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# Phage Display in the Quest for New Selective Recognition Elements for Biosensors

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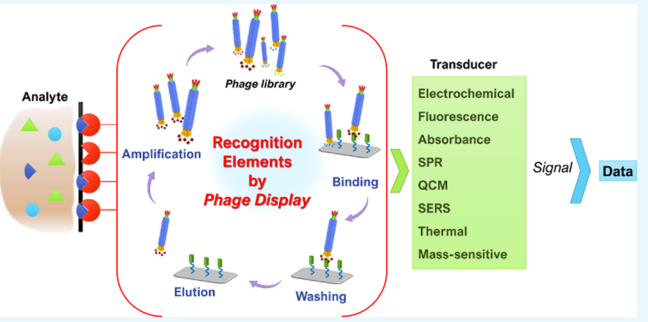
**ABSTRACT:** Phages are bacterial viruses that have gained a significant role in biotechnology owing to their widely studied biology and many advantageous characteristics. Perhaps the best-known application of phages is phage display that refers to the expression of foreign peptides or proteins outside the phage virion as a fusion with one of the phage coat proteins. In 2018, one half of the Nobel prize in chemistry was awarded jointly to George P. Smith and Sir Gregory P. Winter "for the phage display of peptides and antibodies." The outstanding technology has evolved and developed considerably since its first description in 1985, and today phage display is commonly used in a wide variety of disciplines, including drug discovery, enzyme optimization, biomolecular interaction studies, as well as biosensor development. A cornerstone of all biosensors, regardless of the sensor platform or transduction scheme used, is a sensitive and selective bioreceptor, or a recognition element, that can provide specific binding to the target analyte. Many environmentally or pharmacologically interesting target analytes might not have naturally appropriate binding partners for biosensor development, but phage display can facilitate the production of novel receptors beyond known biomolecular interactions, or against toxic or nonimmunogenic targets, making the technology a valuable tool in the quest of new recognition elements for biosensor development.




## INTRODUCTION

Bacteriophages, or phages for short, are bacterial viruses that are abundant in nature but harmless to humans. Their role as the main regulators of the microbial balance among the diverse variety of bacteria existing in the ecosystem makes them naturally eminent, but, owing to many interesting characteristics, phages have also become an exceptional tool for many biotechnological applications.[1] The Greek origin of the name bacteriophage describes them as "bacteria-eaters,"[1] although perhaps more aptly they can be defined as parasites that are capable of infecting bacteria.[2] Upon infection, the phage takes over the biosynthetic machinery of the host cell to replicate its genetic material and subsequently produces more than a thousand identical phage particles.[3]

Naturally, a myriad of different phages exist, but, generally, each phage particle, or virion, encloses its genome of DNA or RNA in a protein coat, or capsid.[4] Phages are ubiquitous in nature, highly specific to bacteria, and, in addition, are extremely robust and can withstand even harsh conditions.[1] They can be classified based on their morphology, their life cycle, and ways to propagate.[1] Lytic or productive phages, such as T4, T7, T3, and MS2 phages, are only capable of replicating their genome, assembling the phage virions, and releasing them by lysing and killing the host cell.[4,5] Lysogenic or temperate phages, such as $\lambda$ phage, can multiply via lytic cycle or incorporate their genome into the host cell genome where it will produce a quiescent state.[5] Filamentous phages, characterized by their long rodlike shape, are lysogenic phages that do not lyse their host cell but secrete the newly assembled virions and continue the process. Filamentous phages can infect a wide variety of Gram-negative bacteria, for example, *Escherichia coli*. The most used phages include M13, fd, and f1 phages, all belonging to the Ff class, so named because they infect the bacterial host via the tip of the F conjugative pilus.[6]

M13 is one of the most used phages for sensing applications,[7] and it consists of a circular single-stranded DNA (ssDNA) genome packaged within the phage particle, a somewhat flexible protein cylinder of about 930 nm in length and 6.5 nm in diameter.[5] The filamentous capsid consists of the phage structural proteins, the major coat protein (pVIII), which appears in approximately 2700 copies alongside the virion, and the minor coat proteins, capping both ends of the phage, pVII and pIX on one end and pIII and pVI on the other (Figure 1A,B).[5,8] The pIII is the largest and structurally most complex of the phage proteins, and it is essential for the infectivity because of its role in the binding to the bacterial pilus and is necessary for the termination of the viral assembly. The remaining six phage proteins are involved in viral

