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Review

Advance in phage display technology for bioanalysis

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Phage display technology has emerged as a powerful tool for target gene expression and target-specific ligand selection. It is widely used to screen peptides, proteins and antibodies with the advantages of simplicity, high efficiency and low cost. A variety of targets, including ions, small molecules, inorganic materials, natural and biological polymers, nanostructures, cells, bacteria, and even tissues, have been demonstrated to generate specific binding ligands by phage display. Phages and target-specific ligands screened by phage display have been widely used as affinity reagents in therapeutics, diagnostics and biosensors. In this review, comparisons of different types of phage display systems are first presented. Particularly, microfluidic-based phage display, which enables screening with high throughput, high efficiency and integration, is highlighted. More importantly, we emphasize the advances in biosensors based on phages or phage-derived probes, including nonlytic phages, lytic phages, peptides or proteins screened by phage display, phage assemblies and phage-nanomaterial complexes. However, more efficient and higher throughput phage display methods are still needed to meet an explosion in demand for bioanalysis. Furthermore, screening of cyclic peptides and functional peptides will be the hotspot in bioanalysis.

Keywords: Biosensor · Microfluidics · Phage display · Selection · Self-assembly

1 Introduction

Sensors, especially biosensors, have become vital tools in food safety control [1–3], environmental monitoring [4, 5], clinical diagnostics [6–8], and bioterrorism inspection [9–11]. A biosensor generally contains two key elements: target recognition and signal output, which converts the chemical, physical and biological interactions into detectable signals, including optical, electrical, thermal, acoustical, and magnetic signals [12–16]. Since transducers are not themselves capable of specific recognition, it is particularly important to introduce recognition probes into the biosensor. On the other hand, analytes are commonly presented in a complex environment, and the signal can be easily disturbed by the background. Consequently, the discovery of highly efficient and specific molecular probes is essential for biosensor development.

To address this issue, several methods based on different types of libraries have been explored for discovery of molecular probes, such as small molecules [17], peptides [18–20], proteins [21–23], aptamers [24–27], nanoparticles [28–32], etc. These methods mimic natural evolution, and the specific ligands are screened and identified through this progress. Among these methods, phage display technology is emerging as a rapid, powerful, economical, and effective approach to develop new molecular probes. It allows screening of specific ligands from a vast random library, and can generate unique peptides, proteins and antibodies that bind to their targets with high affinity and high specificity. The wide range of possible targets

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Abbreviations: cfu, colony forming units; **GNPs**, gold nanoparticles; **LOD**, limit of detection; **MMP**, magnetic microparticle; **QDs**, quantum dots; **ssDNA**, single stranded DNA; **TNT**, trinitrotoluene

varies from ions [33–35], small molecules [36–38], inorganic materials [39–42], natural and biological polymers, and nanostructures [43–45], to cells [46], bacteria [47], and even tissues [48–51]. Since phage display was first reported by G. P. Smith in 1985 [21], thousands of specific molecular probes have been generated and studied by this method. These molecular probes have been widely used as affinity reagents for biosensing, targeted imaging, diagnostics, therapeutics, drug discovery and delivery [52–54], protein purification [55], epitope discovery, mapping and mimicking, etc. [56–58]. The goal of this review is to summarize the advanced molecular probes obtained from phage display for biosensing in recent years, and to highlight their further applications in life science, clinical diagnosis, information science, materials science, and environmental science.

2 Target-specific phages or ligands generated from phage display

Bacteriophages (or phages) are diverse viruses composed by DNA packaged with capsid protein. Phages can infect the host bacteria and utilize the system of bacteria for DNA replication and capsid protein expression, after packaging, releasing the phage out of the cell by crossing the membrane or by lysing the cell. The phage display technique also known as biopanning, is based on the phages, and it has been adequately summarized in several reviews and books [46, 59–65]. With the advantages of simplicity, high efficiency and low cost, phage display is usually used for in vitro selection of target-specific peptides, proteins and antibodies [20, 22]. The general procedure for generating the specific phages or phage-derived ligands is described as follows. First, a library containing a large amount of phage clones each of which carries a unique peptide or protein is prepared and interacted with immobilized targets. After incubation, unbound phages are removed using washing buffer, while keeping the bound ones. Then, the bound phages are removed from the immobilized targets by elution buffer. The collected phages are amplified by infecting *Escherichia coli* (*E. coli*) to generate an enriched pool as the new library for next round of biopanning. In order to obtain the target specific phages, several rounds of selection are needed with variation of selection conditions (often increasing stringency). After that, the enriched pool is characterized by enzyme-linked immunosorbent assay (ELISA), and DNA is extracted from the positive clones for sequencing to identify the encoding sequences that can display target-specific ligands on the surface of the phage.

2.1 Types of phage display systems based on different expression vectors

Bacteriophages have been widely employed as cloning vectors, due to their simplicity in genome and structure. Depending on the type of phage used, the phage display systems can be classified as filamentous M13, T7, bacteriophage lambda (λ phage) or T4 phage display systems.

2.1.1 M13 phage based phage display

Filamentous phages, such as M13, are used not only for many recombinant DNA processes, but also commonly in

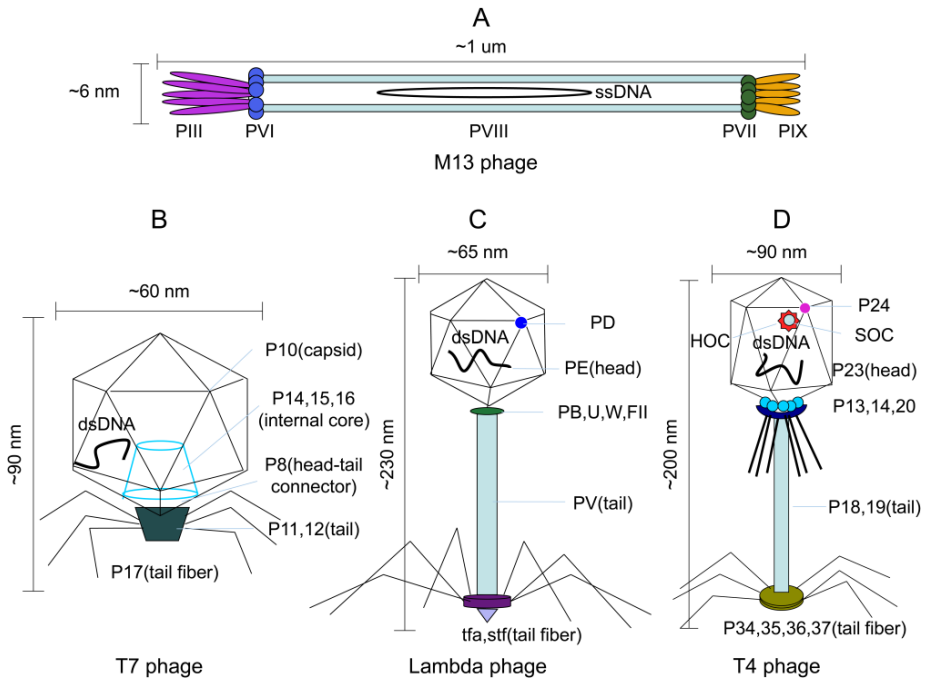

Figure 1. The structures of (**A**) M13 phage, (**B**) T7 phage, (**C**) λ phage and (**D**) T4 phage.

