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REVIEW

Application of bacteriophages in sensor development

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Abstract Bacteriophage-based bioassays are a promising alternative to traditional antibody-based immunoassays. Bacteriophages, shortened to phages, can be easily conjugated or genetically engineered. Phages are robust, ubiquitous in nature, and harmless to humans. Notably, phages do not usually require inoculation and killing of animals; and thus, the production of phages is simple and economical. In recent years, phage-based biosensors have been developed featuring excellent robustness, sensitivity, and selectivity in combination with the ease of integration into transduction devices. This review provides a critical overview of phage-based bioassays and biosensors developed in the last few years using different interrogation methods such as colorimetric, enzymatic, fluorescence, surface plasmon resonance, quartz crystal microbalance, magnetoelastic, Raman, or electrochemical techniques.

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Introduction

Bacteriophages, as well as plants and some animal viruses, have proven to be useful tools in biotechnology, agriculture, and clinical medicine [1, 2]. Although antibodies, enzymes, or nucleic acids have been traditionally used for the development of sensitive and selective analytical methods based on different transduction mechanisms, bacteriophages have recently become of great interest for analytical chemistry to be used as probes for biosensing and imaging.

Bacteriophages or phages are viruses ubiquitous in nature but harmless to humans. Since in some cases phages destroy bacterial cells, they were used in the early 1900s as a medical therapy against some bacterial infections [3, 4]. However, they were later displaced by antibiotics in the 1940s [3, 4]. Phages are extremely robust and stable virus particles, which use bacterial cells as hosts to replicate. Phages are composed of a protein coat that encapsulates its RNA or DNA genome comprising from four to hundreds of genes [5]. Proteins of the phage coat can be easily conjugated or genetically engineered to display peptides, proteins, or antibodies targeting a wide variety of molecules, including biopolymers, toxins, proteins, or even specific contaminant bacterial strains [6, 7]. *Escherichia coli* (*E. coli*) phages are the most extensively used owing to their ease of culture and quick regeneration, making their production fast and economical. Therefore, the easy integration of conjugated or engineered phages into transduction devices, comparable to antibodies and nucleic acids, has boosted their use in recent years for developing phage-based bioassays and biosensors [2, 8, 9].

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Phages can be classified into three major categories, according to their life cycle types and morphology (Fig. 1) [5, 10, 11]:

- *Lytic or productive phages*, such as T4, T7, T3, and MS2 phages. These prolate icosahedron phages are only capable of replicating their genome, assembling phage structured components, and releasing from bacteria after cell lysis and death.
- *Temperate or lysogenic phages*, such as λ phage. These specific phages can multiply via a lytic cycle, as productive phages, or can incorporate their genome into the bacterial chromosome where it will produce a quiescent state (prophage). The cell hosting a prophage is termed a lysogen.
- *Filamentous phages*, such as f1, fd, or M13 are lysogenic phages (Fig. 1), which do not lyse their host cells. After phage assembly, the infected bacteria secrete the phages, thereby continuing the process.

For analytical biosensing and bioassay development, phages are actually used in three main different ways depending on the phage life cycle and morphology and if they have been genetically engineered. Therefore, lytic phages are used as natural recognition elements when they specifically release the content of a bacterial strain [12–14]; non-lytic phages are also used as a scaffold for the immobilization of target molecules by physical and chemical functionalization [15–26]; and genetically engineered lytic and non-lytic phages are predominantly used as target-specific biorecognition elements [27–30].

Despite the use of wild-type phages in biosensing, engineered phages are more advantageous for this application. Intact phages are biologically more active than engineered phages and the host lysis could lead to a loss of signal in downstream biosensing applications [31]. In addition, the enzymatic activity that some natural phages possess could interfere with the detection efficiency on special sensor platforms. In contrast, those bioprobes based on engineered phages exploit the ability of phages to display peptides or proteins on their surfaces that specifically target with high affinity carbohydrates, proteins, small molecules, or even entire cells for their integration into different transduction methods (e.g., optical [22, 32], enzymatic [33–35], electrochemical [36–38], etc.).

In this context, we introduce here briefly the in vitro selection technique used to select engineered lytic or non-lytic phages displaying peptides, proteins, or antibodies targeting a desired molecule, which is known as phage display, and how phage screening and maturation strategies might improve downstream biosensing applications. We then review the use of lytic and non-lytic phages as either natural recognition elements or scaffolds for biosensing and the most recent applications of genetically engineered phages for biosensing.

Phage display technology

Phage display emerged as a synergy of combinatorial peptide libraries fused to phages by genetic engineering [39, 40]. It was first reported by George Smith in 1985, when he successfully displayed peptides of interest at the N-terminal end of the

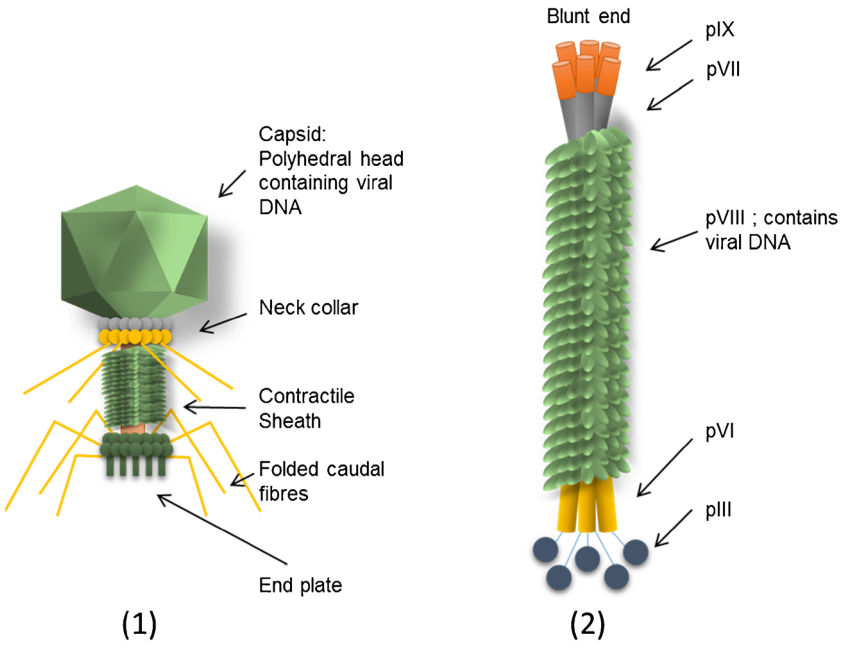

Fig. 1 Schematic diagram of icosahedron (*1*) and filamentous phage (*2*) structures

pIII capsid protein of the M13 filamentous phage [40]. Since then, phage display technology has massively evolved. Indeed, nowadays, large numbers of phage display peptide and antibody libraries containing about 10^{12} different baits each one carrying a different foreign DNA in the genome and displayed as fusion proteins in the coat of the phage have been constructed (Fig. 2) [40–46]. Their subsequent screening to identify specific peptides or antibodies against a desired target molecule has yielded a variety of economical, accurate, and efficient applications in immunology [47–52], pharmacology [53–56], cancer research [57–61], for the construction of nanotubes, nanobatteries, or nanorods [9, 32, 62–64], and in recent years for analytical biosensing through optical, enzymatic, colorimetric, piezoelectric mass, magnetoelastic, surface plasmon resonance, surface-enhanced Raman spectroscopy, or electrochemical phage-based bioassay devices [65–75].

Generalities of bacteriophages used for phage display

Although lytic or filamentous phages or phagemid vectors have been used to display peptide, protein, or antibody libraries [40, 76–81], the most common display systems are based on M13 filamentous phages and their close relatives fd and f1. Filamentous bacteriophage consists of a circular single-stranded DNA genome covered by approximately 2700 copies of the major coat protein pVIII with each end capped by five copies of both pIII and pVI proteins, which permits the infection and the injection of the DNA into the bacteria, and at the other end the same copy number of pVII and pIX proteins [82, 83]. Although all the proteins are reported to be able to display foreign polypeptides or small to large proteins on the surface of the M13 bacteriophage, the minor coat protein pIII is the most widely used, followed by the major coat protein pVIII [39, 41, 84, 85].

The choice of the protein displaying the library determines the display valency, which can vary between less than one and up to about 3000 copies per bacteriophage on average. High-copy display is typically associated with avidity effects and selection of low-affinity peptide ligands, which could be preferred in specific situations, whereas low-copy display is more associated with the selection of high-affinity peptide binders [86]. Phage display can be classified according to the protein used in the display [41]. The pVIII-display systems are able to display up to all of the ca. 2700 copies of pVIII fusions on the surface of the bacteriophage. In contrast, pIII-display systems present low valency with typically between one and three copies per bacteriophage of the pIII fusion, and rarely present the pIII fusion on the five copies of the pIII phage protein. The expression of short peptides on the phage body is usually well tolerated encompassing without many problems a wide range of display valencies. However, despite the ease of manipulation and stability of virions, the main drawback of using M13 phage display is that their non-lytic propagation requires that all components of the virion will be exported through the bacterial inner membrane before phage assembly [87, 88]. Accordingly, the display of large proteins on filamentous M13 phages, whose export depends not only on their length