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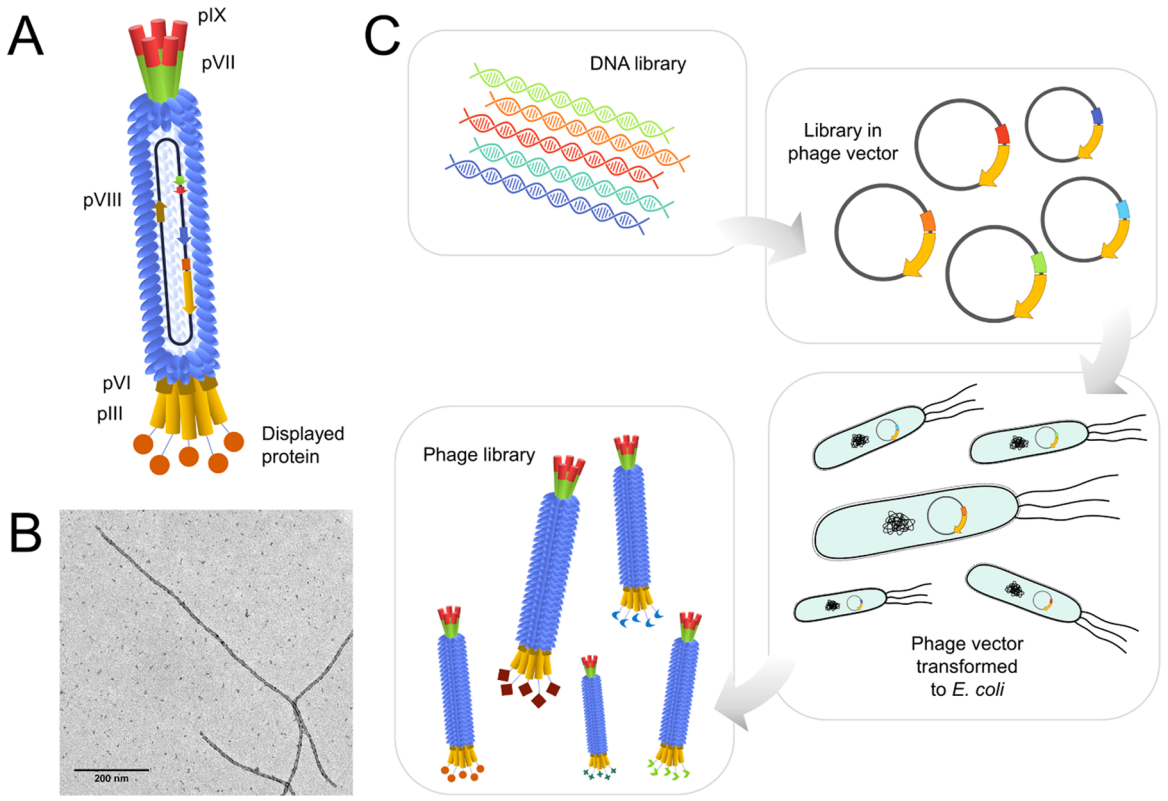


**Figure 1.** (A) Structure of filamentous phage M13 which consists of a protein coat made of the major coat protein (pVIII) and the minor coat proteins (pIII and pVI on one end and pVII and pIX on the other). The genomic DNA encoding for the coat proteins is enclosed within the protein coat. By introducing modified segments into the genomic DNA, the phage can be engineered to display foreign peptides or proteins as a fusion with one of the coat proteins, most commonly pIII. (B) Transmission electron micrograph of the filamentous phage M13 acquired with negative staining using a JEOL JEM-1400PLUS instrument operating at 120 kV, with a LaB6 electron source and a GATAN US1000 CCD camera (2k × 2k). (C) Construction of phage-displayed libraries includes generation of the DNA library encoding for the different variants and introduction of the variable sequences in the phage DNA (typically a phage vector or phagemid system). After transforming the DNA to bacteria, phages are amplified and will display an individual protein or peptide variants outside the virion.

replication and assembly.[6] M13 can infect *E. coli* by the attachment of the pIII to the pilus, which is encoded by the bacterial genes carried on the *F*-factor. After phage binding, the pilus retracts until the phage reaches the bacterial surface and pIII can bind to the membrane proteins and transfer the phage genome into the host cell.

Subsequently, the infecting phage disassembles and the coat proteins insert into the bacterial membrane. Once inside the bacterial cell, the phage genome is converted to double-stranded DNA (dsDNA) by bacterial enzymes and synthesis of new phage protein begins. Newly synthesized proteins finally assemble around the ssDNA genome and extrude from the bacterium. As a nonlytic phage, M13 does not kill the host cell and the infected host continue growing and dividing.[5,8]

The phage structure and its mode of replication have contributed to making phages a valuable tool for biological research.[5] Because of the natural assembly of phage virions, they are rapid and straightforward to produce even in large quantities in bacteria and they are fairly easy to purify in high yields.[6] Modifying the phage genome is relatively simple as foreign DNA sequences can be inserted into the nonessentials regions of the genome, making it a useful cloning vehicle in biotechnology.