phage display [66]. As shown in Fig. 1A, M13 consists of a circular ssDNA and five coat proteins (PVIII, PIX, PVII, PVI, PIII). The ssDNA is 6407 nucleotides long and encoding about 2700–3000 copies of the major coat protein PVIII with five copies each of four different minor coat proteins (PIX, PVII, PVI, PIII) at the ends. The M13 viral lifestyle involves a chronic infection which is not lytic, although M13 can infect bacterial hosts without killing them, a decrease in the growth rate is observed in the infected cells.

Currently, based on the peptide fusing positions of phage, the M13 random libraries can be divided into two major types, PIII and PVIII library. The PVIII library can display thousand copies of peptides along the side wall of phage, and the PIII library can display five copies of peptides at the PIII tip. Therefore, the main difference between the PVIII and PIII phage display systems is that thousands of foreign proteins can be fused to PVIII but only short peptides can be used, since a high density of large foreign proteins increases steric hindrance, which impedes phage assembly and leads to loss of infection ability. In contrast, the PIII library can display large foreign proteins on its surface with one to five copies. Single, double, or triple display of different peptides on a single phage can be realized by site-specifically modification of peptides. However, filamentous phages have limitations for phage display, because they do not display cytoplasmic proteins effectively. Therefore, M13 phage-based libraries only display the peptides or proteins which can pass through the membrane of the bacteria and still keep the right folding [67, 68].

2.1.2 T7 phage based phage display

To select cytoplasmic protein variants, cytoplasmic phages, such as T7 phage, have been used. T7 phage is a DNA virus that has a lytic life cycle, and does not depend on the secretory mechanism of *E. coli*, since T7 phages are assembled in the cytoplasm and released by lysing the bacteria [69]. As shown in Fig. 1B, the T7 phage head is composed of linear double-stranded DNA (39 936 bp) and six capsid proteins (P10A, P10B, P8, P11, P12, P17), for a total of 415 proteins per head. In T7 phage display, the proteins are typically displayed to the C-terminal P10B. However, the T7 phage can be modified to express different amounts of 10B versus 10A proteins, displaying peptide sequences in 1–415 copies. T7 phage has several properties that make it ideal for experimentation. First, large protein fragments (up to 1000 amino acids) can be fused into capsid proteins at low level. Second, it can survive in some harsh conditions, such as high pH, high salt concentration, and even the presence of denaturing agents. Moreover, it grows very rapidly, so that multiple rounds of selection can be performed in one day. In solid medium, a plaque is formed in only 3 h, and in liquid medium, the host bacterium goes from infection to cracking in just 1–2 h. A further advantage is the availability of an efficient packaging system. In addition, compared to the M13 library, Krumpe et al. demonstrated that the T7 library produces greater peptide diversity [70]. However, the T7 system can only display short peptides with 50 amino acids at high density, while display large proteins with up to 1200 amino acids at merely 1–15 copies. In addition, compared to the M13 library, the anti-T7 phage antibodies haven't been commercialized yet.

2.1.3 λ phage based phage display

λ phage was originally discovered in 1951 by Esther Lederberg at the University of Wisconsin, when she serendipitously found it released from the laboratory *E. coli* strain K-12 after ultraviolet irradiation [71]. As shown in Fig. 1C, λ phage is composed of a linear dsDNA and two major coat proteins (D protein and PV protein). The molecular weight of D protein is 11 000, and without D protein the phage assembles to be 18% shorter than the wild-type phage. The fusion of foreign protein and D protein does not interfere with the assembly of λ phage, and the displayed proteins can be close to each other in space. The tubular tail portion of λ phage is constituted of PV protein. The tubular structure consists of 32 disk-shaped structures, each composed of six PV subunits. Therefore, each λ phage has 192 copies of PV. The C-terminus of the PV folded structure (non-functional area) can be extended or replaced with foreign protein sequences. Currently, the PV display system has been successfully demonstrated to express β-galactosidase [72], lectin [73], D protein [74], and green fluorescent protein or alkaline phosphatase [75]. The system shows an average of one foreign protein per phage, indicating that a foreign protein or polypeptide may interfere with the assembly of the λ phage tail. The developments and applications of λ phage display systems have been adequately summarized in several reviews [76, 77].

2.1.4 T4 phage based phage display

Enterobacteria phage T4 is a bacteriophage that infects *E. coli*. The double-stranded DNA genome of T4 phage is about 169 kbp long and encodes 289 proteins (Fig. 1D) [78]. This display system relies on two non-essential coat proteins, SOC and HOC. The foreign protein or peptide is fused with the C-terminus of HOC or the N-terminus of SOC to be displayed on the surface of the phage. SOC site display can be realized by integrating a recombinant vector which incorporates the foreign sequences in the SOC gene of T4, and isolating the lysozyme-independent phages displaying foreign peptides or proteins. HOC site display can be realized through in vitro packaging. The target gene linked to the C-terminus HOC gene to construct a HOC fusion gene expression vector, and the expression of the HOC fusion protein is induced by isopropyl-β-d-thiogalactoside (IPTG). After co-infection with the HOC gene deletion T4 phage, the HOC fusion proteins are packaged into the capsid surface of T4 phage

[79]. Dual displaying phages can also be generated if necessary.

2.2 The history of phage display and the advances in microfluidic-based phage display

Phage display technology was first introduced in 1985 by G. P. Smith [21]. In a typical protocol, the phage provides a direct connection between phenotype and genotype that allows combinatorial screening of specific binding ligands via an in vitro process termed biopanning. In the past 30 years, phase display has undergone a series of advances (Fig. 2), and the technology has shown enormous development in terms of phage display methods and the types of targets [19, 21, 22, 66, 80–85]. This section highlights the development of screening methods based on microfluidic phage display.