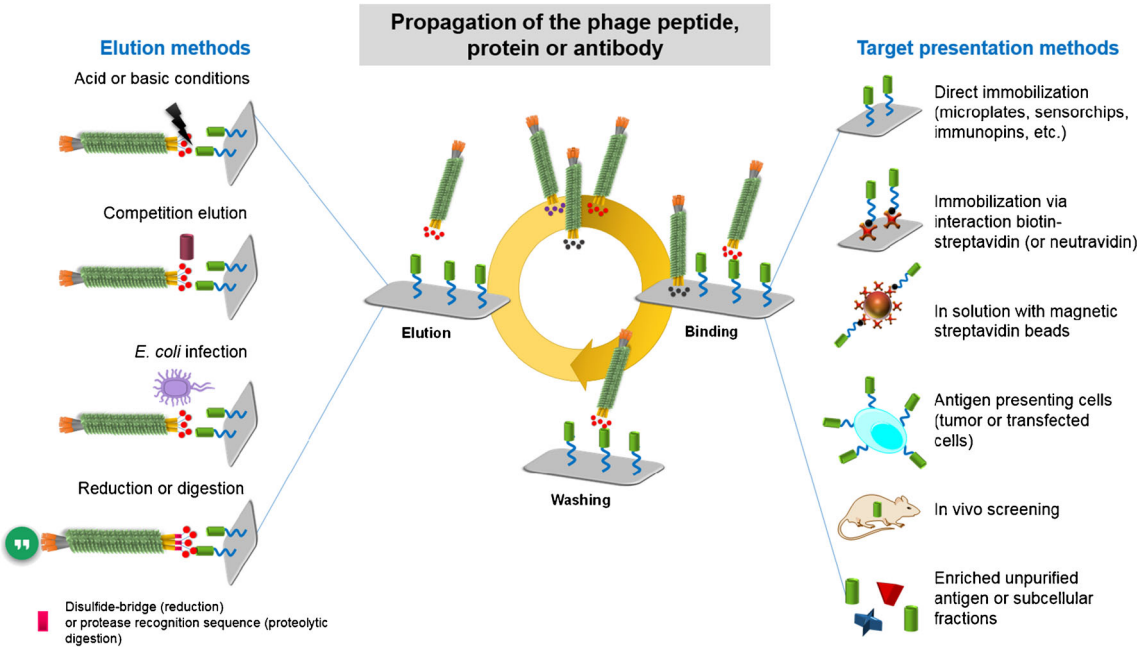

Fig. 2 General scheme of the phage display biopanning procedure. *Left* Depiction of the different elution methods used for the phage elution prior to bacterial infection. *Right* Description of the different antigen presentation methods used to perform the biopanning procedure. Propagation of phages is performed on bacteria, where each eluted phage clone is amplified between 10 to 100 times in each round of biopanning

but also on their sequence and folding properties, cannot be produced. In addition, foreign proteins can disrupt the integrity of the capsid at high valencies because they compromise the structure and function of the phage proteins used for the display of the fusions [89]. These limitations have led to display small antibody fragments such as scFv, Fab, VHH, minibodies, or affibodies instead of large complete antibody molecules of 150 kDa [41, 90–92]. These antibody fragments retain the binding for the antigen, maintain a correct folding conformation, and avoid the display of large molecules on phages, thereby attenuating possible defects on phage structure or function [89, 93].

Therefore, an alternative to overcome the restraints of M13 phage display consists of using lytic phage vectors (i.e., T4 and specially T7). Icosahedral bacteriophage assembly takes place entirely in the cytoplasm and thus eliminates the need to export the fusion proteins prior to phage assembly [87, 94]. Icosahedral phages lyse the host cell after infection of the host and replication of the virion genome [78, 87]. In addition, T4 or T7 icosahedral bacteriophages do not exhibit many aggregation issues and a lower mutation rate or sequence bias of peptide libraries than filamentous phages [79]. Other advantages of T7 icosahedral phages include their robustness to environmental conditions, high valencies (up to 415 peptides or proteins per phage), rapid replication time, and their capacity to accommodate proteins of up to 2000 amino acids in the C-terminus of the coat protein 10B [79, 87].

Enrichment of specific binders

Independently of the phage used to display peptides, proteins, or antibodies, the selection procedure for obtaining small numbers of phage clones with a desired affinity for a specific target from an initial phage library containing about 10^{12} different clones is known as “panning” or “biopanning” (Fig. 2) [40, 41, 76].

The iterative procedure where phages are amplified in *E. coli* bacteria and subjected to subsequent rounds of selection under higher stringency conditions consists of four main steps [41, 95, 96]: (i) binding of specific phage clones to the immobilized target after the addition of the phage display library, (ii) washing to remove unbound phages, (iii) elution of phage binders, and (iv) propagation of eluted phages after *E. coli* infection (Fig. 2). Three or four rounds of selection are usually required to isolate target-specific binders. To minimize the presence of unspecific off-target binders at the end of the procedure, a negative selection step (absence of target) is performed prior to carrying out the target selection. Then, after the last selection, individual clones from the unamplified eluate from the last two rounds of selection are screened for target binding, followed by the amplification and characterization of selected clones for the required biological activity. Finally, the sequence of the displayed peptide or antibody is obtained by sequencing [95, 96].

Remarkably, the major drawback of phage display resides in the non-uniform amplification of libraries [97], which in general favors the propagation of fast-growing clones instead of slow-growing clones after each round of biopanning. This competition during phage amplification leads to a decrease in the diversity of phage clones at the end of the procedure [97], which can limit the identification of useful ligands. This problem is usually not important while screening for ligands of a single binding site like proteins, but can be severe for the identification of ligands against targets with multiple binding sites such as mixtures of proteins, cells, or tissues [97, 98]. On the other hand, these problems have been recently overcome by using microfluidics and monodisperse emulsions in a procedure that can be adapted in academic or industrial laboratories [98]. There, uniform amplifications of phages are produced after each round of biopanning, ensuring the selection of multiple binders against the desired target [98].

Affinity maturation of peptides, proteins, and antibodies by phage display for further biosensing applications

Affinity maturation in vivo during the course of an immune response is the process by which B cells produce antibodies with increased affinity for an antigen. The application of phage display for the preparation of peptides or human antibodies with enhanced affinity for subsequent applications has paved the way for in vitro maturation success [48, 49, 99] that could be necessary for a subsequent construction of phage-based biosensors with improved affinity for a desired target.

To this end, a variety of mutagenesis strategies have proved useful. All these strategies rely on the construction of small to large phage display peptide or antibody libraries based on the desired parental peptide or antibody to be affinity matured by randomization of amino acids, error-prone PCR, parsimonious mutagenesis, cyclization of peptides; or CDR-walking, hot-spot mutagenesis, or in silico guided mutagenesis in the case of maturation of antibodies [48, 86, 96, 99–106].

After construction of the new libraries, the biopanning is performed under highly stringent conditions from the second round of biopanning. The use of different strategies during the biopanning selection has been reported to result in isolation of higher affinity binders; these strategies include using lower and lower antigen concentration in the selection phases from the second round of panning, carrying out selection procedures in solution, or increasing the washing time in the last rounds of selection up to 30 min for each one [49, 105].

Traditional analytical methods, including optical, enzymatic, radiochemical, or electrochemical, can be integrated with the phage-based probes to construct biosensors, while benefitting from using affinity-matured phages displaying peptides, proteins, or antibodies. Therefore, once the desired engineered

phage clone with the required affinity and selectivity is obtained, it can be directly used on subsequent biosensing applications [2, 8, 9].

Classification of engineered phages for biosensing

Although phages can be modified after amplification by chemical functionalization allowing them to participate in the sensing event [17, 18], phages altered by genetic encoding take advantage of the highly flexible and effective means of genetic engineering to develop a wide range of biosensing tools. Engineered phages are able to display peptides, proteins, or antibodies on the coat of the phage [33, 35, 65], to incorporate in their genome exogenous genes able to identify specific bacterial strains [29, 107], to tailor phage receptor-binding proteins with useful tags for signal amplification or for acquiring novel receptor tropisms [108, 109], which makes phages altered by genetic encoding the most interesting phages for the development of biosensing tools. In contrast, modification of phages after amplification mainly restricts their use in biosensing as phage structural elements to amplify downstream signals or to develop nanostructures for accurate geometric control of immobilization of phages for biosensing.

Therefore, depending on the genetic incorporation of functional scaffolds or the display of engineered peptides, proteins, and antibodies, phages as probes for biosensing can be classified as recognition elements, phage structural elements of engineered phage receptor binding proteins, or reporter phages.

Reporter phages

Reporter phages (RPs) are genetically modified phages used as gene carriers to introduce a gene of interest into a specific bacterial genome. The injected exogenous gene is integrated into the bacterial genome and further expressed, coding the bacteria with a fluorescent, colorimetric, or an optical marker that allows pathogen identification [29, 107]. As phages need host cells to replicate for the expression of the reporter gene, reporter phages are indicative of the presence of the infected bacteria.

Typical reporter phages include phages incorporating genes such as *inaW*, *celB*, *lacZ*, or the green fluorescent protein (GFP) for further downstream biosensing pathogen identification by fluorescence, colorimetry, or luminescence [110–112].

RPs have been extensively used in epidemiology for identifying or typing bacterial pathogen strains [13], foodborne pathogenic bacteria [12], or for the identification of specific *E. coli* strains [14].

Phage receptor-binding protein bioprobes

To efficiently attach to the surface of its bacterial host, phages target bacterial cell surface receptors through their receptor-binding proteins (RBPs) located at the phage tail fibers. The binding of these RBPs to carbohydrates or proteins of the host triggers the translocation of the genetic material of the phage into the host cell.

RBPs can be tailored by genetic engineering either to acquire novel receptor tropisms as tailed phages do naturally [108], to modify their binding affinity, or to obtain multivalencies, if desired. Engineered RBPs have better stability against environmental factors, such as pH and temperature, and better resistance against gastrointestinal proteases than antibodies. Particular advantages of using engineered RBPs to get biosensor platforms reside in the possibility to add appropriate tags at appropriate RBP positions without altering their binding affinity for the host pathogen [109].

