular structure with 7 nm in diameter and 700e1200 nm in length. The filamentous phage is constructed with a side wall arranged compactly with 2700e4000 copies of pVIII major coat proteins around the inner linear single-chained DNA and sealed with two blunt ends (five copies of pIII/pVI and five copies of pIX/pVII) (Fig. 2B). The genes of pIII [30,31], pVI [10,32], pVII [33,34], pVIII [35], pIX [36,37] of filamentous phage could be edited to process the phage display. The pIII could be displayed with peptides and proteins, while the pVIII could be displayed with peptides but large proteins due to the space resistance during the phage assembly. The filamentous phage can inject genome into host cells after the pIII recognizes the F factor of bacteria. The DNA replicates to obtain replicative forms, and the proteins translated from the genome wrap the replicated DNA to assemble into progeny phage, which comes out of the bacteria and gets released. Occasionally, it can enter the bacteriolytic cycle spontaneously under the stimulation of some external factors, leading to bacterial lysis. The life cycle of the filamentous phage needs about 30 min longer than the T7 phage [38].

Phage displaying technology

The phage display method is a molecular biology technique in which genes of exogenous peptides or proteins are inserted into phage-specific capsid protein genes by genetic engineering. The peptides or proteins encoded by exogenous genes are presented on the surface of the phage in the form of fusion proteins. It has been widely used in basic and applied research related to biomedicine, new energy, and environmental protection. The peptide or protein displayed can maintain relative spatial structures and biological activities, recognizing and binding the target molecules.

At present, in addition to the most popular and mature filamentous phage display system, there are also T7 phage, T4 phage, and lambda phage display systems. In comparison, filamentous phage has its unique advantages: (1) As a lysogenic phage, it is reproduced and expressed in the host bacteria without destroying the host bacteria. Instead, it continuously synthesizes progeny phage as the bacteria replicate. Therefore, it is convenient to amplify and obtain a large number of phages; (2) Because of its own size, it can stimulate the body to produce an immune response, and it can be directly used as a vaccine when displaying short-chain antigenic determinants; (3) The genome of filamentous phage has two states, ssDNA and dsDNA, and dsDNA can be more convenient for phage display. Filamentous phages are mainly composed of 5 capsid proteins (pIII, pVI, pVII, pVIII, pIX), and DNA encapsulated in them (Fig. 3A). Exogenous peptides or proteins are usually displayed at pIII protein and pVIII protein (Fig. 3BeD) [39,40]. The pIII protein is located at the tip of a filamentous phage, which takes part in the infectivity of the virus. Although the pIII protein has only five copies, it allows the insertion of protein sequences with more than 100 amino acids [41]. The

exogenous protein can be fused to the middle part or the N-terminus of the pIII protein. The peptide displayed at the N-terminus will lower the displayed phage infectivity unless it is assisted by a helper phage. The pVIII protein is the main coat protein of the virus, located on the sidewall of the filamentous phage. The peptide that can be fused at the N-terminus cannot be too long, typically around 10 amino acids long [42]. Only when the helper phage provides the pVIII protein, larger peptide chains can be displayed on the N-terminus. Otherwise, it will affect the assembly of the phage. In addition, the pIV protein exposed on the surface can also be used as a display site for exogenous protein to study the function of the C-terminus structure.

The filamentous phage used in phage display technology has certain limitations in peptide lengths. All the components of the phage structure must be exported through the bacterial inner membrane during the assembly, which is due to the lysogenicity of the phage. Thus, some proteins cannot be displayed because they cannot perform this process. However, the lytic phage display system can overcome this limitation [43]. T7 phage is a typical short-tailed lytic phage. Compared with filamentous phages, T7 phages have the following advantages [44]: (1) T7 phage has a shorter life cycle (only 17 min at 37 C), and can replicate at a high output [45]; (2) T7 phage head protein has small steric hindrance so that either the proteins or long-chain peptides can be fused well on the phage head and retain the natural conformation and biological activity; (3) The C-terminal of the T7 phage capsid protein is exposed on the outside, which is convenient for C-terminal display;

(4) T7 phage is stable and can maintain a strong infection ability under complex conditions. In the T7 phage display system, the peptide-encoding gene fragment is generally inserted into the gp17 gene, and the protein-encoding gene fragment is generally inserted into the gp10 b gene. In the T7 phage display system, exogenous peptides and proteins are generally displayed on gp17 protein or gp10 b protein (Fig. 3EeH).

Phage library

The phage is easy to cultivate and proliferate rapidly, which makes it easy to construct a phage library with a diversity of millions to billions through molecular biology techniques such as random PCR. According to the different exogenous fragments displayed, the library types are divided into peptide and antibody libraries [46].

Peptide library

In the random peptide library, the peptides displayed are generally linear, ranging from a few peptides to dozens of peptides in length [56,57]. Since displayed peptides larger than 20e30 amino acids will affect the infectivity of phage, peptide libraries are more suitable for displaying short peptides. Commercial phage peptide libraries have been out for many years, among which New England Biolabs created three commercial random peptide libraries (7-mer peptide library Ph.D.-7, 12-mer peptide library Ph.D.-12, and 7-mer cyclic peptide library Ph.D.-C7C), which were based on minor pIII protein of M13 phage. The random heptapeptide library can theoretically display 20^7 (approximately 10^9) peptides with different sequences. Modern cloning technology makes it possible to create peptide libraries

containing 10^8 - 10^9 clones. However, creating a library containing 10^{10} - 10^{11} clones is very difficult [58]. Therefore, peptide libraries usually consist of 10^8 - 10^9 independent clones. With the broadening of the application of phage display technology, the exogenous gene fragments inserted into phage have expanded from polypeptides to antibodies, proteins and enzymes [6], which has further promoted the establishment of corresponding phage libraries. Due to the diverse possibilities of phage

Fig. 3. Schematic of phage display technology. (A) Wild type filamentous phage. (B) pIII displayed filamentous phage. (C) pVIII displayed filamentous phage. (D) pIII and pVIII double displayed filamentous phage. (E) Wild type T7 phage. (F) gp17 displayed T7 phage. (G) gp10 b displayed T7 phage. (H) gp17 and gp10 b doubled displayed T7 phage.

surface display, phage libraries have become a powerful tool for screening targeted peptides through phage biopanning.

Antibody library

Antibodies are needed to protect the human body against invading viruses, bacteria, cancer cells, and some other immunogens. The traditional preparation method of the antibodies is purifying the B lymphocytes derived from the human infected by the pathogens, then isolating the cells which secrete the best antibody, and amplifying the cells to prepare the antibodies. The method is time-consuming and expensive. Therefore, the phage antibody library has been designed and prepared via phage display technology and used to select the human antibodies [59]. The phage library contains many variable heavy (VH) and variable light (VL) chains that are rearranged together and displayed on the phage coat protein to form a phage library. Firstly, human cells are purified, and the mRNA needs to be extracted from the cells. Second, the mRNA will be reverse transcribed to get the cDNA. Third, the specific primers are designed to amplify all the VH and VL chains using the over-lap PCR to form the DNA of the single-chain fragment variable (scFv). Fourth, the prepared DNA is inserted into the phage display vector to form a phage scFv antibody library [30,60,61]. The phage Fab antibody library is obtained if the Fab fragment is inserted into the phage vector [62,63].

The phage antibody library has been used to select the antibody to many targets, such as receptors of Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [64,65], SARS-CoV-2 virus [66,67], SARS-CoV virus [68], HIV-1 [69], pemphigus [70], hepatitis A virus [71,72], hepatitis B virus [73], hepatitis C virus [74], rabies virus [75], H5N1 virus [76], tetanus [77], candida albicans [78], cancers (hepatocellular carcinoma [74], breast cancer stem cells [79], colon carcinoma [80], lung adenocarcinoma cells [81], Ewing's sarcoma tumor cells [82]), proteins (gastrin receptor [83], TNF- α [84], aflatoxin [85]), and so on. Compared with the phage peptide library, the protein displayed on the antibody library has better

specificity and stronger affinity to the target as the 3D structure is more complex, and the antibody could be divided from the phage for subsequent use. There are many commercial premade phage antibody library based on the M13 phage (like CAT. #: HuScL-4, Creative Biolabs), T7 phage (like Pre-Made T7Select® Libraries, EMD Millipore) and Lambda phage (like Cat. No. 634144, Clontech).