Typically, the biopanning of the phage display method involves three processes, including binding (to incubate the library with target), washing (to remove unbound or non-specific phages) and amplification (to prepare the library for sequencing or next round). However, a multiplication process in *E. coli* is time-consuming, sometimes requiring weeks to months for displayed peptides to be confirmed. Thus, development of more efficient peptide screening methods with the advantages of speed, high throughput and automation impacts areas ranging from the fundamental research to the further application by integrating phage display with other novel technologies.

Microfluidic-based phage display makes use of one or more of the unique advantages of microfluidics, which can be summarized in two characteristics: first, the high separation efficiency of microfluidics allows rapid screening; second, the unique microfluidic chip structure allows multiple units to be integrated into one chip for constructing high-throughput and high-integrated screening methods. Considering the unique advantages for molecular separation, Persson et al. [86] established an automatable fluidic system that allows highly efficient separation of antigen-bound and unbound phages in a continuous flow format, which is more efficient than conventional separation approaches. For instance, the separation efficiency can be as high as 99.9999% for a double channel chip due to its perfect laminar conditions. More importantly, without artificial operation, the separation of 500 μL sample needs only 8 min with a considerably high throughput of beads (5×10^4 s^{-1}).

Important selection conditions for biopanning include the concentrations of target, library, salt, temperature, incubation time, washing time and strength, as well as the system pH and types of ions, all of which are important but challenging to control. Liu et al. [87] used a microfluidic technique to accurately control the washing stringency in a magnetic separator, demonstrating the direct impact of washing stringency on the diversity of selected peptide sequences. They reproducibly generated magnetic and fluidic forces to precisely control washing conditions for rapid selection of peptide sequences. Their study laid a foundation for constructing automated and rapid selection method for generating affinity ligands.

Although these studies have demonstrated the tremendous potential of performing biopanning in large-scale microfluidic devices, other time-consuming procedures, such as high-throughput multiplex screening have not been addressed. Cung et al. [88] firstly demonstrated a microfluidic chip capable of selecting high affinity peptides. More importantly, four different targets can be selected simultaneously in only one round without bacteria amplification. The total time per one target was shortened to 4 h, which significantly improved the efficiency of the selection. In order to develop a simpler, time-efficient and more conventional method for phage-based selection,

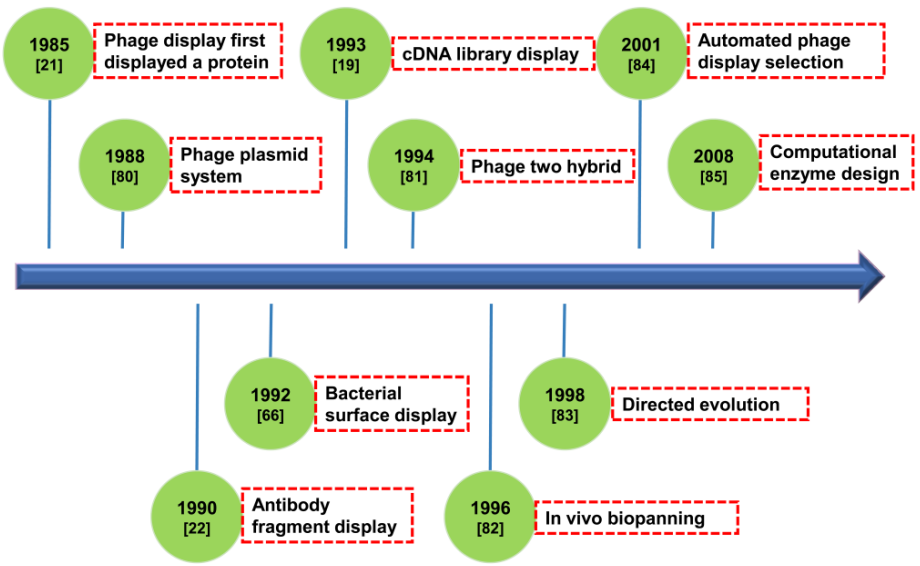

Figure 2. The development history of the phage display.

Wang et al. [89] integrated a novel microfluidic system into the selection procedure, and realized the automated selection of peptides for recognizing ovarian cancer cells. In this system, different microfluidic components, such as micropumps, microvalves, micromixers, were integrated with an *E. coli* culture chamber on a single chip, resulting in a significant reduction of screening time (7.5 h).

3 Biosensors based on phages or specific ligands generated from phage display

Specificity is always a key standard to evaluate a chemical or biological sensor; thus the development of target specific ligands is essential. Fortunately, phage display has provided an ideal tool for generating target specific ligands. In recent years, peptides, proteins, antibodies, etc. generated by phage display have been widely used in biosensors. The phage itself can express a target-specific ligand to serve as an antibody substitute. Furthermore, the phage can crosslink to some molecules or assembled with appropriate nanomaterials for generating a readable output signal in biosensing.

Another way to realize biosensing is based on lytic phages that specifically infect and lyse the bacteria, causing the release of the contents into the medium, which can be detected by fluorescent and electrochemical measurements. As emphasized above, phage or phage-derived probes have been used as recognition elements in biosensors due to their excellent physical and chemical properties, including high specificity and high affinity. In addition, phages such as M13 can be amplified by infecting the host bacteria, providing a novel strategy for signal amplification. Furthermore, phage and phage-derived probes are stable in extreme conditions, such as low or high pH, they are enzymatically resistant, and chemically and thermally stable. Some of these biosensors were presented in Table 1.

3.1 Biosensors based on the biological properties of phages

Since a phage can be modified to construct a readable signal probe and can be amplified by infecting the host bacteria, it is an ideal probe for signal-amplified detection.

In the typical phage-based detection, incubation up to 12 h is generally needed to generate visible plaques on a Petri dish. To speed up this process, as shown in Fig. 3A, Tjhung et al. [90] developed a droplet-based bacteriophage counting assay using a monodisperse emulsion analysis technique [91]. In their work, compartmentalization in droplets accelerated the signal generation from the reporter enzyme. The fluorescence signal is generated by hydrolysis of fluorescein di-β-D-galactopyranoside (FDG) by β-galactosidase, and by counting fluorescent droplets, Tjhung et al. could accurately enumerate phage particles by a fluorescence scanner or iPhone camera. The detection time has been shorten to 2.5 h by amplified signals in monodisperse emulsion , which is much shorter than the traditional agar overlay assays for overnight incubation.