Phage structural elements

Phages themselves can participate in the sensing event, serving not only as support for probe recognition but also by providing phage structural elements (PSEs) that either amplify the signals or develop nanostructures for accurate geometric control. To this end, phages are either functionalized by chemical modification or by genetic engineering to display specific functional motifs for a number of technologically useful materials to construct phage-based biosensors [17, 18].

Phages have been used as PSEs to display specific motifs to form Au phage-based nanoparticles (NPs) for the detection of specific analytes or AgNPs [6, 16], to form hybrid materials (i.e., cationic polymers and M13 bacteriophage virus particles [19]), and to form virus–PEDOT (poly(3,4-ethylenedioxythiophene)) biocomposite nanowires and films for the electrochemical detection of cognate antibodies [15, 21, 23]. Other applications include amplified optical protein detection (where the macromolecular structure of filamentous phages aids in the optical detection of proteins) [22], magnetic microspheres (where filamentous phages are immobilized for serum protein analysis and affinity separation) [24, 26], silica microspheres (merged with DNA-loaded M13 bacteriophages for sandwich immunoassays) [73], or film structures (based on M13 phages undergoing self-templating for colorimetric sensors) [20, 25].

Immobilization strategies of phages for biosensing

Biosensor development requires phage immobilization onto the transducer surface. This is a key step in the design of these devices because it directly affects their sensitivity and long-term stability for real sample analysis. Various phage binding

strategies have been explored for biosensor development so far, including (a) physical adsorption onto the detection platform, based on the characteristic properties of the phage surface proteins, such as net charge; and (b) covalent immobilization of the whole phage, or phage proteins (e.g., P22 tail spike protein [113]), on the substrates. Table 1 illustrates some selected examples of phage biosensors using different immobilization procedures.

Chemical binding usually improves surface coverage density allowing the preparation of reusable biosensors for online analysis [95]. Moreover, this approach usually leads to an improved target capture in comparison to physical adsorption [124]. However, it has been shown that the removal of lysate contaminants from phage suspensions improves planar surface coverage as well as the binding ability of the phages [125]. The use of surface patterning techniques may also allow optimal phage positioning and minimize clustering [126].

Alternatively, phage orientation on the sensor platform has also proved useful to improve target capture and thus to enhance biosensor sensitivity. Phages can be genetically modified to introduce specific binding ligands on their heads to ensure that they are immobilized in the right orientation on the transducer surfaces [127]. This approach has been applied by Tolba et al. to the construction of fully active recombinant T4 bacteriophages expressing a biotin carboxyl carrier protein (BCCP) or a cellulose binding module (CBM) for binding to streptavidin-coated or cellulose-based substrates, respectively, almost irreversibly [128].

Utilizing the charge difference between phage heads and tails is also a simple and useful alternative for oriented immobilization of a wide range of phages [129]. The method is based on charge differences between the bacteriophage head, which exhibits an overall net negative charge, and the tail fibers, which possess an overall net positive charge. The head can interact by electrostatic forces with positively charged surfaces. This approach can be useful for biosensor development.

Applications of phages in bioassays and biosensors

Several analytical detection modes have already been used in combination with phages for biosensing development. Similarly to the immunoassays classification and other (bio)recognition elements [27], the phage-based bioassays included in this section have been separated into different groups depending on the transduction technique. Since phage-based biosensors have been reviewed in great detail before [28–30], we will show a brief overview of the present trends of using bacteriophages as biorecognition elements or nanoscaffold elements for traditional transduction methods. In addition, Table 2 presents a comparison of some recent applications for phage-based detection of *E. coli* and the analytical characteristic of different detection schemes.

Enzyme-based bioassays

The majority of phage display protocols take advantage of phage-ELISA (enzyme-linked immunosorbent assay) methods to survey the panned pools and assess the binding to the target. The most commonly used enzyme is horseradish peroxidase (HRP) that can be coupled with colorimetric, fluorescence, chemiluminescence, or electrochemical detection depending on the substrate. Furthermore, many enzyme-based systems have exploited phage-displayed proteins and peptides to increase the sensitivity compared to conventional ELISA. Some recent examples are presented in Table 3. For instance, Goldman et al. compared phage-displayed single domain antibodies (sdAb) to monomeric solubly expressed sdAb for the detection of ricin [65]. Signal from the phage was generated using an anti-phage HRP conjugate and colorimetric detection. The phage-displayed sdAb instead of the soluble sdAbs in a conventional ELISA improved the detection limit of ricin from 1 ng/mL down to 64 pg/mL [65]. The authors claimed that the large size of the phage as a reporter molecule gives the advantage of incorporating a large number of labels compared to the few modification sites available in the sdAb alone.

Anti-idiotypic antibodies bind to the antigen-binding site of another antibody by mimicking the structure of the original antigen, allowing them to be used in competitive immunoassays. In the study by Xu et al. [34], an anti-idiotypic VHH single-domain antibody was selected from a naïve alpaca VHH library to serve as a surrogate for citrinin. The phage-displayed VHH was used as a signal-amplification carrier to develop an indirect competitive phage-ELISA with HRP-labeled anti-M13 antibody. The half- maximal inhibition concentration (IC_{50}) of phage-ELISA was 10.9 μg/kg, which was 9-fold better than that of conventional ELISA (IC_{50}= 102.1 μg/kg). In a similar way, phage display can be used to select mimotopes for simple competitive phage-ELISAs [33, 35]. For example, Yin et al. developed a competitive phage-ELISA based on phage-displayed mimotopes to detect thiacloprid in environmental and agricultural products [35]. Compared to the conventional ELISA, the sensitivity was improved more than 3-fold. Arévalo et al. used phage-borne mimotopes in an electrochemical phage-ELISA for the detection of molinate [155]. They used protein G functionalized magnetic beads as solid phase together with an anti-molinate monoclonal antibody. The detection was based on phage-displayed mimotopes and anti-M13 antibody coupled to HRP with pyrocatechol as substrate. The benzoquinone produced by the enzymatic reaction was then detected on a carbon screen-printed electrode (CSPE) by square wave voltammetry (SWV). Compared to the conventional ELISA using

Table 1 Representative examples of phage-based biosensors based on different immobilization methods and transducers

Immobilization	Target	Substrate	Recognition element	Transduction	Detection limit	Sample	Ref
Physical adsorption	*B. anthracis* spores	Metglas® 2826 MB ($Fe_{40}Ni_{38}Mo_4B_{18}$) + Cr + Au layers	JRB7 bacteriophage	Magnetoelastic	10^4–10^5 spores/mL for 1 mm or 2 mm sensors, respectively	Water	[114]
Covalent (SAM, MUA +EDC+NHS)	*Listeria* cells	Gold screen-printed electrode	*L. monocytogenes* phage endolysin Ply500	Electrochemical impedance spectroscopy	1.1×10^4 cfu/mL 10^5 cfu/mL	Pure culture Milk	[38]
Physical adsorption	*S. typhimurium*	Metglas® 2826 MB ($Fe_{40}Ni_{38}Mo_4B_{18}$) + Cr + Au layers	E2 filamentous bacteriophage	Magnetoelastic	3.0 log cfu/cm^2	Fresh tomatoes	[115]
Physical adsorption	*S. typhimurium*	Metglas® 2826 MB ($Fe_{40}Ni_{38}Mo_4B_{18}$) + Cr + Au layers	E2 bacteriophage	Magnetoelastic	2.17 and 1.94 log cfu/ spinach for adaxial and abaxial surface	Spinach leaves	[116]
Covalent (SAM, 4-ATP + GLA)	*E. coli B* and *E. coli* μX	Ag n-STFs over Si	T4 bacteriophage	Surface-enhanced Raman spectroscopy	150 cfu/mL (*E. coli B*) 340 cfu/mL (*E. coli* μX)	–	[117]
Physical adsorption	*E. coli K-12*	Fiber optic (SiO_2)	T4 bacteriophage	Refractive index variations (long-period fiber gratings)	–	PBS	[118]
Covalent (APTE + GLA)	*E. coli B*	Fiber optic (SiO_2)	T4 bacteriophage	Refractive index variations (long-period fiber gratings)	10^3 cfu/mL	–	[119]
Covalent (CVR + GLA)	*B. anthracis Sterne*	Screen-printed carbon electrode microarray	*Gamma* phages	Electrochemical Impedance sensor	10^3 cfu/mL	Water Electrolyte media	[120]
Physical adsorption	MRSA	Metglas® 2826 MB ($Fe_{40}Ni_{38}Mo_4B_{18}$) + Cr + Au layers	Lytic phage	Magnetoelastic	3.0 log cfu/mL	Culture	[121]
Physical adsorption	*B. anthracis* spores	Metglas® 2826 MB ($Fe_{40}Ni_{38}Mo_4B_{18}$) + Cr + Au layers	JRB7 bacteriophage	Magnetostricitve milli/micro-cantilevers	104 spores/mL	Water	[122]
Covalent (SAM, DTSP)	*E. coli K12*	Gold	T4 bacteriophage	SPR	7×10^2 cfu/mL	PBS	[123]

SAM self-assembled monolayer, *MUA* 11-mercaptoundecanoic acid, *EDC* 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide, *NHS N*-hydroxysuccinimide, *4-ATP* 4-aminothiophenol, *GLA* glutaraldehyde, *n-STF* nanosculptured thin films, *APTES* 3-aminopropyltris(trimethylsiloxy)silane, *CVR* cyclic-voltammetric reduction, *DTSP* dithiobis(succinimidyl propionate), *SPR* surface plasmon resonance, *MRSA* methicillin-resistant *Staphylococcus aureus*

Table 2 Comparison of phage-based methods and the analytical characteristics with different transduction schemes for the detection of *E. coli*