Phage biopanning technology

Phage biopanning technology utilizes appropriate target molecules to perform affinity screening on the phage library to obtain target-specific proteins or peptides [86]. The phage library is incubated with targets such as antibodies [87], ligand/receptors [11], enzymes [88], cells [89], organs [19,20], nanoparticles [21], compounds [18], semiconductor materials [24], etc. After incubation, the clones that cannot bind to the target are washed away, while the bound phages are eluted with competitive molecules. A portion of the eluted phage is used to measure the concentrations. Then, the residual phages with a strong affinity to targets are amplified and enriched by *E. coli*. The amplified phages are used as an input for the next round of screening. During 3×10^5 rounds of screening, since the amount of the phage input is constant, the concentration of eluted phage is gradually increased, which can prove that the specific binding phage is gradually enriched. After 3×10^5 rounds of enrichment, the proportion of phage that specifically recognizes the target is gradually increased. Therefore, we could obtain the peptide/protein sequences through gene sequencing and employ them to proceed to the following research. The above process is an *in vitro* biopanning technology, and a schematic diagram of its specific operation process is shown in Fig. 4. Biopanning technology *in vivo* is different from *in vitro*, which is to inject a phage-displayed peptide library into mice from the tail vein to screen out peptides that can target tumors or specific organs. All peptide sequences obtained through biopanning technology have been stored in a special free database called BDB [90,91].

Fig. 4. Schematic of the *in vitro* biopanning technology.

A significant number of peptide ligands have been discovered through biopanning technology. Rowley et al. discovered the immunodominant epitope of pyruvate dehydrogenase complex (PDC) E2, which reduces the inhibitory effect of primary biliary cirrhosis (PBC) serum on PDC enzyme activity [92]. Dieltjens et al. revealed the antigenic structure of a patient infected with HIV-1 subtype A with a wide range of cross-neutralizing antibodies, which has laid an essential foundation for developing preventive vaccines containing excellent antigen peptides [93]. Using the actual bacterial inner membrane as a target, Tsuyoshi Tanaka successfully carried out magnetic screening of antimicrobial peptides from the phage library based on bacterial magnetic particles (BacMPs), which may be a promising high-throughput screening method of antimicrobial peptides for various species [94]. Whaley et al. applied this technology to semiconductor materials and determined peptide sequences that were specifically bound to five different single-crystal semiconductors, which can be used for the controlled placement and directional assembly of important materials [95]. In addition, peptides that recognize different tissues and malignant tumor cells can also be screened. Chamarthy re-

ported the sequence of a heptameric peptide, which targets dendritic cells (DCs) in a TNF α -specific manner, provides an efficient gene delivery system for DC responding to multiple immune functions [96]. Kelly et al. discovered peptides targeting cell surface antigens on pancreatic ductal adenocarcinoma (PDAC) cells. Coupling the targeting peptides with magnetic fluorescent nanoparticles has shown specific utility *in vivo* imaging [97].

Phage in nanobiotechnology

Phage as a carrier in drug and gene delivery

Phages, themselves or in combination with some traditional nanocarrier particles, can be used as carriers for drug and gene delivery. Drugs can be directly coupled to the phage surface via chemical bonds and genes can be inserted into phage genome by means of genetic engineering (Fig. 5AeB). The traditional nanocarriers can also be assembled with phages through electrostatic adsorption, chemical bonds or other forms to obtain new composite carriers (Fig. 5CeD).

Drug delivery

Phages can be used as vehicles for delivering cytotoxic drugs. At present, the commonly used methods of administration include gastrointestinal, oral, and transdermal delivery [99]. However, the therapeutic effects of drugs are usually limited by physiological barriers. Besides, due to the high toxicity to the host, more and

more drugs are excluded from treatment. Drug delivery systems are designed to solve problems related to drug targeting ability, toxicity, stability, solubility, as well as bioavailability [100,101]. Chemical groups on phages such as amino, carboxyl, and phenolic groups can be used as linkers for modifying drugs, overcoming the high toxicity of drugs [76] and maintaining their biological activity. As an innovative nanomedicine, drug-loaded phages can be used to treat different diseases. Phages have been used to improve the therapeutic effects of antibiotics, such as chloramphenicol. Antibiotics are connected to the phage through an aminoglycoside linker, which greatly reduces their immunogenicity compared with natural phage particles or particles directly bound to drugs [102]. Phage-based drug delivery systems can also carry anti-cancer drugs. Mao's group combined photosensitizer pyropheophorbide-a with a filamentous phage that targeted breast cancer cells to form a photosensitizer-phage complex, providing a new type of photodynamic therapy for breast cancer [103].

Gene delivery

In addition to delivering drugs, phages can also be designed to deliver genes and be used to treat antibacterial and various diseases. The efficiency of phage for DNA delivery is not high. Different genetic modification methods of phage can make it have targeting ability and promote the delivery of DNA into cells. Larroca et al. displayed FGF2 on the minor coat protein PIII of phage, using GFP as the reporter protein, and published the first article on gene targeting filamentous phage for mammalian cells (COS-1 cells) gene transfer [104]. Two years later, they used multivalent phagemid particles to increase the transduction efficiency to 9%, but this is still far lower than traditional transfection reagents like liposomes [105]. Hajitou et al. designed a new system containing components from adeno-associated virus (AAV) and filamentous phages. This new AAV/phage (AAVP) chimera combines the excellent gene delivery properties of eukaryotic viruses and the non-tropism of phages, which enhances gene delivery efficiency [106]. To further improve the transduction efficiency of the AAVP system, Kia et al. introduced a stress-inducible Grp78 tumor-specific promoter, which combined histone deacetylation inhibitors and DNA methylation inhibitors, to promote gene expression of cancer cells [107]. So far, using phages as a delivery system to introduce genes into animal cells is a feasible solution. The key question is finding out the best solution to improve transduction efficiency.

Cancer therapy

Current cancer treatment methods include surgery, chemotherapy, radiotherapy, hormone therapy, and other emerging nano-

Fig. 5. Phage-based carriers for drug and gene delivery. (A) Phages can be chemically modified to load drugs. (B) Phages can be genetically engineered to carry genes. (C) Liposomes or (D) nanoparticles combined with phages for drug and gene delivery. Reprinted with permission from Ref. [98]. Copyright 2017, Informa Healthcare.

biotechnologies such as immunotherapy. The displayed proteins or peptides could make phage specific for certain cancer-related cells and tissue components and become a key candidate for cancer treatment. To achieve more significant therapeutic effects, the displayed proteins and peptides can be further combined with other therapeutic reagents (e.g., drugs and nanoparticles). In addition, the specific peptides obtained by phage biopanning can be designed to modify other drug carriers such as liposomes for targeted cancer therapy.

Many groups have studied the interaction between phages and cancer cells. Dabrowska et al. [108e110] conducted a similar study on T4 phage. They found that T4 phage could interact with $\beta 3$ - integrins receptors on cancer cells, then regulate human immune response, and inhibit tumor expansion. The high level of reactive oxygen species (ROS) can accelerate cancer cell proliferation and tumor growth in the tumor microenvironment. It has been reported that phages could reduce the level of ROS produced by phagocytes in the presence of bacteria [111,112]. The phage-mediated ROS inhibition is due not only to the interaction between phages and phagocytes but also to the interaction between phages and lipopolysaccharides on bacteria. Meanwhile, the phage can trigger an immune response as it is an exogenous antigen, so the immunogenicity and retention time of the phage needs to be considered when continuously using the phage as a therapeutic vector [113].