In selective bacteria detection, Martelet et al. [92] first reported the detection method combining unlabeled phage amplification with mass spectrometry (LC-SRM analysis) for bacterial detection in complex food matrices. They have achieved 1×10^5 cfu/mL *E. coli* in broth medium and 5×10^5 cfu/mL in orange juice, which were comparable to the previous methods using MALDI-MS20 and ESI-MS22, but were still insufficient for food safety risk assessment. To further improve the sensitivity, Martelet et al. [93] developed a highly sensitive method for detection of bacteria by targeted mass spectrometry. First, phages were amplified by infecting host bacteria,

Table 1. Various phage display-based probes for biosensors

Probe Type	Target	Signal Mode	LOD	Reference
Nonlytic phage	*E. coli*	TC-phage-FlAsH, Fluorescent	1 cfu/mL	[95]
	E. coli	Capture phage, Electrochemical	10^4 cfu/mL	[102]
	TNT	Self-templating assembly, Colorimetric method	300 ppb	[118]
Lytic phage	*E. coli*	T4 phage amplification, LC-SRM	1 cfu/mL	[93]
	miRNA	Counting plaques, Fluorescent	3 aM	[96]
	E. coli	Lysed cells, Electrochemical	0.01 cfu/mL	[105]
Peptide or protein	Spores	Capture peptide, Fluorescent	34 in 2 μL	[97]
	LPS	Peptide-GO, Fluorescent	130 pM	[101]
	Antigen	Biligand peptide, Electrochemical	2 pM	[103]
	PSA	Capture probe, ELISA	0.16 ng/mL	[109]
	M. tuberculosis	Phage D29 inhibited, QCM	100 cfu/mL	[112]
Phage-nanomaterial complex	*E. coli*	Phage-QDs, Fluorescence	10 cfu/mL	[126]

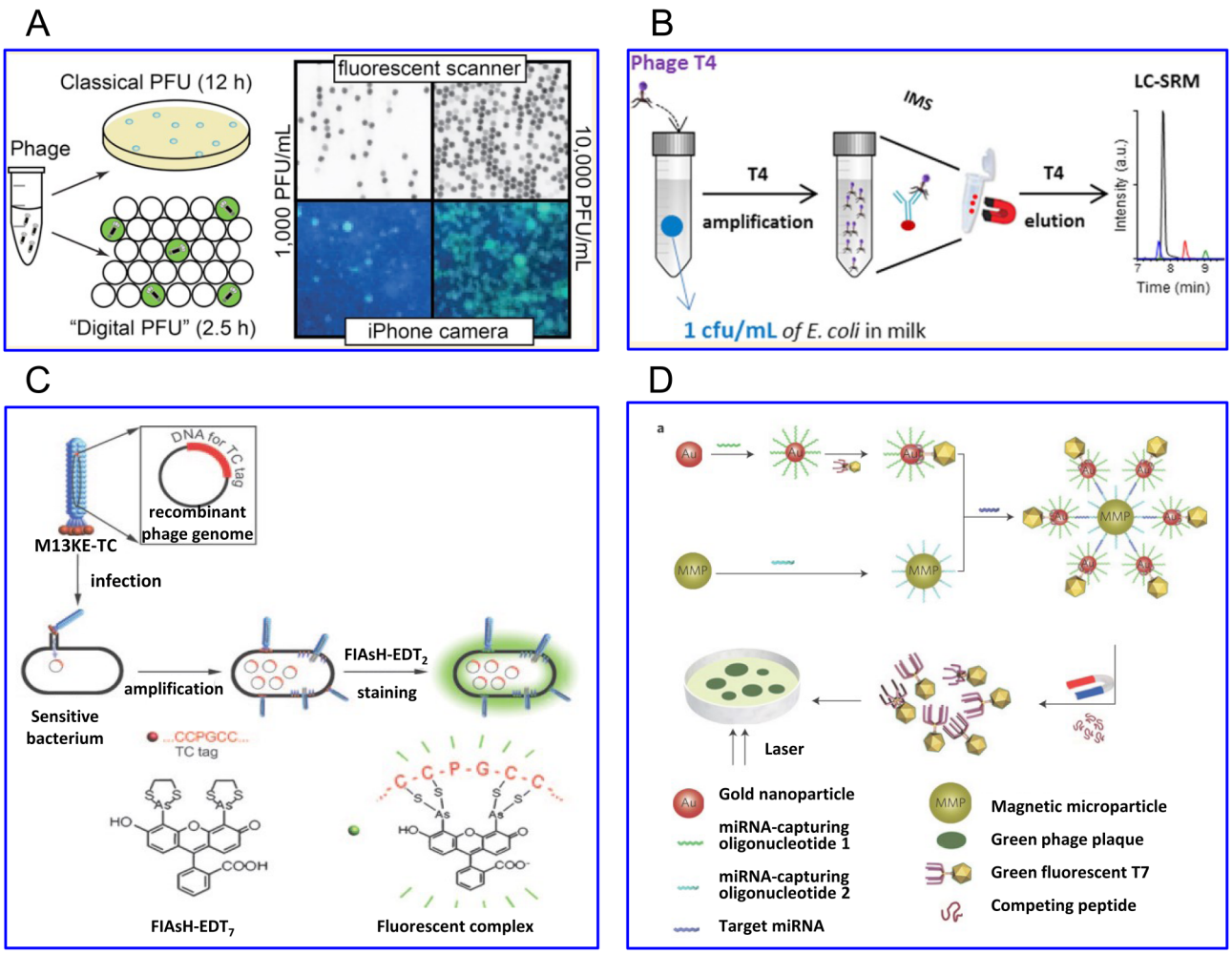

Figure 3. Examples of biosensors based on the phage amplification. (**A**) The phages were encapsulated in monodisperse droplets, then the fluorescence generated from hydrolysis of FDG by β-galactosidase, can be used for rapid enumeration of phage (adapted with permission from [90]). (**B**) Phage-MS approach for detection of bacteria in food stuffs includes three steps, the phage amplification, immunomagnetic separation of progeny phages and targeted MS (adapted with permission from [93]). (**C**) The TC tag genes were inserted into the M13 phage to express the TC tag on the surfaces of M13 phages. After incubation with host bacteria, the phage was amplified and detected by the fluorescence due to the staining by biarsenical dye (adapted with permission from [94]). (**D**) Schematic shows the strategy of miRNA detection, including the preparation of the probes, magnetic separation and counting. GNPs were modified with capture DNA and T7 phage. MMPs were modified with another miRNA-capturing DNA. In the presence of the target miRNA, it hybridized with two probes to form a sandwich complex. After magnetic separation, the phage can be counted by plating in the Petri dish, which related to the amount of miRNA (adapted with permission from [96]).

and then captured by the antibody modified magnetic nanoparticles. Finally, the biomarker of the phage, the structural protein, was quantified by targeted mass spectrometry (Fig. 3B). The LOD was as low as 1 cfu of *E. coli* in 1 mL of milk, which enabled the evaluation of the contamination level of food in less than 12 h.