Assay scheme	Detection technique	*E. coli* strain	Phage	Detection limit	Assay time	Sample	Comments	Ref
Biotinylated phage and streptavidin-QDs	Fluorescence (QD)	Wild-type *E. coli*	T7 engineered for in vivo biotinylation	10 cells/mL	1 h	River water samples	Measured with flow cytometry and fluorescent microscopy	[130]
Homogeneous assay detects bacterial cells lysed by the phage	Time-resolved fluorescence	*E. coli* B (ATCC 11303)	Wild-type T4	1000 cells/mL 10,000 cells/mL	25 min	Buffer Urine	No prior labeling required	[131]
Detection of released β-galactosidase upon phage-mediated cell lysis	Colorimetry and bioluminescence	*E. coli* B	*lacZ* T4 phage	10 cells/mL	5.5 h	Water	Paper-based portable culture device (See also Fig. 3)	[107]
SAM-modified sensor surface, phage immobilization using physisorption and covalent immobilization	SPR	*E. coli* K12	T4	1000 cfu/mL	20 min	Buffer	No prior labeling or enrichment steps required	[132]
Biosensor with nanosculptured thin films of silver	SERS	*E. coli* B (ATCC 11303)	T4	150 cfu/mL	–	Buffer	Low sample volume (10 μL)	[117]
Screen-printed carbon electrode microarray with covalently immobilized phages	Impedimetric	*E. coli* K12	T4	10,000 cfu/mL	25 min	Buffer	Direct and label-free measurement	[68]
Screen-printed carbon electrode microarray integrated with magnetic manipulation	Impedimetric	*E. coli* K12	T4	1000 cfu/mL	Few minutes	Buffer Milk	Improved sensitivity using magnetic beads to concentrate the bacteria	[36]
Capture of *E. coli* with immobilized phage, detection based on the impedimetric/ LAMP dual-response	Impedimetric LAMP + LSV	*E. coli* B	T4	800 cfu/mL 100 cfu/mL	15 min 40 min	Buffer, water, milk	LAMP-based detection of *tuf* gene after cell lysis	[37]
Filter-based assay	Colorimetry (*β*-gal and colorimetric substrate)	K12 (*lacZα*-complementing)	M13KE with αGal peptide	5 cfu/L 50 cfu/L	Overnight 4 h	Water (orange juice, milk)	Requires specific *lacZα*-complementing strain of *E. coli*	[133]

QD quantum dot, *SAM* self-assembled monolayer, *SPR* surface plasmon resonance, *SERS* surface enhanced Raman spectroscopy, *LAMP* loop-mediated isothermal amplification, *LSV* linear sweep voltammetry

Table 3 Representative examples of phage-based biosensors based on different transduction methods

Target(s)	Recognition element	Sensing scheme	Measuring technique	Detection limit	Analytical characteristics	Ref
Enzyme and colorimetric bioassays						
Citrinin	Phage-displayed AId, anti-phage-HRP	Indirect competitive phage ELISA: anti-citrinin capture antibody and anti-phage-HRP with colorimetric substrate	ELISA (absorbance)	1.2–1.5 μg/kg	Linear range 2.5–100.0 μg/kg; IC_{50}=10.9 μg/kg	[34]
Antibodies against *Strongyloides stercoralis*	Mimotopes for *S. stercoralis* antigen, anti-human IgG	Capture by phage-displayed mimotope, goat anti-human IgG-peroxidase for detection and colorimetric measurement	ELISA (absorbance)	–	Sensitivity 87.5 % Specificity 80 % Diagnostic accuracy 82.5 %	[33]
Thiacloprid	Anti-thiacloprid antibody, phage-displayed peptide	Anti-thiacloprid capture antibody, phage-displayed peptide and anti-phage-HRP with colorimetric substrate	ELISA (absorbance)	0.7 μg/L	IC_{50}=8.3 μg/L	[35]
rTNFα	Target coated into wells, genetically engineered bacteriophage	Genetically engineered bacteriophage with multiple biotin groups on the phage coat proteins, avidin-HRP with colorimetric substrate	ELISA (absorbance)	2.5 nM	–	[134]
Atrazine	Anti-atrazine MAb, phage-displayed anti-immunocomplex peptide, protein G functionalized magnetic beads as the solid phase	PhAIA electrochemical immunosensor: anti-phage Ab-HRP, pyrocatechol as substrate, product of the enzymatic reaction, benzo-quinone, is detected on a CSPE by CA	ELISA (EI)	0.2 pg/mL	0.2–20 ng/mL; SC_{50}=1.3 pg/mL	[135]
Free prostate-specific antigen (fPSA)	Landscape phage library, anti-PSA antibody and HRP-IgG for detection	Sandwich ELISA with phage-displayed peptide as capture probe in	ELISA (absorbance)	0.16 ng/mL	0.825−165 ng/mL	[136]
Candida albicans antibody biomarkers	HRP-IgG	Phage displaying two functional peptides, one responsible for recognizing and another for binding magnetic nanoparticles, colorimetric substrate for HRP	ELISA (absorbance)	1.1 pg/mL	–	[137]
Fluorescence bioassays						
Ricin and botulinum A	Phage-displayed single domain antibodies, phage labeled with fluorescent dye	Sandwich immunoassay with microspheres in fluid array format	Fluorescence	300 pg/mL for botulinum A 0.1 ng/mL for ricin	–	[66]
MS2 virus	Fluorescently labeled phage displaying a biotinylatable peptide, anti-virus capture antibody	Lateral flow assay with epi-fluorescence microscopy imaging	Fluorescence	ca. 10^2 pfu/strip	–	[138]
Core antigen of hepatitis B virus	Phage-displayed peptide for detection	Phage display mediated immuno-PCR: phage-displayed peptide for detection and template for the PCR, fluorescent detection of the PCR product	Real-time PCR	10 ng	–	[139]
Ochratoxin A	Phage-displayed antibody fragment	Phage display-mediated immune-PCR: phage-displayed antibody fragment works as both the detection antibody and template after heat lysis; fluorescent detection of the PCR product	Real-time PCR	3.7 pg/L	0.001–1000 pg/mL	[140]

Table 3 (continued)

Target(s)	Recognition element	Sensing scheme	Measuring technique	Detection limit	Analytical characteristics	Ref
Chemiluminescence and bioluminescence bioassays						
Ochratoxin A (OTA) (in corn, rice and instant coffee samples)	Phage-displayed dodecapeptide mimotope; monoclonal anti-OTA and anti-phage-HRP antibodies	Inhibition competitive phage assay using immobilized anti-OTA antibody; anti-phage-HRP with luminol substrate	ELISA (chemiluminescence) + qualitative dipstick assay (QDA) (colorimetric)	ELISA 0.005 ng/mL QDA (cut-off): 1 ng/mL	EC_{50} (ELISA): 0.04 ng/mL 0.006–0.245 ng/mL	[141]
P. cannabina pv. *alisalensis* (in cruciferous vegetables)	PBSPCA1 reporter phage "light tagged" with *luxAB* genes	Measuring luminescence of crucifer leave samples contaminated with *P. cannabina* pv. *alisalensis* and inoculated with PBSPCA1::*luxAB* phages	Bioluminescence assay	–	Sensitivity $>10^3$ cfu/mL Negligible cross-reactivity with non-*alisalensis Brassica* pathogens	[142]
B. anthracis (in milk and ground beef)	Wβ reporter phage "light tagged" with *luxAB* genes	Measuring luminescence of food samples contaminated with *B. anthracis* Sterne and inoculated with Wβ::*luxAB* phages	Bioluminescence assay	Milk: 8 cfu/mL (16 h incubation) Ground beef: 3.2×10^2 cfu/g 16 h incubation)	–	[143]
Shigella dysenteriae type 1 strain and *Shigella flexneri* strain (in human stool)	*Shigella flexneri* reporter phage (Shfl25875) "light tagged" with *luxAB* genes	Measuring luminescence of human stool samples contaminated with *S. flexneri* and inoculated with Shfl25875::*luxAB* phages	Bioluminescence assay	*S. flexneri:* 10^3 cfu/g	Positive cross-reactivity with *Shigella dysenteriae* type 1 strain Negligible cross-reactivity with: *Shigella boydii*, non-type 1*S. dysenteriae* and non-*Shigella enterobacteriaceae*	[144]
vRifampicin resistance (in clinical isolates of *Mycobacterium tuberculosis*)	*M. tuberculosis* reporter phage (phAE85) "light tagged" with *luciferase* genes	Measuring luminescence of samples contaminated with *M. tuberculosis* and inoculated with phAE85 phages	Bioluminescence assay Minimum inhibitory concentration (MIC)	–	Sensitivity and specificity: 91 % (CI 0.75, 0.98) and 99 % (CI 0.95, 1.00), respectively	[145]
Surface plasmon resonance bioassays						
Zearalenone (ZON) (in corn)	Phage-displayed dodecapeptide mimotopes Monoclonal anti-ZON and anti-phage-HRP antibodies	Inhibition competitive phage assay using immobilized anti-ZON antibody and phage-displayed dodecapeptide mimotopes as antigen substitutes	ELISA (for binding specificities) SPR (for reaction kinetics)	PhDI: 50 ng/g	Negligible cross-reactivity with: zearalanone, α-zearalanonl, β-zearalanol, α-	[146]

Table 3 (continued)