Many therapeutic drugs, including anti-cancer drugs, antibiotics, cytokines, and radionuclides, have been excluded from anti-cancer candidate drugs due to their high toxicity and large doses. Using phage-mediated drug delivery carriers and combining these therapeutic drugs with nanoparticles can achieve targeted delivery of drugs, exert therapeutic efficacy, and reduce toxic and side effects [11,114].

Breast cancer

Breast cancer has the highest morbidity, especially for women [115]. Mao group [116] allowed anionic phages to electrostatically self-assembled with cationic liposomes. Then, the photosensitizer zinc phthalocyanine (ZnPc) was loaded in the lipid bilayer of the liposomes, enabling more efficient photodynamic therapy (PDT). Torchilin's group [117,118] used an 8-mer phage library to screen for MCF-7 breast cancer cell-specific peptides. By directly integrating the peptide-fused phage pVIII protein into liposomes with the anti-cancer drugs (Dox) to prepare phage-Doxil nanoparticles, they created a novel nano-drug for targeting treat breast cancer tumors. Mao group [11] double-displayed the breast tumor homing peptide and angiogenin-binding peptide on the fd phage using the fd388 phage vector.

The results showed the engineered phage could inhibit the angiogenesis of the vessels within the tumor and

then prohibit the tumor growth. The targeting peptides on phage were combined with therapeutic peptides to reduce the side effects of the off-target effects of the traditional anti-angiogenesis medicine (Fig. 6AeC).

Colorectal cancer

Zheng et al. [119] developed a phage-mediated targeted nanomedicine, which was successfully used to regulate the intestinal flora and effectively enhance the effect of tumor chemotherapy. They used the irinotecan-loaded dextran nanoparticles, which were covalently linked to phages modified by azide, for oral and intravenous administration in mice with in situ colorectal cancer (CRC) and spontaneous CRC. Therapeutic results showed that the nanomedicine significantly inhibited the growth of tumor-promoting *Fusobacterium nucleatum* (F.n), and promoted the production of butyric acid by anti-tumor bacteria, thereby greatly improving the efficiency of first-line chemotherapy for CRC patients (Fig. 6DeE). Genetically engineered phages can also be directly chemically coupled with anticancer drugs [33,120].

Based on the targeting peptide selected via phage biopanning, many other targeted delivery vehicles (e.g., liposomes) for anti-cancer drugs have been constructed. With endothelial progenitor cells (EPCs) involved in tumor angiogenesis as the target of anti-cancer drug delivery, Tatsuya et al. [121] screened the tumor vascular targeting peptide ASSHN. ASSHN peptide modified PEGylated liposomes (ASSHN-Lip) can be used as a targeted delivery carrier for DOX. Compared with drug-loaded liposomes without peptide modification, ASSHN-Lip coated with DOX can significantly inhibit mouse tumors growth.

Brain carcinoma

The mortality of brain cancer, neither neuroglioma nor meta-static tumor, is very high because of the brain blood barrier (BBB). The BBB stops the large molecular medicine and hydrophilic drug. Arap group [106] displayed the RGD peptide on the M13 phage and inserted the GFP gene into the adeno-associated virus (AAV) vector. After that, the authors recombined the phage-RGD4C gene and AAV-GFP gene to prepare the AAV-GFP/phage-RGD4C (AAVP) nanoparticles and used the AAVP to image the tumor. Years later, the same group [122] displayed an octreotide peptide on the M13 phage and inserted the tumor necrosis factor (TNF) into the AAV, then recombined them to construct the Oct-AAVP-TNF virus nanoparticles. The octreotide peptide could induce the Oct-AAVP-TNF nanoparticles to enter the neuroendocrine tumors and inhibit the tumor growth. Recently, they inserted the Herpes simplex virus thymidine kinase (HSVtk) to form the RGD4C-AAVP-HSVtk, then combined the RGD4C-AAVP-HSVtk and ganciclovir (GCV) for a suicide gene therapy to the glioblastoma [123]. In 2020,

Fig. 6. (A) The dual-functional fd388-AR-WV phage displayed with tumor-homing peptide and angiogenin-binding peptide. (B) The tumor growth curve after treatment with fd388-AR-WV phage. (C) The influence of the engineering phage to the tumor vessels. (D) Phage-guided biotic/abiotic nano drug delivery system and its treatment principle schematic. (AeC) Reprinted with permission from Ref. [11]. Copyright 2020, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (E) Tumor targeting mechanism of A-phage (azide-modified phage) and D-IDNPs (azodibenzoicyclooctyne (DBCO) modified irinotecan-loaded dextran nanoparticles) (F) Fluorescence imaging of the covalent linkage efficiency between A-phage and D-IDNPs. (DeE) Reprinted with permission from Ref. [119]. Copyright 2019, Springer Nature.

Hajitou group [124] used the RGD4C/AAVP-GFP to image the Glioma (9 L).

Hepatocarcinoma

Hepatocarcinoma is a unique malignancy with high mortality. The liver cancer can always be induced by the HAV, HBV, HCV and other elements like fat, alcohol, aflatoxin and so on. Therefore, the researchers are looking for a new method of detection and therapy. Du et al. [125] selected the liver cancer-specific binding peptide AGKGT PS LETTP (A54), and chemically coupled the synthetic peptide directly with DOX for animal experiments. Experimental results showed that compared with DOX associated with mutant

polypeptideAGKGTAALETTP(A54M),A54-DOXpresented a more significant effect of targeted therapy for liver cancer.

In 2020, Jinping and colleagues [126] used the T7 phage library displayed with 56-amino acid (aa) peptide to process the phage immunoprecipitation and sequencing (VirScan PhIP-seq) of serum samples of 899 individuals. The results indicated that this VirScan PhIP-seq could identify individuals at a median follow-up 8.8 years prior to a clinical diagnosis of hepatocarcinoma. This is very important to early detection of malignancy and could provide more opportunities for treatment and improve the cancer-related five-year survival rate.

Lung carcinoma

Lung carcinoma has the second-highest incidence rate, just lower than breast cancer deaths [115]. Ren et al. [127] displayed mouse Flt4 (mFlt4) on the surface of T4 phage to elicit antitumor immune responses. The results showed that T4-mFlt4 recombinant phages could inhibit tumor metastasis. In 2014, two groups [128,129] constructed the T7-phage cDNA library using the lung cancer patients' RNA and picked up the common clone by sequencing analysis. The picked phage clones could be used to detect the biomarker of early lung cancer. In 2019, the Ren group constructed virus-like nanoparticles by displaying the mouse VEGFR2 on the surface protein of T4 phage. The engineered T4 phage nanoparticles could inhibit tumor angiogenesis and subsequent tumor growth [130].

Other cancers

Stomach cancer is a fifth-highest morbidity cancer, and its strongest singular risk factor is *Helicobacter pylori*. The *Helicobacter pylori* are not sensitive to a certain antibiotic and some antibiotic-resistant bacteria appeared after treatment with many antibiotics [131]. So the phage treatment of *Helicobacter pylori* is a unique approach. In 2021, SaraKhosravi et al. [132] isolated the phage, which could specifically lyse the *Helicobacter pylori*.

Nisaraporn et al. [133] used M13 phage as a shell to encapsulate polymer nanocores. M13 phage was chemically coupled with folic acid (FA) through an amino group (FA-M13) to target folate receptor-positive nasopharyngeal cancer cells. The poly (caprolactone-b-2-vinylpyridine) (PCL-P2VP) nanoparticles, loaded with the hydrophobic anticancer drug doxorubicin (DOX), achieved pH-responsive release in the tumor microenvironment with low pH value. The DOX-loaded FA-M13-PCL-P2VP self-assembled nanoparticles have a significantly higher anti-tumor therapeutic effect than free DOX.