Wu et al. [94] reported a sensitive and rapid method for detection of bacteria using genetically engineered TC-tagged phages for target recognition and signal amplification (Fig. 3C). TC-tagged phages can be specifically amplified in host bacteria and stained with biarsenical dye, resulting in fluorescent detection of the bacteria by flow cytometry or fluorescence microscopy. Furthermore, because phages only propagate in living host cells, the TC-phage strategy can distinguish the live bacteria from dead bacteria and other non-target bacteria [95].

Zhou et al. [96] developed an ultrasensitive phage-based counting strategy for miRNA detection at attomolar levels in a Petri dish by naked eye (Fig. 3D). This strategy of miRNA detection contains three steps: the preparation of the probes, magnetic separation and counting. One probe consists of gold nanoparticle (GNP) functionalized with fluorescent T7 phage (specific recognition of GNP) and oligonucleotide (complementary to part of target miRNA) simultaneously. Another probe is the magnetic microparticle (MMP) modified with oligo-

nucleotide complementary to another segment of target miRNA. In the presence of target miRNAs, the two probes recognize the target and hybridize to form a GNP-miRNA-MMP sandwich complex. Then a gold binding peptide is introduced to compete with T7 phages bound on GNPs and release the T7 phages. After magnetic separation, the fluorescent T7 phages are plated on a host bacterial medium to develop fluorescent plaques for counting. Because the number of T7 phages is equal to the number of target miRNAs as well as the number of developed plaques, the phage in this work established a direct relationship between the number of target miRNAs and the number of plaques in the Petri dish. The sensitivity of this strategy is comparable to that of qPCR, but it is less costly, simpler and easier.

3.2 Biosensors based on affinity ligands identified by phage display

Various affinity ligands generated by phage display have become ideal probes in biochemical analysis, clinical diagnosis and targeted therapy, due to their unique characteristics, including high affinity, high specificity, ease of synthesis and labeling, and good stability. This section focuses on biosensors based on the probes generated from phage display. These biosensors are summarized and highlighted according to the type of signal output.

3.2.1 Optical biosensors

This section focuses primarily on fluorescence biosensors based on the different properties of the probes generated from phage display.

Short peptides are used in biosensors as specific recognition elements with several advantages. Short peptides can withstand extreme environments that would denature proteins and antibodies. Short peptides can be easily modified and synthesized with simple procedures. Acharya et al. [97] developed an optical sensor array on a glass wafer and coated with gold ring, which enables the laser to penetrate through the inner circle of array. Short peptide ligands were used as the specific recognition elements for *Bacillus anthracis* spores, and immobilized on the surfaces of the sensor for capturing the target spores. When the spores bind to short peptides, part of the laser beam can be blocked by the captured spores, leading to the reduction of optical signal intensity. As low as 34 spores in a 2 μL solution can be detected by this optical sensor.

The immobilization of peptides with high density in each spot during a peptide microarray fabrication process decreases performance greatly. To address this problem, Qi et al. [98] developed a novel peptide immobilization method using the orderly structures of phages. Using endoglucanase I (EG I) as a model, the EG I specific peptide EGSDPRMV was first selected by phage display. Thereafter, the phage EGSDPRMV was used to construct a peptide microarray. Compared with the traditional fabrication method, the phage microarray was reproducible with higher density, can simplify the procedure of microarray construction and improve the sensitivity of the biosensor.

Goldman et al. [99] demonstrated a FRET-based biosensor using antibody fragments attached to QDs for trinitrotoluene (TNT) detection (Fig. 4A). A dye-labeled TNT analogue prebound to the antibody binding site and quenched the QDs via proximity-induced FRET. The introduction of soluble TNT displaced the dye-labeled analogue, eliminating the FRET effect and resulting in concentration-dependent recovery of QD photoluminescence. The LOD of the method was 20 ng/mL. The use of an antibody fragment instead of a full antibody provides a more compact QD conjugate for better FRET signal, due to the short distance between donor and antibody-bound acceptor.

Phages are not only used for target recognition but signal output as well. Sometime, traditional signal amplification alone is generally insufficient for detection of trace protease activity. In order to further increase signal, Capek et al. [100] introduced a second stage amplification utilizing post-cleavage propagation of filamentous phage. Bacteriophages from a solid support were specific cleaved by protease and further quantified by infectivity or quantitative PCR. The LOD is 2 pM, 850 pM and 690 pM for botulinum neurotoxins (BoNT)/A, BoNT/B, and lethal factor, respectively.

A peptide can physically adsorb to negatively charged graphene oxide (GO) via electrostatic interactions and/or π–π stacking in solution. Based on this property, Lim et al. [101] developed a peptide-GO assembly sensor for fluorescence detection of lipopolysaccharide (LPS), a toxic inflammatory stimulator released from the outer cell membrane of Gram-negative bacteria, causing millions of casualties annually through septic shock. They selected a LPS-binding peptide (KC-13, sequence: KKNYSSSISSIHC) using the phage-display method and labeled the peptide with tetramethylrhodamine (TMRho). The fluorescence of the TMRho-labeled peptide is quenched upon interaction with GO. Specific binding to LPS triggers the release of the peptide-LPS complex from GO, resulting in fluorescence recovery. This fluorescent turn-on sensor offers an estimated LOD of 130 pM, which is the lowest ever reported among all synthetic LPS sensors to date.