## PRINCIPLES OF PHAGE DISPLAY

Phage display refers to the expression of peptide or protein variants on the surface of the phage virion by cloning the corresponding encoding gene as a fusion with one of the phage coat proteins (Figure 1).[5,9] This technique has been recognized as a powerful tool to screen and select binders on the basis of molecular recognition from phage-displayed libraries, assemblies of about 10 billion of phage clones each harboring a different variant of the displayed entity.[6]

Filamentous phages are the most common ones used for phage display because they are rather ideal for inserting various-length DNA segments into the genome that is relatively small in size and accommodates well such modifications. Moreover, cloning and construction of fusions are facilitated by the possibility of isolating both ssDNA and dsDNA. Being nonlytic, filamentous phages do not kill the host cell but can be amplified in high quantities.[10] On the other hand, one of the drawbacks of using M13 for phage display is that owing to the nonlytic propagation all of the compounds of the virion must be exported through the bacterial inner membrane prior to the phage assembly. Occasionally, this has been a limitation for displaying large proteins whose size, sequence, or folding might prevent the translocation or disrupt the integrity of the capsid. In principle, this limitation can be obviated by using lytic phages, such as icosahedral T4 and especially T7, which assemble entirely in the cytoplasm.[11,12]

Nevertheless, based on filamentous phages, numerous display systems have been developed using different coat proteins for the display together with phage or phagemid vectors. These phage display systems can be more specifically



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classified according to the arrangement of the coat protein genes, which in the end affects the display valency, i.e., whether the foreign peptide or protein is displayed on all or only part of the copies of the coat proteins.[2] The N-terminus of pIII was the first location used for the display of a foreign peptide,[13] and it is still the most commonly used, although all five capsid proteins have been used to this aim.[6,10] The major coat protein, pVIII, can be used for both N- and C-terminal fusions, but only short peptides (six to eight residues) can be displayed without disturbing the phage's ability to replicate. Fusion with pVIII will typically lead to multivalent display with even thousands of copies, whereas lower valencies, usually one to five copies per phage, can be obtained with pIII fusions.

Commonly, phage display is based on operating either on phage or phagemid vectors.[14] In the phage vector display, the foreign DNA sequence to be displayed is cloned into the phage genome fused with the gene of one of the coat proteins. As a result, the phage will express the foreign entity, peptide or protein, as a fusion with the coat protein. In some cases, for example, if the size of the target is too large, such display may interfere with the phage assembly and the phagemid system is preferred. Phagemids are phage-derived vectors that do not encode for all of the structural and functional proteins of the phage but carry only the necessary replication origins and one kind of coat protein that is used for the display. Phagemid usually contains an antibiotic resistance gene and two origins of replication, a plasmid replication origin that allows them to replicate in a high copy number in the host cell and a filamentous phage replication origin that is activated once the phagemid-bearing cell is superinfected with the helper phage. Thus, the phagemid by itself can replicate within a bacterial cell and maintain itself as a plasmid, but it is not able to finish the assembly of phage particles independently. Only when a helper phage vector containing the genetic elements for phage packing is present, the host cell harbors both the phagemid and the helper phage genome, and thus, phage particles can be produced. In fact, superinfection results in two types of infective virions, particles carrying the helper phage DNA and particles with the phagemid DNA.[6,15]

Generally speaking, the phagemid vectors are preferred, especially when displaying entire proteins or antibody fragments. As the size of the phagemid is considerably smaller than that of the phage vector, they are easier to manipulate, maintain, and propagate, and they usually allow higher transformation efficiencies and improved genetic stability. Moreover, cloning is relatively simple in comparison with phage vectors that have a somewhat complex structure with overlapping genes, promoters, and terminators.[10] In addition, the phagemid system is required if a monovalent display is desired. Nevertheless, the phagemid system requires an additional infection with a helper phage to provide all of the proteins needed to make ssDNA and new virion particles.[8] The helper phage usually has a modified packing signal to favor the packing of the phagemid,[16] and further modifications can allow, for example, multivalent display or inhibition of wild-type phage infection with trypsin-cleavable pIII display.[17,18] The tryptic helper phage (KM13) is sensitive to trypsin and loses its infectivity when incubated with this protease. Therefore, trypsin treatment after the selection can eliminate the majority of the helper phages that do not display the foreign peptide of interest. In other words, only those phages carrying the fusion protein can maintain their infectivity and continue to propagate in the bacterial host.[5,6]