Phage-based vaccines

The infection (especially bacterial, viral, and parasitic infection) and cancer have been claimed to be the two of ten broad human diseases. The previous scientific research and clinic trials have confirmed that many of the two diseases could be effectively prevented and treated by the vaccine. Although the common vaccine (e.g., the live attenuate vaccines, inactivated vaccines, DNA vaccines, etc.) have been widely used, some bottleneck problems restricted its clinic application. The first problem is the low temperature, even 70 C, is essential in the transport and storage of the common vaccines to maintain the biomedical function [134]. The second problem is that the common live attenuate vaccines and inactivated vaccines have a risk of infection as they are derived from the real pathogen [135]. The third problem is that the complex human structure decreases the immune effect of the DNA vaccine, which did not have the targeted delivery property [136].

The phages could be applied to solve the three bottleneck problems due to its unique advantage. The phages are chemically and physically stable to the environmental conditions (e.g., pH value, temperature, salt concentration, etc.), making it easy to transport and store the phage vaccines [137]. The phages specifically infect the prokaryotes not eukaryotes for amplification [52], so they are safe and friendly to human body while they are used in vaccine development. The phage display technology could impart the targeting property (derived from the displayed peptide or protein), and the phage itself could work as the immunologic adjuvant of vaccine to stimulate the immune response [138]. T4 phage is considered a platform for arraying multiple antigens from pathogenic organisms, and the resulting virus-like nanoparticles (VLPs) are highly immunogenic and can be used for vaccine design [139]. In 2019, Bao and colleagues [140] summarized the phage-based vaccine used in preventing fungal/viral/bacterial infection, cancer, and parasites.

The recent infection disease COVID-19 is carried out by the SARS-CoV-2. Pasqualini et al. [141] displayed the SARS-CoV-2 spike (S) protein and a ligand peptide (CAKSMGDIVC), which could make the phage transfer through lung epithelium into blood, on the pVIII and pIII of filamentous phage, respectively. Meanwhile, the authors knocked the entire S protein gene in the AAVP gene to make the engineered AAVP could induce the antibody response to the spike (S) protein. Then, the authors used the two vaccines to perform the immunization (Fig. 7).

The number of patients with bone defects and bone injuries due to various reasons is increasing every year. Effective treatment of bone defects is one of the clinical problems in orthopedics. Repairing bone defects and rebuilding bone function is an urgent task for scientific researchers. In tissue engineering, an ideal scaffold should not only have suitable pores to accommodate adult stem cells and other growth factors but have good biodegradability to facilitate tissue regeneration [142]. Genetically engineered phage has a modifiable surface, which has been widely applied in bone tissue regeneration.

The RGD (Arg-Gly-Asp) peptide in the extracellular matrix can specifically recognize the integrins on activated endothelial cells (ECs) to regulate migration and adhesion of ECs, and then induce angiogenesis and bone regeneration. Kim et al. [143] designed a composite scaffold composed of RGD-M13 phage/alginate and poly (ϵ -caprolactone) (PCL), presenting the potential of biomedical scaffolds for bone tissue regeneration. Among them, PCL imparts good mechanical strength to the scaffold, while phage/alginate provides excellent biological activities such as osteogenic differentiation, biomineralization, and protein absorption capacity. Han et al. [144] developed a hybrid nanofiber sheet obtained by electrospinning a mixed solution of poly (lactic-co-glycolic acid) (PLGA) and RGD-M13 phage, which shows significantly enhanced cell adhesion. Our team electrostatically integrated the negatively charged RGD phage into chitosan-decorated 3D printed bone mineral scaffold [13]. The resulting scaffold was inoculated with MSCs and implanted in the bone defect site. Eventually, it was found that the phage-integrated 3D-printed scaffold could induce the formation of vascularized bone tissue (Fig. 8).

Bone scaffolds containing hydroxyapatite (HAP) have properties close to those of natural bone. The presence of HAP-binding peptides helps introduce HAP into the scaffold to obtain better bone conductivity and osteoinductivity. The use of phage biopanning technology can successfully identify HAP binding peptides. Sarikaya et al. [145] used Ph.D.-C7C phage library to screen out the HAP binding peptide HAPB1. The same group [146] combined HAPB1 peptide with a self-assembling peptide, VKVKVKVKVDPPTKVEVKVKV (MAX8), to construct a bifunctional

Fig. 7. Schematic of the phage- and AAVP- based nanoparticle used as vaccines. Reprinted with permission from Ref. [141]. Copyright 2021, The National Academy of Sciences.

peptide MAX8-HAPB1. The MAX8 part gives the combination peptide the ability to self-assemble and form a hydrogel, while the HAPB1 fragment can guide the biomineralization of the hydrogel. This three-dimensional HAP-hydrogel structure provides a new idea for bone tissue engineering. Kohn et al. [147] used phage display technology to identify human mesenchymal stem cells (MSCs) specific binding peptide (DPI). Then, they combined DPI with the previously identified peptide sequences (VTK), containing high affinity to apatite, to produce a peptide made of two motifs, DPI-VTK [148,149]. DPI-VTK improved the adhesion strength of human MSC to the surface of specific biological materials, subsequently increasing the cell proliferation and differentiation.

Bone morphogenetic protein 2 (BMP-2), an essential factor, in bone development, can effectively induce the differentiation of osteoblasts in many cell types [150]. Phage biopanning technology can be used to screen out peptides that specifically bind to these targeting proteins. These peptides can modulate the function of their target protein, or help the protein to be fixed on the scaffold to promote bone regeneration. Hodges et al. [151] identified the peptide sequence of the BMP-2 binding domain. This peptide was further combined with the collagen affinity peptide. The resulting bifunctional peptide could promote bone formation and increase osteogenic activities. Stupp et al. [152] used a 12-mer peptide library to successfully identify peptide with high affinity binding to

Fig. 8. RGD phage-filled scaffold promotes bone regeneration. (A) Schematic diagram of scaffold structure design. (B) The Micro-CT image of bone repair contains (B-a) normal control, (B-b) blank control, (B-c) scaffold, (B-d) negative control (wild-type phage), (B-e) experimental group (RGD phage) and (B-f) positive control (RGD phage and VEGF). (C) Immunofluorescence staining (a, c, e) and H&E staining (b, d, f) images of negative control (C-a, b), experimental group (C-c, d) and positive control (C-e, f) newly formed blood vessels. (D) Number of newly formed vessels. Reprinted with permission from Ref. [13]. Copyright 2014, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

BMP-2. In a follow-up study, they assembled the peptide into peptide amphiphile nanofibers to enable it to recruit BMP-2 and reduce the dosage of exogenous BMP-2 [153]. Puzas et al. [154] used tartrate-resistant acid phosphatase (TRAP) (type V) as a biopanning target and identified transforming growth factor- β (TGF- β) receptor-interacting protein (TRIP-1) from the T7 phage library. TRAP takes part in many biological processes, including bone development. After TRAP specifically binds to TRIP-1, it activates the TGF- β differentiation signaling pathway, thereby regulating the differentiation of osteoblasts.

Biosensors

Researchers have used phages as biosensors for ultrasensitive detection of nucleic acids, proteins, organic compounds and metal ions. The signal generation principles of phage-based bio-monitoring methods include the following aspects: (1) Phages are separated from the complexes and infect the host bacteria to form plaques, which are further counted to measure the target numbers;

(2) chemically or genetically modified phages are integrated with nanomaterials, and analyzing the optical properties of nanomaterials can result in the determination of the target concentrations; (3) phages are engineered to display fluorescent proteins, and collecting the fluorescence signal intensity can lead to target detection; and (4) phage-specific gene bands are PCR-amplified, and the intensity of the bands reflects the number of target molecules captured in the complexes.