3.2.2 Electrochemical biosensors

Applications of phage display in electrochemical sensors can be divided into two types in terms of different detection mechanisms. On the one hand, target-specific detection is achieved by the immobilization of a target-specific phage probe onto the surface of the working electrodes. On the other hand, a cell-lytic phage encounters a target

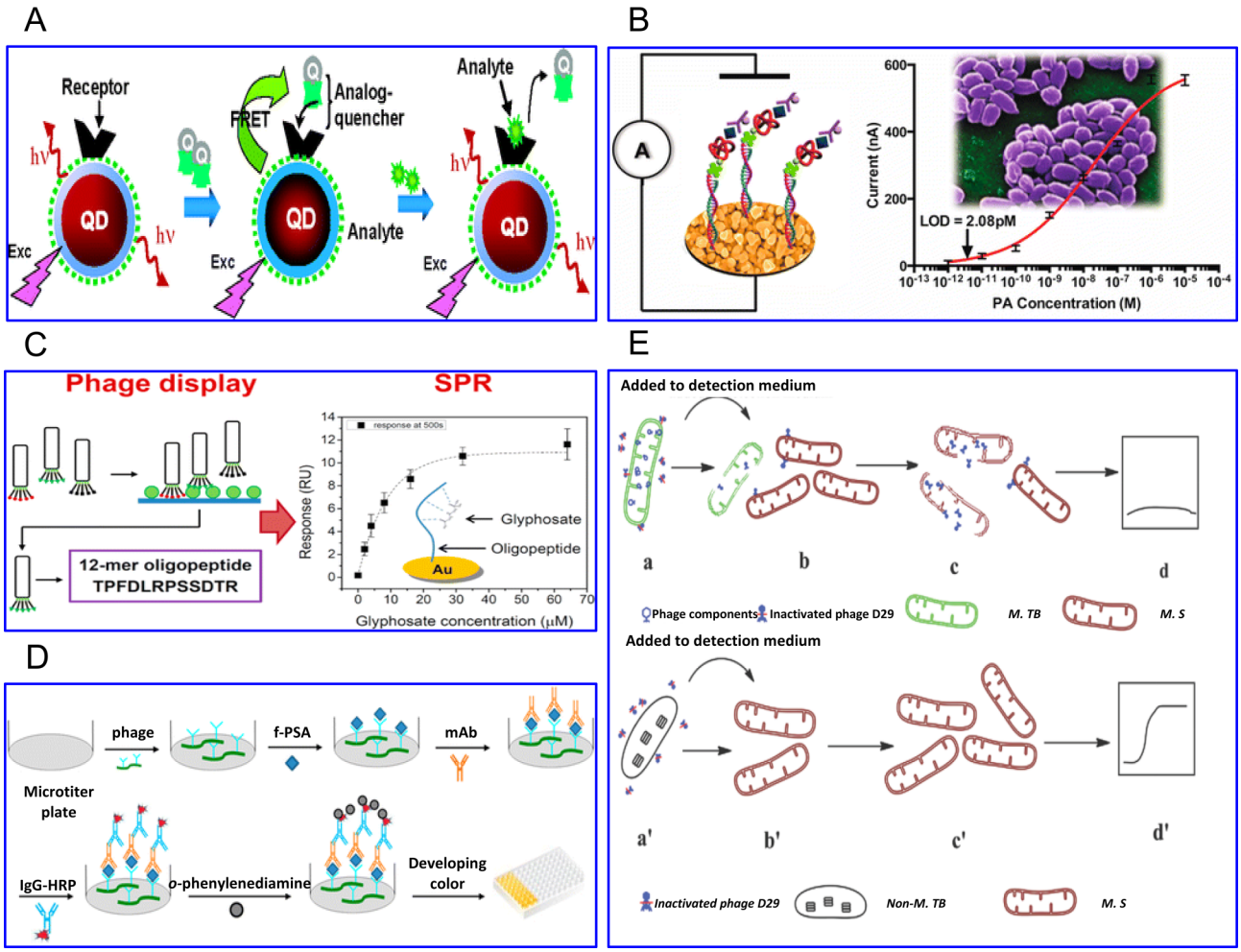

Figure 4. Probes derived from phage display associated with different signal readout systems, such as (**A**) Fluorescence (adapted with permission from [99]). (**B**) Electrochemistry (adapted with permission from [103]). (**C**) SPR (adapted with permission from [107]). (**D**) ELISA (adapted with permission from [109]). (**E**) QCM (adapted with permission from [112]).

bacterial cell and releases intrinsic cell-marker enzymes, leading to catalysis of some substrates to produce electrochemically active species.

Bacteriophages are small viruses that have recently been used as recognition elements in bacterial biosensors, due to their robust response, low cost and ease of production. Shabani et al. [102] developed a bacteriophage-based biosensor for direct impedimetric detection of *E. coli*. Phages were immobilized onto a functionalized screen-printed electrode, resulting in a significant change in impedance due to the arrival of intact bacteria at the phage-modified electrode surface. Concentration response curves yielded a LOD of 10^4 cfu/mL for 50 μL samples.

In terms of practicability, the assay must be adaptable to complex conditions, while maintaining good sensitivity and selectivity for complex samples such as serum. Farrow et al. [103] reported a sensitive method for the detection of *B. anthracis* exotoxin protective antigen (PA) in complex environments (Fig. 4B). In this method, a biligand peptide was conjugated to a cDNA through biotin-streptavidin interaction, and captured by ssDNA on the gold electrode. In the presence of *B. anthracis* exotoxin protective antigen, the peptide, the antigen and the antibody against PA (labeled with alkaline phosphatase) formed a sandwich-type electrochemical ELISA assay. The LOD of the electrochemical assay was sharply decreased to 2 pM, much sensitive than optical ELISA assay (10 nM).

The improvement of probe affinity is another strategy to improve the detection sensitivity. Mohan et al. [104] described an electrochemical sensing method with high sensitivity for prostate-specific membrane antigen (PSMA), resulting from a dual-ligand phage. The special phage contains two different ligands for the target PSMA. One is the peptides displayed on the surfaces of phages, and the other is the artificially synthesized ligands to wrap around the phage by electrostatic interaction. By

further integrated with poly(3,4-ethylenedioxythiophene) (PEDOT) films, the dual-ligand phage probe can greatly increase the affinity between the PSMA and the phage and achieve a LOD as low as 100 pM of PSMA in synthetic urine without any amplification.

The λ phage is not only used for recognition of *E. coli* but also as the releasing agent for detectable bacterial markers. Neufeld et al. [105] used this principle for the detection of *E. coli* K-12 and MG1655. Upon binding with *E. coli*, the λ phages lysed the *E. coli* and released the contents of *E. coli* into the medium, which contained β-galactosidase. Then the β-galactosidase hydrolyzed the substrate *p*-aminophenyl-b-d-galactopyranoside (PAPG) to produce *p*-aminophenol (PAP) for measurable current signal. Filtration and preincubation before phage infection resulted in a wide detection range with as low as 1 cfu in 100 mL within 6–8 h.

3.2.3 Surface Plasmon Resonance (SPR) biosensors

Changes in the refractive index near a metallic surface induced by molecular adsorption, enable the highly sensitive, rapid, and label-free detection of biomolecules. Phages or phage-derived ligands can serve as the specific probes for biosensors.