Target(s)	Recognition element	Sensing scheme	Measuring technique	Detection limit	Analytical characteristics	Ref
			Phage-dot immunoassay (PhDI) (colorimetric)		zearalenol, β-zearalenonl aflatoxin B1, fumonisin B1, deoxynivalenol, ochratoxin A	
Salmonella	Salmonella-specific phage (MSA1020417) molecular engineered	Label-free direct assay Phage immobilized covalently onto carboxymethylated dextran gold chips (CM5)	SPR	8.0×10^7 cfu/mL	Negligible cross-reactivity with: *L. monocytogenes* *E. coli K12* *E. coli O157:H7*	[147]
–	Phages as structural elements: Phage displayed gold binding dodecapeptides (Au-PΦ3 and Au-PΦ81)	Label-free direct assay Au-PΦ3 and Au-P Φ81 immobilized onto gold chips	SPR Circular dichroism (CD) Molecular dynamics (MD) Steered molecular dynamics (SMD)	–	Binding constants: K_{eq} (Au-PΦ3)/Au (0.49–0.82)$\times10^6$ M^{-1} K_{eq} (Au-PΦ81)/Au (0.49–0.82)$\times10^6$ M^{-1}	[148]
NIH3T3 mouse fibroblast cells (Cell proliferation and morphology studies)	Phages as structural elements: Phage displayed integrin binding peptide (RGD) in pVIII and biotin-like peptide (HPQ) in pIII	Cysteamine gold activated substrates and coated with: (1) RGB-phage (2) RGB/HPQ phage	SPR	20 cells/cm^2	20–1280 cells/cm^2	[149]
2′,3′,4′,5,5′-Pentachlorobip-henyl (PCB106)	(1) Phage displayed DSNKLSPE in pVIII (2) Phage displayed LSFAADRT in pVIII	Label-free direct assay Phage immobilized covalently onto carboxymethylated dextran gold chips (CM3)	SPR	–	K_D (PCB106): (1) 57±18 μM (2) 71±20 μM	[150]
SERS bioassays						
Prostate-specific antigen (PSA)	Phage-SERS probe based on bifunctional M13: • Single chain variable fragment (scFv) antibody anti-PSA in pIII • Proline-aspartic dipeptide (PD) in pVIII	Magnetic bead (MB)-based sandwich assay Raman reporter 4-chlorobenzenethiol (4-CBT)	SERS	1 pg/mL	–	[73]
E. coli	T4 phage wild type	Direct assay T4 phages immobilized on nanosculptured thin films (nSTFs) of silver over silica substrates Raman reporter 4-aminothiophenol (4-ATP)	SERS	1.5×10^2 cfu/mL	Negligible cross-reactivity with: *C. violaceum*, *P. denitrificans*, and *P. aeruginosa*	[117]
L. monocytogenes	*L. monocytogenes*-specific phage (A511)	Polyclonal anti-A511 antibodies immobilized covalently onto thiol-modified SERS nanoparticles	SERS + lateral flow immunochromatogr-aphy assay (LFI)	6.1×10^7 pfu/mL	–	[151]

Table 3 (continued)

Target(s)	Recognition element	Sensing scheme	Measuring technique	Detection limit	Analytical characteristics	Ref
Piezoelectric mass bioassays						
MRSA	Phages as structural elements: • Phage 12600 structural converted to spheroid • Anti-penicillin-binding protein (PBP 2a) antibodies	Sandwich assay Spheroid physically immobilized onto the gold-coated quartz pieces and latex beads decorated with anti-PBP 2a	QCM	–	Phage capture efficiency: 5.0×10^7 cells/cm^2 Negligible cross-reactivity with: *S. aureus* species (MSSA)	[73]
Tetragonal zirconia polycrystal (TZP)	Phage-displayed dodecapeptide mimotopes	Direct assay	QCM	–	PΦ17 showed a 300-fold, significantly higher binding affinity ($p<0.01$) to TZP	[152]
Electrochemical bioassays						
E. coli K12	*E. coli*-sensitive wild-type phages	Direct assay T4 bacteriophage coated magnetic beads to capture and concentrate the cells Screen-printed carbon-based impedimetric sensor	Impedimetric	10^3 cfu/mL	–	[36]
E. coli	*E. coli*-sensitive wild-type phages	Phages immobilized covalently onto functionalized gold disk electrodes, LAMP amplification of *E. coli tuf* gene after cell lysis and detection using LSV	Impedimetric	8×10^2 cfu/mL	10^2–10^7 cfu/mL	[37]
Listeria cells	Phage endolysin	Direct assay: bacteriophage endolysin coupled to gold SPE	Impedimetric	1.1×10^4 cfu/mL	10^4–10^8 cfu/mL	[38]
Glucose	Silver nanoparticle-binding bacteriophages displaying three methionine peptides	Bio-nanowire network for electrochemical detection	Electrochemical		10^{-7}–10^{-4} M	[16]
Magnetoelastic bioassays						
MRSA	Lytic phage	Unlabeled phage immobilized on the sensor, SEM analysis	ME	10^3 cfu/mL	10^1–10^8 log cfu/mL	[121]
B. anthracis	Phage	Phage-coated micron-size ME sensor, pulsed wave excitation system with signal amplification	ME	5×10^2 spores/mL	–	[153]
S. typhimurium	Phage	Phage-coated ME sensor, real-time, in situ detection	ME	1.5×10^3 cfu/mm^2	–	[154]

ELISA enzyme-linked immunosorbent assay, *AId* anti-idiotypic antibody, *HRP* horseradish peroxidase, *Mab* monolonal antibody, *PhAIA* phage anti-immune complex assay, *CSPE* carbon screen printed-electrode, *SWV* square wave voltammetry, *CA* chronoamperometry, *EI* electro-immunoassay, *LAMP* loop-mediated isothermal amplification, *LSV* linear sweep voltammetry, *MRSA* methicillin-resistant *Staphylococcus aureus*, *SEM* scanning electron microscopy, *ME* magnetoelastic, *QCM* quartz crystal microbalance, *SERS* surface-enhanced Raman spectroscopy

the same antibody, the electrochemical immunosensor was faster, performed with a much wider linear range, and the detection limit was 4.4 pg/mL, about 2500-fold lower than that of the ELISA assay [155].

The use of anti-immunocomplex recognition molecules is an attractive choice to develop noncompetitive assays for small molecules. Anti-immunocomplex peptides can also be selected from phage display libraries to recognize not only the small molecule target alone but in a complex with the capture antibody. The noncompetitive ELISA-phage anti-immune complex assay (PhAIA) has been developed using HRP-labeled anti-phage antibody for colorimetric detection of molinate [156] and clomazone [157]. The PhAIA resulted in approximately 10-fold increased sensitivity compared to the conventional competitive assay, with a remarkably enhanced specificity. A similar assay with electrochemical detection has also been developed for the detection of atrazine [135]. The phage-displayed anti-immunocomplex peptide was detected by HRP-labeled anti-phage antibody, and the benzoquinone produced by the enzymatic reaction was detected on CSPE by chronoamperometry (CA). The electrochemical assay format improved the sensitivity 200-fold and extended the linear range 10-fold compared to a colorimetric measurement. Some efforts to even further improve the sensitivity of phage-ELISA by genetically modifying the phage have been developed. Recently Brasino et al. modified the phage coat protein 8 (pVIII) with biotin groups allowing them to attach approximately 70 avidin-conjugated HRP enzymes to each phage [134]. This approach increased the signal approximately 3- to 4-fold compared to the assay with biotinylated antibodies alone.

The phage can also be used not as part of the recognition but as the capture probe of the assay. Lang et al. selected a phage probe from the f8/8 landscape library for f-PSA (free prostate-specific antigen) binding clones and used it as the capture molecule to establish a sandwich ELISA for the detection of f-PSA [136]. The detection was based on anti-PSA antibody and IgG-HRP. The limit of detection for the phage ELISA array (0.16 ng/mL) was lower than or comparable with other conventional methods. Wang et al. genetically engineered the phage to display two functional peptides on a single phage [137], one responsible for recognizing the biomarker and the other that can bind to magnetic nanoparticles. The resultant phage was used to capture the target biomarker from sera and detect it using HRP-labeled antibody. The detection limit of the method was approximately 1.1 pg/mL, about two orders of magnitude lower than that of the traditional antigen-based method.

Colorimetric-based bioassays

Colorimetric examinations are simple and sensitive. Quantifying the color changes enables the use of non-sophisticated detection systems such as spectrophotometers, or even smartphones, both relatively popular and achievable. Mostly all phage-based colorimetric biosensors reported before are based on the use of reporter phages carrying genes which encode for enzymatic reporters. One of the first colorimetric phage-based assays reported is a *Salmonella* ice nucleation diagnostic assay that uses the reporter gene *inaW* [29]. Upon infection, the expression of an ice nucleation protein (INP) is induced, which freezes the cell, and is subsequently monitored through the introduction of an orange-colored indicator dye [74]. Other useful reporter genes are the *celB* and *lacZ* sequences encoding β-glycosidase and β-galactosidase [29, 110, 111], which have been used with a variety of colorimetric substrates. For example, Derda et al. developed a very sensitive and simple filter-based assay for detection of *E. coli* strain that expresses high levels of ω-domain of β-galactosidase. Upon infection with genetically modified phage, a small peptide is delivered to the bacteria, and combination of this peptide and the ω-domain will result in formation of an active β-galactosidase. Filtering the sample through a syringe filter captured the bacteria and improved the detection of colored product of the enzyme reaction. The detection limit of 50 cfu/L was obtained in only 4 h [133]. However, to be used in real samples for detecting *E. coli* strains without the ω-domain, the method requires further development to introduce the full-length enzyme gene into the phage genome.