Detecting nucleic acids

Detection of specific nucleic acids is of great significance for disease diagnosis. MicroRNAs (miRNAs) are actively secreted by tumor cells and are closely related to the development of tumors, so they can be used as tumor biomarkers. The Singapore research team successfully discovered 12 miRNAs, which can distinguish gastric cancer patients from ordinary patients with an accuracy of over 92% [155]. Based on this set of miRNA biomarkers, the team successfully developed a gastric cancer blood detection kit that meets clinical standards, bringing miRNA detection to clinical application for early cancer screening. Zhou et al. [28] developed a molecular diagnostic method capable of quantifying cancer marker miRNA with naked eyes. The constructed genetically engineered green fluorescent T7 phage can bind to gold nanoparticle (GNP) in a one-to-one manner. GNP and magnetic microparticle (MMP) were functionally modified by the complementary oligonucleotides of two fragments of the miRNA. According to the principle of base complementary pairing, target miRNAs were captured by modified T7-GNP and MMP to form a sandwich complex. The complex was separated with a magnet, and the gold-binding peptide (GBP) was used to compete with T7 phage for binding to GNP. The separated T7 phage infected the host bacteria in the solid medium, and the number of fluorescent plaques produced were used to quantify target miRNAs (Fig. 9).

Detecting proteins

In addition to detecting nuclei acids, molecular diagnostic methods targeting proteins can also help assess a patient's disease risk, further advancing the development of precision medicine. The research of Siti Salwa Hasmoni and colleagues [156] has proved that the recombinant M13 phage with the peptide sequence of WSFFSNI has the ability to specifically bind the core antigens (HBcAg) of hepatitis B virus (HBV), which can be further used for detection and purification of HBcAg. Wang et al. [51] designed a bifunctional fd phage that can detect the anti-secreted aspartyl proteinase 2 IgG antibody (anti-Sap2-IgG), a biomarker of *Candida albicans* (*C. albicans*) infection, in serum. The bifunctional fd phage

displayed peptides capable of targeting anti-Sap2-IgG and peptides specifically binding magnetic nanoparticles (MNPs) on pIII and pVIII proteins, respectively (Fig. 10A). Magnetic phages formed after incubation of functionalized phages with MNPs were added to serum samples to capture target proteins, and the complexes could be separated under the action of magnetic force (Fig. 10B). Phages were eluted from the complexes and finally the marker proteins were detected in patient serum by ELISA analysis (Fig. 10C). Sano et al. [157] first reported an antigen detection system based on immuno-PCR technology with a higher sensitivity. Guo et al. [158] introduced a recombinant phage with surface-fused single chain variable fragment (scFv) capable of specifically binding to two viral proteins. The DNA of the phage was then amplified by PCR to achieve sensitive detection of viral antigens. Hou et al. [159] displayed a customized scFv at the end of M13 phage that could specifically bind to the carcinoembryonic (CEA) antigen expressed by colon cancer cells such as Caco-2 and HT29.

Detecting organic compounds

Organic compounds such as explosives, drugs, spoiled food, etc. are harmful to human life and health. The volatile organic compounds (VOCs) produced by them can be detected for early prevention. Oh et al. [160] reported a biomimetic colorimetric sensor consisting of genetically engineered M13 phage fused with a trinitrotoluene (TNT) affinity peptide, which can detect TNT down to 300 ppb and discriminate chemical molecules of similar structure. Similarly, Moon et al. [161] prepared biomimetic colorimetric sensors for the detection of endocrine disrupting chemicals such as benzene, phthalic acid, and chlorobenzene derivatives. As a VOC, benzaldehyde (C_6H_5CHO) can be used as a potential biomarker of lung cancer [162]. Tanaka et al. [22] screened three peptides with high affinity for benzaldehyde from the phage library, which can be applied in the design and development of benzaldehyde sensors. Lee et al. [23] designed an electronic nose based on recombinant phages, which can be used to monitor the freshness of fruits.

Detecting metal ions

Phage-based biosensors can also be used to detect metal ions. Low levels of mercury (Hg^{2p}) can cause serious threats to human health and the ecological environment [163]. Therefore, it is necessary to develop highly sensitive and selective Hg^{2p} detection biosensors. Wang et al. [164] obtained M13 phage with high affinity for Hg^{2p} by biopanning with a 7-mer peptide library. Gold nanoparticles (GNPs)-phage networks were constructed by in situ growth of GNPs on the surface of the Hg^{2p} -binding M13 phage. After adding Hg^{2p} , the engineered M13 phage specifically bound to it, which was subsequently reduced to $Hg(0)$ by the abundant reducing amino acid residues of the phage coat protein and deposited on the surface of GNPs. Hg^{2p} was quantitatively detected by the blue shift of surface-plasmon resonance (SPR) peak of GNPs and the change of absorbance, leading to a limit of detection (LOD) being 8×10^{-8} mol/L. Yang et al. [165] prepared silver nanoparticles (AgNPs)-M13 phage complexes under alkaline conditions. In the presence of Cr^{3p} , Cr^{3p} could be quantitatively analyzed by measuring the absorbance of AgNPs with high selectivity. Due to the high content of Cr in dental alloys, the content of Cr^{3p} was related to the degree of corrosion of dental alloys, indicating the importance of detecting this species by the phage-based probes.

Detecting bacteria

According to the natural ability of phages to infect host bacteria, bacterial detection methods based on phage have been developed for many years. Meanwhile, in the presence of dead bacteria, advanced molecular detection methods such as mass spectrometry, polymerase chain reaction (PCR), and enzyme-linked

Fig. 9. T7 phage-based biosensor for quantifying cancer marker miRNAs. (A) T7-GNP and MMP were combined with miRNA to form a sandwich structure, which was then separated by a magnet, and the green fluorescent plaques were counted with the naked eyes to determine the amount of miRNA. (B) Image of the recombinant green fluorescent T7 phage. (C) Plaque formation on the petri dish. (D) Green fluorescent plaques under fluorescence scanning in the same petri dish. Reprinted with permission from Ref. [28]. Copyright 2015, Springer Nature. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

immunosorbent assay (ELISA) tend to show false positives results. In addition, compared with antibodies, phages cost less, and are also resistant to extreme pH, temperature and organic solvents [166,167].

So far, two design types of phage-based biosensors have been developed in detecting bacteria. One is to capture bacteria and generate sensing signals on the surface of the sensing element or on the phage probe. Xu et al. [168] developed a T4 phage based micro electrochemical biosensor (T4B-MES) to detect viable *E. coli* B cells. The phage immobilization scheme optimized by external electric field and chemical functionalization can achieve an extremely LOD of about 14 CFU/ml, and has a wide testing range from 1.9×10^1 to

1.9×10^8 CFU/ml. Farooq et al. [169] achieved a good balance between LOD and analysis time by reaching a LOD of 3 CFU/ml in 30 min. In this study, the electrochemical biosensor contained phages in a surface-modified bacterial cellulose (BC) matrix with a high density, and such biosensors could be used in food safety.

In comparison, methods that use phage to infect bacteria to amplify signals have lower LOD. Hinkley et al. [170] customized a phage genetically engineered with a reporter enzyme for *E. coli* and developed a membrane filtration assay based on T7 phage. The highly active reporter enzyme was fused with the cellulose-specific carbohydrate binding module (CBM) gene to fix the reporter probe. After the bacteria were lysed, the *E. coli* could be quantitatively determined by the naked eye or by the bioluminescence imaging of the reporter enzyme. This method could reach 1 CFU/100 ml LOD in about 10 h. The same team [171] used the same method to create the reporter phage NRgp5, which was combined with syringe filtration to reduce the LOD by half and detected *E. coli* with less than 20 CFU/100 ml in 5 h. The study by Duong et al. [172] showed

that the LOD reduction of T4 phage-based biosensors can be achieved by optimizing the expression of reporter enzymes.

Antibacterial materials

Antibiotics are traditional reagents of fighting bacterial infections. As the antibiotics abuse increases the drug resistance, this method has limitations. The new

nanostructured antibacterial materials based on phages are promising to replace antibiotics to prevent and control bacterial infections.