Target-specific phage can be directly used in the SPR sensor to improve the sensitivity and specificity of sensors. For example, Arya et al. [106] reported a SPR-based method for detection of *E. coli* K12 using T4 phage as the recognition element. Using glutaraldehyde as a crosslinker, T4 phages were attached onto the gold substrate via a cysteine and cysteamine self-assembled monolayer. Then the host *E. coli* K12 were captured by T4 phages onto gold substrates. This biosensor can be used for *E. coli* K12 detection in the range of 7×10^2 to 7×10^8 cfu mL^{-1}.

Peptides or proteins selected by the phage display technique can be used in SPR sensors. For example, Ding et al. [107] described a modified phage display method for generating specific ligands against glyphosate. After three rounds, the peptide TPFDLRPSSDTR was identified, and the extended peptide TPFDLRPSSDTRGGGC was immobilized on the gold surface to construct SPR sensor for glyphosate detection (Fig. 4C). The sensitivity of this peptide-functionalized SPR biosensor is 1.02 RU/μM, with an LOD of 0.58 μM.

3.2.4 ELISA biosensors

ELISA, which requires a capture reagent that can be immobilized on a solid phase plus a detection reagent that binds specifically with a labeled enzyme for signaling quantification, is often a method of choice for detecting biological molecules. For example, Wang el al. [108] used aflatoxin as a model to generate an aflatoxin-specific phage by phage display. In the ELISA assay, aflatoxin is captured by the antibody 1C11, and then recognized by an aflatoxin-specific phage to form a sandwich complex. Addition of the anti-M13 phage antibody conjugated with HRP leads to a color change. The sensitivity of the assay is 14 μg/kg with a linear range of 4–24 μg/kg, which meets the standard of current regulatory limits of aflatoxin in most countries (20 μg/kg).

PSA has been regarded as a tumor marker in clinics by the US Food and Drug Administration for early diagnosis of prostate cancer. Lang et al. [109] used peptide ERNSVSPS as the capture probe and anti-PSA mAb to establish a sandwich ELISA of "phage/peptide/anti-PSA mAb" (Fig. 4D). At the optimal phage ELISA conditions, the absorbance at 492 nm is linear for PSA concentrations of 0.825–165 ng/mL with a low LOD of 0.16 ng/mL.

The development of nanobodies (Nb) is much easier compared with conventional recombinant antibodies. He et al. [110] constructed a phage-displayed library for screening Nb of aflatoxin B1 (AFB1), and two AFB1-specific nanobodies, Nb26 and Nb28, were selected and used for ELISA. The linear range of nanobodies Nb26 and Nb28 is 0.117–5.676 μg/kg and 0.171–6.431 μg/kg, respectively, which is much wider than that of monoclonal antibody 1C11.

3.2.5 Quartz crystal microbalance (QCM) biosensors

The QCM is a simple, label-free, cost-efficient, high-resolution mass sensing technique for determining the nature of binding interactions in real time. In order to improve the sensitivity for biosensing, some researchers immobilized the specific phages or peptides isolated from phage display on the QCM transducer surface.

To provide a universal approach by using the peptides selected by phage display for biosensor applications, Wu et al. [111] used troponin I (TnI) binding peptides generated from phage display for TnI detection. The selected peptides were immobilized on the gold surface via the amino acid cysteine at the C-terminus of the peptide. Response curves were obtained for different concentrations of TnI ranging from 0 to 10 μg/mL with LOD of 0.11 μg/mL.

One of the problems for the *Mycobacterium tuberculosis* detection is the difficulty of meeting the requirements for clinical diagnosis due to its long turnaround time (30 days). To address this issue, Mi et al. [112] developed a novel phage-based method for rapid detection of *M. tuberculosis* (Fig. 4E). The phage D29 inhibits the growth of both *M. tuberculosis* and *M. smegmatis*. *M. tuberculosis* can protect phage D29 inside from being killed by ferrous ammonium sulfate (FAS). Therefore, the detection procedure was as follows. D29 infected *M. tuberculosis* and survived from FAS treatment. Then, the sample solution was added to *M. smegmatis* solution, and the growth of *M. smegmatis* was inhibited by D29 released from *M. tuberculosis*. Through this design, *M. tuberculosis* detection was converted to *M. smegmatis* detection, and the turnaround time was shortened to 30 h, which is much faster than the clinic method BACTEC960 MGIT, with the LOD of 100 cfu/mL.

3.3 Biosensors based on phage self-assembly or phage composites

3.3.1 *Self-assembled supramolecular materials based biosensors*

Phages have recently been highlighted as unique natural building blocks for the self-assembly process [113–115]. Moon et al. [116] discussed recent advances in the application of M13 bacteriophage self-assembly structures and the future of this technology in their review. This self-templating method plays an important role in practical applications as well as fundamental studies of biomacromolecules in-vivo assembly under complicated and dynamic conditions.

Many natural materials change their colors in response to the environmental stimuli, which makes them ideal substrates for applications in sensing. However, they can only response to certain physical changes, while lack the specificity towards chemicals. It is a challenge to introduce such selectivity into these materials. Chung et al. [117] described a colorimetric biosensor based on the M13 phage assembly for detection of specific targets (Fig. 5A). This biosensor mimicked the skin of a turkey. The phage assembled into bundle nanostructures to form the phage litmus with viewing-angle independent color. Upon the target introduction, the interaction between target and phage can induce the change of distance between phages, leading to the color change. Finally, the target concentration can be quantified by the color patterning.

In the previous colorimetric biosensors, the affinity of the ligands was insufficient for the target, leading to poor selectivity of the biosensors. To enhance selectivity, Oh et al. introduced a TNT-binding peptide selected by phage display into the colorimetric biosensors (Fig. 5B). As low as 300 p.p.b. TNT was detected by TNT-binding phage litmus. The color of phage litmus was collected by handheld device, such as iPhone, and converted to the signal intensity by the software. In addition, the biosensor can distinguish TNT from its analogues, dinitrotoluene (DNT) and mononitrotoluene (MNT) [118].