Recently, several authors reported new phage-based detection systems that integrate colorimetric coupling of surface plasmons of noble metal nanoparticles with engineered phages. The novelty of this approach is the integration of these two emerging technologies that permits fast and simple phage-based assay developments using simple colorimetric readouts. For instance, Oh et al. reported the development of colorimetric sensors using M13 genetically engineered phages that were able to self-arrange into phage-bundle microstructures, exhibiting macroscopic color changes triggered by the exposure to various volatile organic chemicals (VOCs) [25]. To increase the selectivity, a trinitrotoluene (TNT)-binding peptide, identified previously by phage display, was incorporated to the pVIII moiety of the phage by genetic engineering techniques. The new material is readable with a common smartphone distinguished TNT down to 300 ng/mL. In addition, no cross-reactivity was observed when the TNT-phage matrix was exposed to 20 μg/mL of 2,4-dinitrotoluene (DNT) and 300 μg/mL of 4-nitrotoluene (MNT) [25]. Although no tests were carried out using liquid samples, which would have boosted this approach as an outstanding colorimetric sensor in the near future, the authors claimed that it could be implemented to detect many other target molecules. Relevant colorimetric phage biosensors developed in the last few years are summarized in Table 3.

Fluorescence-based bioassays

Improved sensitivity in phage-based assays can also be obtained with fluorescent labels in a similar approach as the phage-ELISA. Table 3 summarizes some relevant bioassays based on phages and fluorescent detection. For example, Goldman et al. developed sandwich assays with sdAbs or the same sdAbs displayed on phages covalently attached to the surface of color-coded microspheres [66]. Both biotinylated phage or biotinylated soluble sdAb were used as reporters, and the signal was generated using fluorescent phycoerythrin coupled to streptavidin. The phage displaying sdAb antibodies showed about five times improved detection over the soluble sdAb. In the work of Edgar et al. a phage-based immunoassay was combined with quantum dots (QDs) as labels [130]. Compared to common fluorophores QDs are highly photostable and have low autofluorescence, which provides enhanced sensitivity in the assay. A genetically engineered phage was biotinylated in vivo and conjugated to streptavidin-coated QDs for bacterial detection. The QD-based method provided specific detection of 10 cells/mL in experimental samples.

Some applications that do not require labeling of the reporter molecule have also been described. Kulpakko et al. presented a homogeneous lanthanide chelate-based method for the detection of *E. coli* [131]. The method utilized lytic-specific phages to break the *E. coli* cells in order to release the cell contents to the surroundings. To detect these leaked molecules a lanthanide chelate complex Eu^{3+}/NTA/TOPO and citrate ions were used. In the presence of lysed cells, unspecific interactions of the chelate and the released cell content provided protection from the citrate ion, enabling the formation of the luminescent chelate complex. The detection limit was 1000 and 10,000 cells/mL in buffer or urine, respectively, which is relatively high compared to other systems [130, 133], but the short assay time of 25 min and the homogeneous assay format might be an advantage in some applications.

Another fluorescent method for bacterial detection was developed by Wu et al. [158]. They constructed a recombinant phage by cloning a DNA sequence encoding a tetracysteine (TC) motif into a phage coat protein gene. If bacteria sensitive to these phages were present in the sample, the phages replicated within the bacteria, and each progeny phage bore a TC tag on the surface protein of pIII. In the final step, the cell-membrane-permeating biarsenical dye, which specifically recognized TC tag, was added and the bacteria could be fluorescently detected by flow cytometry and fluorescence microscopy.

Phage sensors have also been coupled to PCR-based detection. In conventional immuno-PCR an antibody and chemically bound DNA are used to initiate the PCR reaction. In phage display mediated immuno-PCR (PD-IPCR), first introduced by Guo et al. [159], the target is captured by the immobilized capture antibody and the recombinant phage particle with the surface-displayed antibody fragment is then bound to the target. The phage DNA is subsequently released by heat lysing and then amplified and detected by real-time PCR carried out with TaqMan probes. The detection sensitivity of the PD-IPCR was 1000- to 10,000-fold higher compared with conventional ELISA but lower than in monoclonal antibody-based IPCR [159]. Later the PD-IPCR was also used for the detection of the hepatitis B virus core antigen to obtain a 10,000-fold better sensitivity than the phage-ELISA [139], and the detection of mycotoxins using phage-displayed variable domain of heavy-chain antibodies (VHH) in a competitive assay format [140].

Furthermore, the reporter phage system can also be used with fluorescent labels by inserting for example the GFP gene into the phage genome. Several genetically engineered GFP-phages have been established to detect for example pathogenic *E. coli* by fluorescence microscopy or flow cytometry [112, 160, 161]. The availability of fluorescent proteins with distinguishable spectra can offer the possibility for multiplexed detection of various different target pathogens in one sample [162].

Chemiluminescence- and bioluminescence-based bioassays

The use of chemiluminescence (CL) and bioluminescence (BL) phage-based sensing systems has been of great interest as they feature very sensitive detection owing to the high luminescent and low background signal. This approach results in an excellent signal-to-noise ratio even in comparison to photoluminescence detection. However, the required addition of a substrate to activate measurement, in combination with its irreversible consumption, can be a disadvantage in routine analysis [67].

Biosensors based on phages and CL or BL have been successfully used for the detection of live bacterial cells, including *E. coli* O157:H7, *Salmonella*, *Listeria* spp., or *Staphylococcus aureus* in both food and environmental samples [142, 163]. These biosensing systems involved several experimental approaches for the use of phages as capturing and sensing recognition elements. In general, one approach is based on the lytic property of some phages. Therefore, after infection and phage-mediated cell lysis, the intracellular metabolites, such as ATP, are liberated and monitored by bioluminescence analyses. Unfortunately, the methods based on this approach have high limits of detection ($1–6\times10^3$ cfu/mL within 120 min) and require expensive reagents and equipment [164].

Another approach relies on the ability of engineered phages to insert a gene into a target cell that expresses a reporter protein, which can be monitored by a detectable luminescence signal. The most used reporter genes for CL and BL are the

*lac*Z gene, which encodes for β-galactosidase, and bacterial (*lux*) and firefly (*luc*) luciferase genes. The methods based on this reporter phage approach significantly improved the limits of detection, which ranged from 1 to 1000 cells, and demonstrated ability to perform rapid and simple analysis [165]. For example, Burnham et al. designed a reporter phage assay based on the *lac*Z gene and its insertion into T4 bacteriophage [107]. The assay was performed on a paper-based portable culture device suitable for on-site detection of *E. coli* in water (Fig. 3). Application of the bioluminescent substrate 6-*O*-β-galactopyranosyl-luciferin, demonstrated detection limits below 10 cfu/mL within 5.5 h. Besides, the specificity was proved using several microorganisms including *Aeromonas hydrophila*, *Enterobacter cloacae*, and *Salmonella typhimurium* [107].

Bioluminescent reporter phages carrying the *lux* reporter genes cause their particular host bacteria to emit at 490 nm. The *lux* operon (*luxCDABE*) is typically derived from *Vibrio fischeri*; the *luxAB* genes are responsible for the production of luciferase that features a non-toxic luminescence and precise and sensitive signal-to-noise ratio. Ulitzur and Kuhn described one of the first attempts to use *lux*-based reported phages for monitoring *E. coli* [166]. They inserted the *luxAB* genes into the phage λ and showed a detection limit of 10 cells/mL within 1.5 h in milk samples. However, substrates should be added exogenously, to compensate for the lack of the *luxCDE* genes, which makes the biosensing system more laborious and susceptible to user error [30]. The inclusion of the entire operon *luxCDABE* would avoid this limitation but its size (ca. 6 kb) prevents the appropriate incorporation into a phage genome, because of the reduced phage-head capacity [167]. Alternatively, Kim et al. described an advanced bioluminescent reporter *Salmonella* temperate phage [168], SPC32H, whose genome was genetically modified by replacing non-essential genes with the *luxCDABE* operon. The SPC32H-CDABE detection assay was able to detect 20 cfu/mL of *S. typhimurium* in food samples within 2 h in lettuce, sliced pork, and milk [168], without appreciable matrix interferences. Although the construction of the recombinant SPC32H-CDABE *lux* reporter phage requires costly reagents and highly trained personnel, it is a promising tool for the development of portable diagnostic phage-based kits with bioluminescence detection. Recent chemiluminescence and bioluminescence phage biosensors are summarized in Table 3.

Surface plasmon resonance (SPR)-based bioassays

Surface plasmon resonance (SPR) is a label-free detection method that measures changes in the refractive index occurring in the field of an electromagnetic wave supported by the optical structure of the sensor [75]. The technique enables real-time and cost-effective detection of analytes binding to a receptor immobilized on the sensor surface. Therefore, it is very useful for evaluating the affinity and binding kinetics of immobilized phages [150, 169]. The measuring format in SPR sensors depends on several factors including the size of target analyte molecules, the binding characteristics of the biomolecular recognition element, range of concentrations of analyte to be measured, and the sample matrix [75].

Most phage-based SPR sensors described so far are based on the direct detection of the target compound after selective recognition by the phage immobilized on the SPR. This approach is limited to those applications where the binding of the analyte, at the concentrations of interest, results in a sufficient refractive index change, which is usually the case for bacterial detection. Covalent attachment of the phages onto the SPR sensing platforms can be labor-intensive and cumbersome but it usually allows an improvement of biosensor sensitivity.