The skin of patients with inflammation and burn wounds is particularly susceptible to bacterial infections. Phage-based wound dressings can overcome the problem of antibiotic resistance, improve skin infections, and regulate the skin microbiome. Sarhan et al. [173] used honey, polyvinyl alcohol, and chitosan (HPCS) nanofibers as raw materials for electrospinning and loaded them with bee venom (BV), propolis (Pr) and/or *P. aeruginosa* phage (PS1). In three new types of nanofibers wound dressings (HPCS-Pr, HPCS-BV and HPCS-BV/PS1), HPCS-BV/PS1 demonstrated the strongest antibacterial ability against all tested strains, especially the multi-drug resistant *P. aeruginosa*. Compared with HPCS-BV, the addition of PS1 phage enhanced the wound healing effect and biocompatibility of nanofiber dressings, making HPCS-BV/PS1 extremely competitive in wound dressings. In the same year, Nogueira et al. [14] demonstrated the potent antibacterial activity of vB_Pae-Kakheti25 phages against *P. aeruginosa* and developed polycaprolactone (PCL) covalently fixed with vB_Pae-Kakheti25 phages through amide bonds to maximize the exposure of its tail, greatly improving the antibacterial properties of the wound dressing (Fig. 11). Cheng et al. [174] incorporated T4 phage into

Fig. 10. Detection of marker proteins of *C. albicans* infection in serum by bifunctional phage. (A) Construction of anti-Sap2-IgG-binding MNPs-binding bifunctional PK-ASIT-phage and formation of magnetic ASIT-MNP-phage. (B) Magnetic phages capture the target protein in serum samples, and the phages are eluted after separation with a magnet. (C) ELISA assay to detect the marker proteins bound on the eluted phages. Reprinted with permission from Ref. [51]. Copyright 2015, American Chemical Society.

polycaprolactone/collagen I (PCL-CollI) membrane by electrospinning, which showed an antibacterial efficiency of more than 90% against *E. coli*. The membrane also had a good hemostatic effect.

Choi et al. [175] covalently immobilized T4 phages that specifically infect *Escherichia coli* on a polycaprolactone (PCL) film, and the T4 phage-modified PCL film can be used as an antibacterial food packaging material. Fixing phages on a surface-modified cellulose (BC) film can be used not only for food safety testing [169], but for food packaging. Anany et al. [176] attached negatively charged phage heads to the surface of a positively charged modified cellulose membrane to achieve directional high-density immobilization of phage, which effectively killed the two pathogenic bacteria to prevent their growth in meat. Lone et al. [177] studied three

different methods of introducing phages into food packaging antibacterial materials. One is to immobilize the phages on a surface-modified cellulose membrane, the second is to impregnate the paper with a phage suspension, and the other is to encapsulate the phages in alginate beads followed by coating the beads on the paper. No matter what kind of antibacterial packaging material is used, it can reduce the number of pathogenic bacteria to ensure food safety, and provide a reference solution for the safety problem of the food industry. One of the main obstacles to the application of phages as biologically active antibacterial agents in food safety is their long-term antibacterial activity. Leung et al. [178] reported the use of a pullulan/trehalose film to maintain the phage activity and suggested avoiding the presence of buffers that caused crystal formation in the film. In another study, they once again studied the

Fig. 11. (A) Antibacterial principle diagram of vB_Pae_Kakheti25 bacteriophage-PCL nanofiber. (B,C) The percentage of microbial inhibition of vB_Pae_Kakheti25 phage-PCL after three times of rinsing (no washing cycles), 5 and 25 washing cycles at 0 h (B) and (C) 24 h, compared with the natural PCL control group. Reprinted with permission from Ref. [14]. Copyright 2017, Elsevier.

protective effect of the preparation and storage conditions of the film on the phage as an antibacterial agent [179]. According to the research results, phage-based pullulan/trehalose antibacterial films need to be prepared under low humidity conditions and vacuum dried, and they should be sealed or stored in a vacuum before use. Alves et al. [180] added the 4IBB-PF7A phage to the calcium chloride cross-linked sodium alginate film to prevent meat spoilage caused by the growth of *P. fluorescens*.

The harmlessness of phages to humans has led to the emergence of edible antibacterial materials. Huang et al. [181] developed an edible whey protein isolate (WPI) coating on the fish feed. The coating was designed to improve the loading efficiency of phages and maintain the antibacterial activity. The research provides an economical and effective method to solve bacterial infections in fish and seafood, meeting the increasing safety requirements of the aquaculture industry. Amarillas et al. [182] used the natural polymer chitosan as an edible coating and a carrier of phages to protect fresh vegetables such as tomatoes from contamination by pathogenic microorganisms. Tomat et al. [183] evaluated an edible whey protein concentrate (WPC) film supplemented with six lytic bacteriophages, which showed high stability, effective release and antibacterial activity against pathogenic *E. coli* strains. To sum up, several kinds of phages have been used to prevent the bacterial infection in meat, fish, sea food and fresh vegetables.

Bacterial infections are believed to be responsible for the 5e10% yield loss of many important food crops [184]. Traditionally, antibiotics have been used to eliminate pathogenic bacteria in crops. However, the dose of anti-bacteria for plants is very large and can induce the bacteria to become resistant to antibiotics. As a biological control agent for plant protection, phages have attracted the attention of both academia and industry. In many pathological systems, seeds infected by bacteria are considered to be one of the main methods of infection [185]. The seed antibacterial coating can protect the seed from bacterial plant diseases. Kimmelshue et al. [186] reported that a bioactive polymer coating based on CN8 phage reduced the biological control ability of *Clavibacter michiganensis* subsp. *nebraskensis* (Cmm) in maize seeds. Cmm can cause corn to suffer from a widespread Goss's wilt, which may reduce corn production by 50%. This study mainly evaluates the effect of

using different polymer carriers and stabilizers on the stability of biological control seed coatings. In the process of controlling bacterial fruit blotch (BFB) in melons, Rahimi et al. [187] found that coating seeds with the newly isolated *Siphoviridae* family's ACPWH phage could increase the germination rate of seeds from 13% to 96%. In addition, they also showed that ACPWH phages could be transported from the soil to the leaves of melon plants, protecting plants from being infected by *Acidovorax citrulli*, the pathogen of BFB [188].

Energy storage and generation

Recently, phages have also made great contributions to energy storage and generation. The combination of phages and nanoparticles can produce unexpected effects: 1) The phage particles have simple geometric shapes and uniform sizes in the nano-scale range, representing monodisperse nanoparticles; 2) The surface modification of phages has a wide range of possibilities. The target functional groups can be obtained by genetic modification or chemical modification of the phages to obtain new characteristics. Recently, the use of phage particles to develop electrochemical devices has aroused great interest. In the process of nanomaterial assembly, phages play a critical role in the control of the shape, density, and orientation of materials, as well as micro-scale devices and applications, along with the many remarkable achievements that have been made in the fields of electrode material batteries, solar cells, fuel cells, and piezoelectric materials.

Electrode material batteries

Scientists continue to pursue smaller and more flexible high-capacity battery materials, and new materials and unconventional methods may promote their research. Phages, as a type of bio-organic materials used in Li-ion battery electrode field, provide new inspirations. Thus, genetically modified phages as biological templates for nano-material synthesis came into being. Belcher's team [189] took the lead in using the genetically modified M13 phage as a template for the synthesis of cobalt oxide (Co_3O_4) nanowires and used the nanowire to build the anode of the lithium-ion battery. A peptide that consisted of four glutamic acid residues was displayed

Fig. 12. (A) Schematic of the experimental design for the M13 phage-templated catalyst nucleation. (B) At $120,000\text{ h}^{-1}$ and $450\text{ }^\circ\text{C}$, the M13 phage-templated catalyst (1% Rh/10% Ni@ CeO_2) exhibited thermal stability over 52 h measurements. (C) The untemplated catalysts showed a decrease in conversion over time. (D) After exposure to air for 1 h, the test

on the major coat proteins of M13 phage. Experimental test results showed that the reversible capacity of lithium-ion batteries using phage as a template ranged from 600 to 750 mA h/g, indicating that the virus template alleviated the capacity attenuation problem of Co_3O_4 powder itself. This result indicated that the phage-templated nanowires could be used to construct lithium-ion batteries on various flexible and rigid substrates in the future, although self-organized peptides not on phage could also template the synthesis of materials [190]. In addition to Co_3O_4 , porous iron phosphate ($\alpha\text{-FePO}_4$) has also become a promising candidate material due to its low cost, low toxicity, and stable structure [191]. In order to solve the shortcomings of the inherent poor conductivity of $\alpha\text{-FePO}_4$, the combination of transgenic M13 phage and silver nanoparticles (Ag NPs) is used to increase the conductivity of the material. The experimental best result is achieved in the high-rate charge-discharge mode so far (the battery capacity is 134 mAh/g in the 3C charge-discharge test, and at that time, the biggest reported capacity of $\alpha\text{-FePO}_4$ is 85 mA h/g [192]). Besides, after at least 50 charge and discharge cycles, there is no significant capacity degradation.