3.3.2 *Phage-nanomaterial composites based biosensors*

Recently, phage-based nanomaterial designs have attracted considerable attention [119]. Nanoparticles (NPs) [40], QDs [120, 121], nanowires [122, 123], and nanotubes [124,

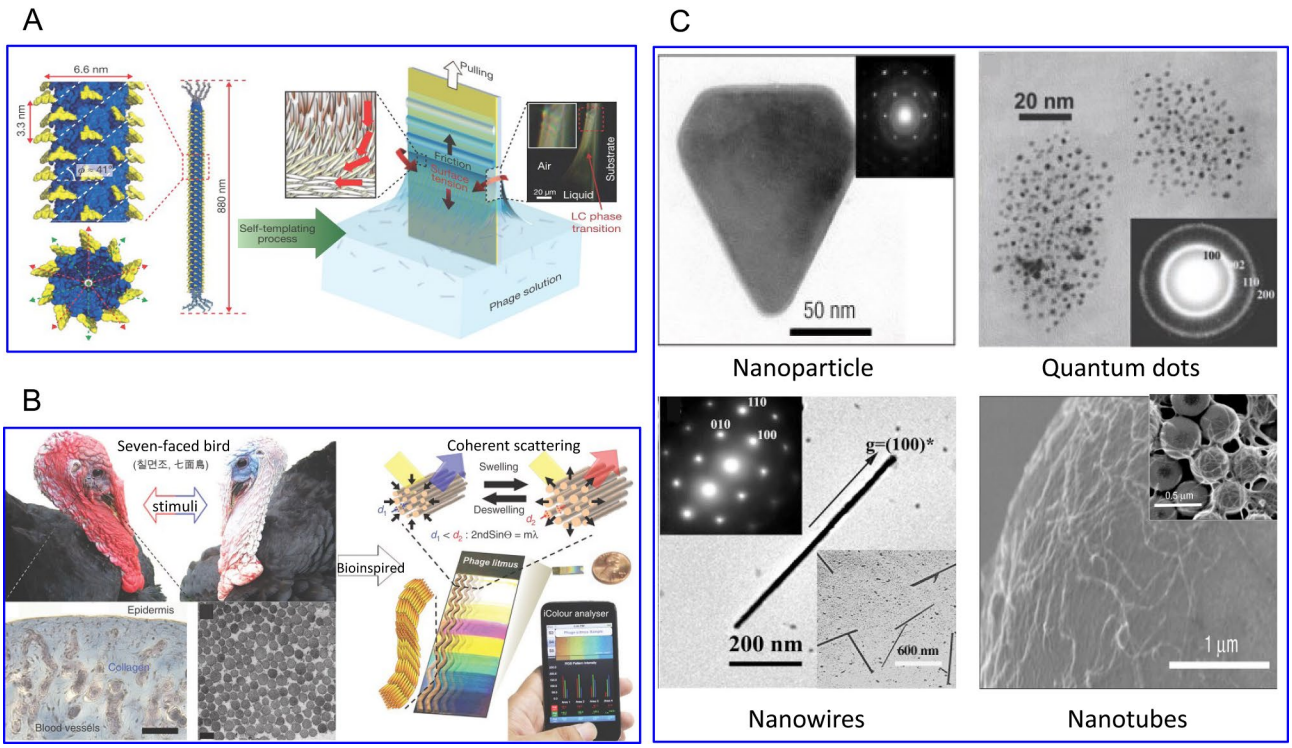

Figure 5. Phage assembly-based biosensors and phage-nanomaterial complex. (**A**) The scheme shows the nanostructure of M13 phage and the phage-based self-templating process (adapted with permission from [117]). (**B**) The color of the turkeys' skin changes autonomously when excited, which inspired the development of colorimetric biosensor based on the color change of phage assembly. In the presence of target molecules, the color changes due to change of bundle spacing and coherent scattering. Finally, the color can be analyzed by the software iColour Analyser in iPhone, and translated to the quantification of target molecules (adapted with permission from [118]). (**C**) Some typical types of phage-nanomaterial complex are shown, such as nanoparticles (upper left) (adapted with permission from [40]); quantum dots (upper right) (adapted with permission from [121]); nanowires (lower left) (adapted with permission from [122]); and nanotubes (lower right) (adapted with permission from [125]).

125] have unique physical and chemical properties that enable them to become attractive materials in sensor development with enhanced sensitivity (Fig. 5C).

Sensitivity and rapidity are the two main obstacles in detection of pathogenic bacteria. To overcome these shortcomings, Edgar et al. [126] constructed an in vivo biotinylation engineered phage to conjugate with streptavidin-coated QDs. This complex can provide a sensitive and rapid method for pathogenic bacteria detection by flow cytometry. The bacteria specific phage could be amplified approximately 100-fold as detectable signal by QDs in 1 h. As a result, the method achieved the detection of limit of as few as 10 bacterial cells per milliliter.

4 Concluding remarks

Phage display, with characteristics of simplicity, high efficiency and low-cost, is a powerful tool for generating target-specific ligands. Phages and target-specific ligands screened by phage display have been widely used as affinity reagents in imaging, therapeutics, diagnostics, bioassays and biosensors, protein purification, epitope discovery, mapping and mimicking, drug discovery and delivery, etc. However, the throughput and efficiency of traditional phage display cannot meet the growing needs of specific ligands. Thus there is a great need for more efficient and higher throughput phage display methods.

As described in this review, microfluidics technology provides a novel way to simplify and integrate the procedure of phage display or to improve the efficiency of separation. On the one hand, it is more convenient to control the conditions of selection, such as the concentrations of target, library, salt, temperature, incubation time, washing time and strength, as well as the system pH and types of ions. On the other hand, it is easier to realize the automatic selection, and utilize the advantages of high separation efficiency, high throughput and high integration. However, the potential of phage display using microfluidics technology is largely untapped and leaves a huge developing space.

Furthermore, the screening of cyclic peptides will be attractive because cyclic peptides have more types of 3D structures. It's worth noting that the access to functional peptides will be hot spots and trends in the future. Since peptides not only own the ability of specific binding, but also have specific function, which is useful for target therapy or cell regulation, such as the brain penetrating peptides or signal path switching peptides, etc. Hence, it will be a novel yet important area to develop methods of direct screening of functional peptides. In addition, with the emergence of new technologies, there is still much room for developing biosensors with higher sensitivity and selectivity. Thus, we believe that phages display will continue to play a very important role in bioanalysis.

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Cover illustration

This special issue, in collaboration with the Asian Federation of Biotechnology and edited by Professors Eiichi Tamiya and Chunhai Fan, covers advances in biosensors. This issue includes articles on optimization of biosensors for better sensitivity, exploration of the graphene interface for DNA analysis, aptamers for developing biosensors, etc. Image: © kentoh – Fotolia.com

Biotechnology Journal – list of articles published in the June 2016 issue.

Research Article

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Lu Xiao and Yu Xiang

http://dx.doi.org/10.1002/biot.201500432

Biotech Method

Label-free optical detection of C-reactive protein by nanoimprint lithography-based 2D-photonic crystal film

Tatsuro Endo, Hiroshi Kajita, Yukio Kawaguchi, Terumasa Kosaka and Toshiyuki Himi

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Biotech Method

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Rapid Communication

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