Tawil et al. compared the efficiency of several immobilization strategies for phage immobilization to gold surfaces for further sensor development [170]. Several immobilization strategies, including physisorption, the use of glutaraldehyde, L-cysteine, 11-mercaptoundecanoic acid (MUA), 1-(3-

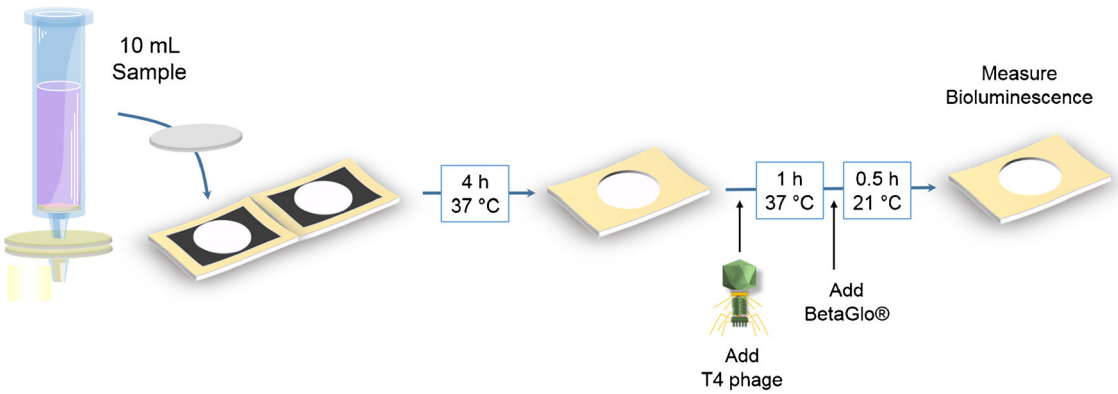

Fig. 3 General scheme of the simple disposable paper-based test and device for rapid specific detection of *E. coli* in environmental samples. Upon the addition of substrate for the bacteriophage and β-galactosidase, the accumulation of color or bioluminescence signal (4 and 1.5 h, respectively) is acquired. Adapted from [107]

dimethylaminopropyl)-3-ethylcarbodiimide (EDC)/*N*-hydroxysuccinimide (NHS) or their combination, were tested to determine which strategy would best preserve the biofunctionality and lytic efficiencies of methicillin-resistant *Staphylococcus aureus* (MRSA) phages after immobilization onto gold substrates. They found that the use of mixed self-assembled monolayers (SAMs) of L-cysteine and 11-mercaptoundecanoic acid allowed the recognition and disruption of bacterial membranes. The authors applied the method to the development of bacteriophage-based sensor for the detection of *E. coli* and MRSA. The authors used the T4 bacteriophage to detect *E. coli*, and a novel and highly specific phage (BP14) to detect MRSA at concentrations less than 10^3 cfu/mL, without prior labeling or enrichment steps in less than 20 min [132].

In a different approach [123], T4 phages were covalently immobilized onto the gold platforms using SAMs of dithiobis(succinimidyl propionate) (DTSP) for the detection of *E. coli* K12 bacteria. The use of DTSP allowed phage immobilization in one step, in comparison to the two-step procedure followed when using cross-linkers such as glutaraldehyde [124], resulting in stable substrates with chemically anchored phages that were successfully regenerated by treatment with 20 mM NaOH and thus could be reused for multiple SPR cycles. This aspect is of particular interest for the development of phage-based biosensors for online detection of food and water borne pathogens.

The attachment of phages to gold nanoparticles for enhanced SPR detection has received much attention in recent years. The use of localized surface plasmon resonance (LSPR), where the plasmon is produced by metal nanoparticles (NPs), usually results in an amplification of the detection signal. However, it has been reported that the contact area between a bacterial cell and a single NP may be too small to result in a detectable plasmon peak shift [171]. To overcome this limitation, Tawil et al. prepared gold-phage hybrids using gold nanoparticles (AuNPs) [172]. The authors stabilized (PEGylated) the hybrids using heterobifunctional polyethylene glycol (PEG) molecules, containing a thiol anchor group on one end to stably anchor it to the gold surface and a carboxylic acid group on the other end for further coupling to the amine groups at the phage surface by carbodiimide chemistry.

In a different approach gold nanoparticles have been capped with carboxymethyl chitosan (CMC-AuNPs) for the determination of femtomolar concentrations (2 fM) of T7 virus (bacteriophage) [173]. In the presence of the T7 virus, the spherical structure of the CMC-AuNPs is changed into chain-like nanostructures as a result of the strong coordination (affinity) of CMC-AuNPs with T7 virus. This approach resulted in a decrease of the absorption intensity of CMC-AuNPs at 520 nm followed by the growth of a new absorption band at approximately 610 nm in the presence of the T7 virions enabling colorimetric biosensing detection. Recent SPR phage biosensors are summarized in Table 3.

Surface-enhanced Raman spectroscopy-based bioassays

Surface-enhanced Raman spectroscopy (SERS) is an advanced Raman technique that enhances the vibrational absorbance of certain molecules by a factor of several orders of magnitude when they are near the surface of nanostructured noble metals. The enhanced intensity of this phenomenon depends on the molecules' ability to emit a Raman signal and the localized fields of plasmons in their vicinity.

A SERS nanoprobe is composed of a metallic nanostructure and a recognition element. The conjugation of both elements in a single unit is difficult and still remains a challenge. However, phages can be genetically modified to display biorecognition elements by the pIII end and functional peptides along with the long pVIII coat protein. Thus, fabrication of phage SERS nanoprobes that simultaneously display biorecognition elements and peptides for binding noble metal nanoparticles is a promising approach for SERS-based biosensor development.

For instance, Lee et al. presented a novel SERS nanoprobe based on the bifunctional M13 virus [73]. For its assembly, the pIII protein was genetically engineered to express a single chain variable fragment (scFv) antibody against the prostate-specific antigen (PSA) and the pVIII coat protein was modified with a proline-aspartic dipeptide (PD) that binds cetyltrimethylammonium bromide (CTAB)-coated Au nanocubes (AuNCs) of 50 nm in size (Fig. 4a). The AuNCs were also decorated with 4-chlorobenzenethiol (4-CBT) and used as a Raman reporter. To demonstrate the performance of the phage-SERS probe in PSA detection, a magnetic bead (MB)-based sandwich assay was performed using anti-PSA monoclonal antibodies, bound to the MBs, and the phage-SERS probe as the detection recognition element (Fig. 4b).

The 541 cm^{-1} peak was selected to plot the intensity of the SERS signal and construct the calibration plot. The limit of detection achieved was 1 pg/mL, slightly higher than those obtained previously using SERS biosensors [174]. However, the authors claim that this M13 SERS nanoprobe features strong and uniform signal and could offer, after some improvements, a powerful and robust tool for further multiplexed detection of any antigens [174].

In a different approach, Srivastava et al. reported a SERS biosensor for the detection of *E. coli* using T4 phages immobilized on nanosculptured thin films (nSTFs) of silver over silica substrates (Fig. 5) [117]. The enhancement of Raman bands was monitored using 4-aminothiophenol (4-ATP) as reporter molecule. The specificity was checked by exposing the sensor to three different bacteria strains: *Chromobacterium violaceum*, *Paracoccus denitrificans*, and *Pseudomonas aeruginosa*. As expected, the cross-reactivity was

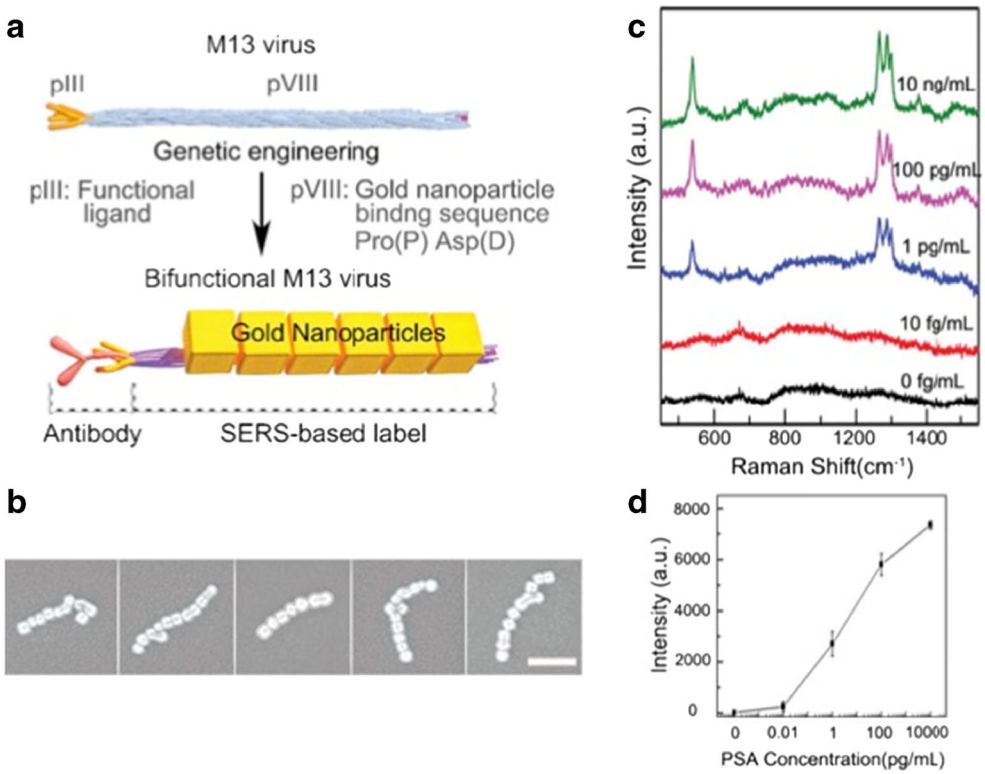

Fig. 4 **a** Phage-based SERS nanoprobe: PD sequence was expressed on the pVIII part for binding cetyltrimethylammonium bromide (CTAB)-coated gold nanoparticles. The pIII part was functionalized with an antibody for capturing target analyte; **b** scanning electron microscopy images of phage-probes at a concentration level of 10^9 pfu/mL (*scale bar* 200 nm); **c** SERS spectra of the MB-based sandwich assay acquired at different concentrations of PSA (10 ng/mL, 100 pg/mL, 1 pg/mL, 10 fg/mL, and 0 fg/mL); **d** plot of the SERS signal at 541 cm^{-1} as a function of PSA concentration (n=5). The SERS spectra were obtained using a 647-nm line from a Kr ion laser with 10 s of acquisition time. Copyright Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

insignificant in all cases. Moreover, the *E. coli* target was not lysed by the T4 phage on the sensor chip and thus the results were not time-dependent. The authors attributed this behavior to the fact that the absorption of the phage to the surface prevented viral DNA injection because its tails had undergone a large conformational change. The SERS biosensor showed a detection limit down to 1.5×10^2 cfu/mL, which is an order of magnitude lower than others recently reported with SERS [175] or SPR sensors [132]. Other examples of phage-SERS bioassays are collected in Table 3.