Phages have also played a crucial role in the development of lithium-sulfur batteries. Studies have shown that pVIII, the phage shell protein, is used in the electrode material of lithium-ion batteries by taking advantage of the specific binding characteristics of phage shell protein and sulfur element [193]. Oh et al. [194] demonstrated that phages could benefit electrode material synthesis if they were genetically modified to bear good adhesion to the graphene sheets for providing good electrical conductivity. In addition, another advantage of using phages is that temperate synthesis conditions, non-poisonous reagents, and low temperatures can be used in the synthesis of electrode materials. It was also reported [21] that the M13 phage displaying the BaTiO_3 binding peptides could be utilized as the template with a higher specific surface to induce the BaTiO_3 nanoparticle synthesis at room temperature to form piezoelectric materials. This work indicates that displaying inorganic specific peptides on phage could enable phage to guide the synthesis of a wide variety of inorganic electrode materials at environmentally benign conditions.

Fuel cells

As for fuel cells, the anti-deactivation of catalysts is often the critical factor. It has demonstrated [195] that catalyst materials based on phage templates can effectively enhance the catalytic activity (Fig. 12A). Compared to the untemplated catalyst, M13-templated catalyst could improve the thermal stability by combining resistance to surface deactivation on rhodium and less phase segregation (Fig. 12B-E). Thus, the biological templating of phages can be a very promising approach in this field. The methanol fuel cell electrode converts methanol into water and releases electrical energy through electrons circulating in the electrode material. The electrocatalyst used in fuel cells is usually dispersed on a traditional carbon support, which affects the catalytic activity through the interaction of the metal and the support. The Laberty-Robert team [196] used modified M13 phage as nucleation sites and templated the formation of nanoparticle (NPs) arrays. Such a method contributes to the formation of 3D nano-structured electrodes, which exhibited a high-efficiency electro-catalytic activity and provided a new direction for efficient electrode materials. In addition, Belcher et al. [197] synthesized gold nanowires (Au NWs) with uniform diameters at room temperature by selectively interacting Au-binding M13 phages and Au

ions in an aqueous solution, with a yield greater than 98%. Due to the templating effect of phages, the diameter of Au nanowires was controlled in between 10e50 nm, improving the efficiency of Au particle catalysis.

Solar cells

Phages have also found use in the development of solar cells. In such an application, one way is to effectively collect light-generating electrons by rationally designing phage-templated nanocomposites with high electron mobility, consequently promoting the performance of photovoltaic devices. For example, Belcher et al. [198] synthesized single-walled carbon nanotubes- TiO_2 nanocrystalline core-shell nanocomposites by using genetic engineering phages as a template. By using the phage-templated nanocomposite materials as photoanodes in dye-sensitized solar cells, an energy conversion efficiency of 10.6% was achieved. In recent years, many reports have revealed the applications of the M13 phage templated retractable electrodes in solar cells (Fig. 13) [25]. Compared with other templated materials, M13 phage templated metal nanowires can reach well-controlled sizes and shapes. The stretchable electrodes prepared based on this method showed a good stretchability of 11%. In another case, the M13 viruses can serve as a multifunctional 3D scaffold by making a crosslinked viral template to construct continuous porous titania networks and composite metal nanoparticles. Changes in phage morphology control the porosity of the titania network, which in turn optimizes the photovoltaic properties of thin-film solar cells. The recombination of phage and nanoparticles further enhances the performance of the device [199].

Piezoelectric materials

Phages play an important role in producing piezoelectric materials for energy harvesting. In the future, wearable, flexible devices will lead the mainstream of science and technology and put forward new requirements for the power generation and storage of microwatt and nanowatt sources. Because of their non-toxic nature and genetic modification technology, phages are highly biocompatible and multifunctional. Lee et al. [200] firstly used the piezoelectric and liquid crystal properties of M13 phages to generate electricity. By genetically engineering the main coat protein of the phage, the dipole intensity of the phage can be changed to adjust the piezoelectric response. As biotechnology makes it possible to produce transgenic phages on a large scale, piezoelectric materials based on phages may provide a simple and reliable material. Shin et al. [201] showed that phage nanopillars could be aligned vertically, through infiltration and the piezoelectric response of the resulting materials, generating an output voltage and current of $\sim 140.8\text{ mV}$ and $\sim 9.5\text{ nA}$, respectively. Lee et al. [202] used rod-shaped M13 phage as piezoelectric biomaterials and designed its tail shell protein to immobilize it on the target substrate. The capillary force induced by the template is used to induce the vertical arrangement of the phage to exhibit unipolarization. The obtained phage nanostructure shows significantly enhanced piezoelectric properties (Fig. 14).

Assembly into higher-ordered structures

Liquid crystal formation

Nature has given creatures unlimited possibilities. The diversity and accuracy of biological structures are incomparable to any synthetic materials, and filamentous phages are such a typical

showed faster deactivation. (E) The XRD pattern of the M13 phage-templated catalyst after 52 h testing showed peaks of NiO, Rh₂O₃ and CeO₂. (F) The XRD pattern of the untemplated catalyst showed peaks of CeO₂, NaCl and more complex oxide NiRh₂O₄ after two tests. Reprinted with permission from Ref. [195]. Copyright 2010, American Chemical Society.

Fig. 13. (A) Schematic of the synthesis of M13 phage-templated gold nanowire stretchable electrodes. (B) Schematic of stretchable electrodes structure. (C) J-V curves of stretchable perovskite solar cell (PSC) based on phage-gold nanowires under forward and reverse biases. (D) MPPT data of stretchable PSC based on phage-gold nanowires. (E) Stretchability data of phage-gold nanowires electrode. (F) Stretchability data of stretchable PSC based on phage-gold nanowires. Reprinted with permission from Ref. [25]. Copyright 2021, Royal society of chemistry. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

example. Compared to the rod-shaped particles synthesized in the laboratory, the filamentous phage suspension has a uniform size and shape. This remarkable property has a significant impact on soft matter physics, leading to countless influences on the structure of matter, self-assembly pathways, and the study of the unknown state [203]. The liquid crystal composed of anisotropic rod-shaped molecules is a typical example of soft matter research [204]. A disordered state was maintained under the low concentration, and the rod-shaped molecules are in an oriented arrangement under the high concentration. The higher the concentration, the more ordered the phases [204].

Phage concentration is the main factor regulating the liquid crystal behavior of filamentous phages. Cao et al. [205] chemically modified the surface of M13 phage with phenylboronic acid (PBA) to prepare a pH-responsive PBA-M13 phage. PBA-M13 phage can form chiral nematic liquid crystalline phases under suitable conditions and further induce the formation of a pure nematic phage in the presence of aromatic diol compounds. Zan et al. [206] chemically grafted poly (ethylene glycol) (PEG) on the surface protein of fd phage. Due to the steric hindrance effect, high-density grafted PEG-fd phage can only form a pure nematic phase, while low-density grafted PEG-fd phage and wild type fd phage can form a chiral nematic liquid crystalline phase.