Electrochemical-based bioassays

Electrochemical detection has also been applied to a few sensor systems for bacterial detection. Some recent applications are summarized in Table 3. For example, Shabani et al. developed an impedimetric sensor for bacterial detection [68]. The phages were covalently immobilized onto functionalized carbon screen-printed electrode (SPE) microarrays and were used to detect *E. coli* with a detection limit of 10^4 cfu/mL [68]. Later the same group developed another impedimetric sensor with improved sensitivity of 10^3 cfu/mL [36]. The system used phage-coated magnetic particles to concentrate the *E. coli* cells from samples before detection. Also Tlili et al. employed phages as the recognition element in label-free electrochemical impedance spectroscopy for the detection of *E. coli* [37]. This dual-response biosensor used a covalently immobilized phage for capturing and lysing the target cells. The released intracellular *tuf* gene was then amplified by the loop-mediated isothermal amplification (LAMP) method and monitored by

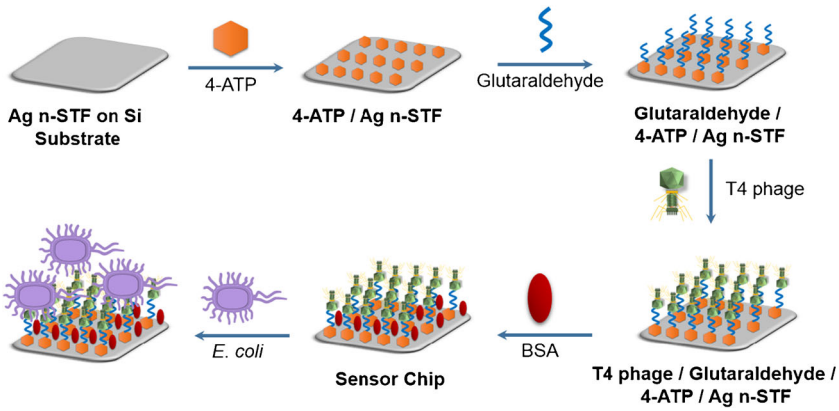

Fig. 5 Workflow of the SERS sensor chip development utilizing metallic nanosculptured thin films. The Ag surface is functionalized using self-assembled monolayer of 4-ATP and glutaraldehyde to immobilize the T4 phage for detection of *E. coli*. Adapted from [117]

the linear sweep voltammetry (LSV) technique as a complementary approach for the impedance measurement. The sensor had a detection limit of 8×10^2 cfu/mL and 10^2 cfu/mL for the impedimetric and LAMP methods, respectively.

Tolba et al. developed a biosensor for detection of *Listeria* using the cell wall binding domain (CBD) of phage-encoded peptidoglycan hydrolases (endolysin) [38]. The phage CBD was immobilized on a gold SPE and the bacterial cells were subsequently detected by electrochemical impedance spectroscopy (EIS) in pure culture and artificially contaminated milk, with detection limits of 1.1×10^4 cfu/mL and 10^5 cfu/mL, respectively [38].

Piezoelectric mass-based bioassays

Piezoelectric mass (PM) sensors are largely used for pathogen detection in microanalytical technologies although few of them are based on phages as recognition elements. These methods rely on measuring the changes in vibrational frequency induced by a mechanical oscillation, which results from changes in mass on the piezoelectric crystal oscillator's surface. Common configurations used for piezoelectric crystal biosensors are quartz crystal microbalance (QCM) devices, which vibrate in bulk, and surface acoustic wave (SAW) devices [69].

Unlike other biosensor approaches, QCM and SAW platforms are mainly used for characterization purposes, including (a) examination of bacterial–phage interactions [70], (b) investigation of phage-based coatings when used as nanoscaffolds [176], and (c) implementation of phage display processes. Nevertheless, some interesting PM sensors have been recently reported (Table 3). For instance, Guntupalli et al. presented a phage-based QCM biosensor for discrimination of MRSA from sensitive *S. aureus* species (MSSA) [177]. In the first step, the lytic phage 12600, structurally converted to spheroid, was physically immobilized onto the gold-coated quartz pieces and used as a sensor probe for *S. aureus* identification. In the second step, latex beads decorated with penicillin-binding protein (PBP 2a) antibodies were perfused through the sensing area. When MRSA was tested, anti-PBP 2a beads were bound to PBP 2a cell wall protein and yielded a positive signal, whereas the QCM sensor, exposed to MSSA, gave no response. The phage capture efficiency found in this work was 5.0×10^7 cells/cm^2, demonstrating its potential for making effective antimicrobial surfaces [177].

Magnetoelastic-based bioassays

Magnetoelastic (ME) sensors are made of magnetoelastic materials that contract or lengthen when they are excited by an AC magnetic field. The resonant frequency is dependent on the mass or viscosity surrounding the surface of the resonator material. ME sensors have been used to detect chemical and biological targets by incorporating (bio)recognition elements (antibodies, phages, enzymes, etc.) on the sensor surface and could be operated in either gaseous, liquid, static, or flowing environments.

ME biosensors have been established as powerful wireless screening methods; among these, phage-based ME biosensors have been broadly reported. In general, the detection of foodborne pathogens with on-site screening capabilities has been described, offering stable, reproducible, and inexpensive biosensing approaches [71, 72]. Moreover, phage-based ME biosensors are simple, sensitive, and selective low-cost devices that are currently, or in the near future, being used at multiple points in the food supply chain offering robustness in monitoring for bacterial contamination outbreaks.

For instance, filamentous phage (E2 phage) has been genetically engineered to specifically detect *S. typhimurium* in food samples [178], such as spinach leaves [116] and tomato, milk, or apple juice [179]. All these E2 phage-based ME biosensors showed excellent specificity and selectivity for *S. typhimurium* (5×10^2 cfu/mL) even when the platforms are exposed to high concentrations of both *E. coli* and *Listeria monocytogenes* (5×10^7 cfu/mL). Furthermore, E2 phage-based ME biosensors show excellent stability upon exposure to harsh environments [180]. The longevity is demonstrated by comparison of their performance with an antibody-based ME biosensor. The authors claimed that after 5 days of storage at +65 °C, the antibody-based biosensor showed no affinity activity whereas the phage-based biosensor maintained its binding properties with good sensitivity [181].

Another type of receptor phage used in ME biosensor is the JRB7 phage. This engineered filamentous phage detects *Bacillus anthracis* spores with high sensitivity and selectivity. For example, Chai et al. demonstrated the in situ and real-time detection of *S. typhimurium* and *B. anthracis* spores on watermelon surfaces [182]. E2 phage and JRB7 phage ME biosensors were simultaneously monitored during exposure to *B. anthracis* or *Salmonella* at a concentration of 1.5×10^6 cfu/mm^2. Some recent examples of ME biosensors based on phage-based recognition elements are summarized in Table 3.

Future trends

Bacteriophages are increasingly applied in a variety of chemical and biological sensors and assays. This evolution is the result of their versatility, continuously implemented by the discoveries and innovations in molecular and chemical engineering. Phage display has contributed to the widespread application of phages and shifted their uses from traditional methods, all based on detection of pathogens, to promising prospects in biosensing development.

The future of biosensing platforms will depend on several factors. Some of them have received a great deal of attention in recent years such as the creation of new recognition elements, implementation of signal amplification strategies, or development of nanostructures for accurate geometric control. In this regard, engineered phages are covering a wide area of these topics through the simple manipulation of their genome, which allows the expression of desired proteins and peptides on their surface with varying functions (i.e., specific biorecognition reporters or detection protein probes). In fact, the appropriate understanding of the phages has allowed their production with multifunctional capabilities in a single unit, representing a clear advantage over other biomolecules used in biosensing (DNA, antibodies, enzymes, etc.). Additionally, since phages are stable in harsh environments, phage-based biosensing systems can be applied not only to measurements with industrial and clinical purposes but also and more importantly in developing countries, where medical diagnosis devices have typically limited use because of their sensitivity to storage conditions.

Moreover, one of the most promising applications of phages in biosensing is their combination with emerging materials and nanostructures (magnetic, metallic, polymer nanoparticles, quantum dots, etc.) to produce novel analytical nanodevices or bioinspired sensing materials. The facile synthesis and tunable sensor properties of those hybrids can be useful for the detection of a wide variety of target molecules. Nevertheless, the research in this area will require special efforts to address some critical limitations of phages in biosensing applications. For example, the application of phage-based biosensing systems to in vivo environments should be preceded by toxicity examinations to avoid any safety concerns. Indeed, care should be taken to avoid any potential social alarm that could be created by the use of viruses for medical diagnosis and foodborne controls.

In conclusion, although advances in this field have been impressive in the last few years, the future of phages as sensing elements will require a close collaboration between experts in key areas such as biochemistry, molecular biology, engineering, physics, biology, chemistry, and material science to boost their analytical applications to the same level as other common biorecognition elements.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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