One-dimensional assembly

Due to the large aspect ratio and liquid crystal properties of filamentous phages, researchers have successfully fabricated one-dimensional assemblies. Lee et al. [207] prepared one-dimensional M13 phage microfibers by a wet spinning process using glutaraldehyde as a cross-linking agent. In addition, using 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) as the electrospinning solvent, the authors obtained continuous and uniform electrospinning one-dimensional M13 phage nanofibers under the condition of a suitable concentration of polyvinyl pyrrolidone (PVP). The M13-PVP fibers retained the complete structure and biological activity of the phage.

Two-dimensional assembly

Phages have been shown to self-assemble into membrane materials on a variety of different substrate materials. Lee et al. [208] developed ultrathin membranes based on genetically engineered M13 phage. Due to the hydrogen bonding and electrostatic interactions between the peptides on the pIII protein and the edges of graphene-oxide (GO) nanosheets, the phage ends were aligned at the edges of the GO nanosheets. Under the external shear force, unidirectionally aligned phage layers were formed. Each layer was vertically aligned into a highly selective ultra-thin

Fig. 14. Schematic diagram of (A) the axial and radial dipole moments of a monolayer of phage and (B) the dipole intensity of a single PVIII protein, which can be adjusted by the number of N-terminal glutamate (E). (C) The relationship between the out-of-plane PFM amplitudes and the AC peak-to-peak voltage (V_{pp}) of the engineered phage with $1e4$ glutamic acid added. (D) The relationship curve between the effective piezoelectric coefficient (d_{eff}) of the 4 E-phage film and the film thickness. (E) Comparison of piezoelectric response of phage membrane with PPLN and collagen membrane. Reprinted with permission from Ref. [202]. Copyright 2012, Springer Nature.

film of $\sim 10e30$ nm. Sawada et al. [209] obtained the M13 phage with an affinity for isotactic poly (methyl methacrylate) (it-PMMA) by biopanning. The it-PMMA film was dipped and swept up from the phage solution to achieve the directional arrangement of the phage. The pulling speed and the concentration of phage have crucial effects on the arrangement. In 2019, we used the filamentous phages to generate an unprecedented ordered nano-to-micro hierarchical nanoridge-in-microridge (NiM) structure via another unique method (termed dip-pulling) [26]. We found that the pulling speed affected the length and width of nanoridges, nanogrooves, microridges, and microvalleys (Fig. 15AeB). The pulling at 1.5 mm/s could make the film the most regular and have a stable NiM structure along the pulling direction. The influence of the phage concentration, pH value, salt concentration, and polylysine pre-coating on the NiM structure was also identified

(Fig. 15CeF). Using a phage concentration of 3.5×10^{13} pfu in a 0.01 M salt concentration (pH = 7.0) and a pulling speed of 1.5 mm/s,

films with good NiM structures could be formed on different substrates (e.g., glass, gold, titanium, plastic, and silicon) through the dip-pulling method.

Three-dimensional network assembly

Novel three-dimensional network assemblies have been developed through interactions between phages and inorganic nanoparticle surfaces. Sawada et al. [210] displayed hemagglutinin (HA) peptide on the minor coat protein pIII of M13 phage, and immobilized the antibody (anti-HA peptide) on the surface of gold nanoparticles (GNPs). HA-M13 phage and anti-HA-GNPs were self-assembled to obtain metal-phage hybrid hydrogels. The GNPs form a regular three-dimensional network structure, and the phages are

in a liquid crystal state. Phages can also self-assemble into templates for the precise fabrication of silica nanostructures. We [211] prepared highly ordered mesoporous silica with uniform pore size using the self-assembled hexagonal lattice structure of filamentous phage as a template.

Perspectives and current challenges

Phage display technology occupies an increasingly important position in nanobiotechnology. Researchers not only have had a clear and profound understanding of the advantages of phages but have also discovered areas to be perfected in advancing the application of phage in nanobiotechnology.

The basis of applying phages to various fields in nanobiotechnology is the ability of phages to display peptides with affinity for the target on their surface. The phage display biopanning method can help researchers obtain peptides with relatively high affinity against target molecules. However, because the peptides are relatively short and linear ligands, their target affinity still needs improvement. With the development of bioinformatics tools, computer-based prediction and simulation should be used to analyze and optimize the affinity peptide sequences after biopanning to develop the best binding peptide for a certain target. Bioinformatics software could be employed to identify the best peptide out of the multiple peptides after several rounds of biopanning.

The natural host recognition ability and genetically tunable molecular display of phage make it possible to design phage into biosensors, which can detect various substances such as biomolecules, compounds, metal ions, and harmful bacteria. However,

Fig. 15. (AeB) The influence of the pulling speed on the NiM structure of phage film. (CeE) The influence of the phage concentration, pH value, and salt concentration on the NiM structure. (F) After the substrates are pre-coated with polylysine, the film could be prepared on the surface of different substrates (glass, titanium, gold, plastic, silicon). Reprinted with permission from Ref. [26]. Copyright 2019, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the detection efficiency of phage-based biosensors needs to be further improved. For filamentous phages, target affinity peptides or proteins with larger molecular weights such as scFv can only be fused to the pIII protein on the tail. Due to the large aspect ratio of filamentous phages, the proportion of their tails is small. Although T7 phage does not have such a disadvantage, the coat protein of T7 phage can only display a small number of peptide fragments. Therefore, in order to improve the accuracy of phage in biosensor

applications, the length of filamentous phage can be reduced as much as possible on the premise of retaining the pIII tail of phage. At present, researchers have tried to produce shortened M13 phage [212]. However, this modified shortened phage has not been studied in the field of biosensors.

Although the antibacterial effect of phages can be accepted and applied in the food and feed industries, phages, as a kind of microorganisms, still have many problems worthy of deep

consideration. The realization of the antibacterial effect needs to ensure that the phage maintains its biological activity, which is affected by various environmental factors such as temperature, pH, and humidity. Deviation from the appropriate living environment will lead to the reduction of phage activity or even inactivation, greatly reducing its effect on inhibiting bacterial growth. Therefore, when preparing phage-based antibacterial materials, their physical and chemical properties should be studied at the same time, and the materials should be designed according to the specific occasions so that the phage can be stabilized in a suitable environment for as long as possible.

Employing phages as a nano-biomaterial in developing nanobiotechnology relies on the validation of the phage preparations. Enhancing the stability of phages under extreme conditions, improving quality evaluation standards, promoting affinity of peptides to target, and optimizing the size of phages are essential for developing phages-based nanomaterial science. Unfortunately, few studies have been done on the above aspects. These temporary challenges cannot conceal their vast application potential. The research space of phages is still huge. It is no doubt that with the continued improvement of phage-based biotechnology, phages will have a more profound impact in the field of nanobiotechnology.

Conclusion

Phages as naturally occurring nanomaterials are becoming increasingly important in various aspects of nanobiotechnology. Phage display technology can identify peptides or antibody fragments that can target specific molecules or nanomaterials with high affinity. It could link biology and nanotechnology and provide

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an unprecedented approach to synthesizing and assembling new nanomaterials. The successful applications of phages to technologically important fields such as biosensors, antibacterial materials, and vaccines demonstrate the power of phages as a nano-biomaterial in advancing nanobiotechnology. In addition to the use of phages in disease diagnosis and therapy, the key role of phages in non-biomedical fields such as batteries and energy storage cannot be ignored. In the medical field, in addition to cancer diagnosis and therapy, novel phage-based nanomaterials can also be used to fabricate biomaterials for applications such as modulating cellular behaviors and regenerating tissues. Moreover, filamentous phage proved to be a monodispersed unit in higher-ordered assembled materials due to their uniform size and shape. This lays a foundation for using phages to build nanostructured materials, ranging from electrodes in batteries to scaffolds in tissue engineering. Overall, being both a biomacromolecule and a nano-biomaterial, phages are unique building blocks in developing a wide variety of nanoscale devices such as probes in biosensors, therapeutics in disease treatment, and electrodes in energy storage.

Data availability

All figures from other sources are cited. The figures made by the authors are available from request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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