## PHAGE-DISPLAYED LIBRARIES

**Peptide Libraries.** Phage-displayed libraries are made of billions of phage clones, each one carrying a different foreign DNA insert and therefore displaying a unique peptide or protein sequence on its surface (Figure 1C).[2] The foreign DNA sequence coding for the displayed entity can be derived from a natural source, or it can be deliberately designed and synthesized chemically.[6]

The use of peptide libraries is perhaps inferior to antibody libraries, but nevertheless, they have been widely used for various applications, for example, for epitope mapping,[19] immunotherapy,[20] antiviral research,[21] vaccine development,[22] and protein−protein interaction studies.[23] Moreover, peptides can provide information about interactional motifs, epitopes or binding sites of antibodies and other proteins. Phage-displayed peptides can also be useful intermediates in the development of small-molecule drugs.[24] Remarkably, peptides can be isolated against almost any protein target, including, for example, antibodies, enzymes, receptors, transcription factors, and protein interaction domains.[6] Experience has shown that peptides identified by phage display commonly bind to protein functional sites rather than randomly or nonspecifically to the surface of the target. Such binding sites are often grooves or depressions in the protein surface with exposed hydrophobic groups, making them suitable for specific target binding.[6] If the peptide binds to the functional site of the target protein, it will also most likely inhibit binding of others to the same site. Taking advantage of this feature peptides have been used as surrogate ligands in a variety of competition-based assays for drug discovery.[6] Similarly, antibody binding peptides have been applied as epitope mimics in competitive immunoassays.[25]

Many peptide libraries are made by joining the DNA sequences coding for the peptide directly into the phage genome at, or near, the N-terminus of gIII. As a result, potentially five copies of peptide−pIII fusions are displayed at one end of the phage particle. Fusion to the gVIII can result in more than 2000 copies of the peptide per phage. It should be noted that as the strength of the binding is defined not only by the affinity of the interaction but also by the avidity, i.e., the overall strength of the binding, which is affected by the number of binding sites, may have substantial effects.[1] Thus, the multivalent nature of the display will have significant avidity effects and potentially low-affinity binders might end up selected due to the high avidity.[24] The size of the peptide is limited in such systems but can be increased if a phagemid system is used. Moreover, the phagemid system allows reducing the avidity effects by a monovalent display. The diversity of peptide libraries, in terms of the number of distinct peptide sequences, is usually limited by bacterial transformation efficiency. Typically, the diversity of peptide libraries vary between $10^7$ and $10^9$ phages. Thus, the size of completely randomized peptides (for example, 20 different codons, at $n$ different positions) is limited to $n = 6-8$. Despite these limitations, also longer peptides, although incomplete in terms of nucleotide randomization, have been used to construct libraries with sufficient variability.[2,24]

Random peptide libraries can be derived by oligonucleotide-directed mutagenesis from "degenerate" oligonucleotides that are synthesized chemically by adding mixtures of nucleotides to a growing nucleotide chain.[2,6] Random peptide libraries can be described as naïve, in the sense that they are designed to be



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as unbiased as possible and, thus, they can provide specific binders for many different targets. However, selections from naïve libraries often result in binders with low or mediocre affinities, especially if the diversity of the library is not sufficient.[26] Randomness in the sequence can be introduced at the nucleotide level varying all of the nucleotides of the codons (NNN), resulting in highly biased diversity since some amino acids are represented by several codons and furthermore three codons are stop codons. Thus generally, degeneracy at the codon level is preferred as it gives a less biased representation of the amino acids. In fact, even less redundant diversity where only the third position of the codon is allowed to vary either as G/C or G/T (NNK or NNS) is sufficient to cover all of the 20 amino acids.[6]

In some cases, randomization is restricted to certain regions, thus creating a constrained library opposed to an unconstrained one. In general, constrained peptide libraries present less three-dimensional shapes than an unconstrained library and, therefore, the probability for target binding is reduced. However, those with appropriate conformations may possess far higher affinities than any unconstrained peptide because the loss in entropy on target binding is likely less than for unconstrained peptide.[2,26] Common constraint on displayed peptides is a disulfide bond between two cysteine residues at fixed positions in an otherwise random sequence, resulting in cyclic peptides. Similarly, coordination bonds between histidine residues and metal ions can be used as constraints.[2,26] Alternative, constrained peptides can be presented in the context of a protein scaffold, such as $\alpha$-helices, $\beta$-sheets, or other secondary structure elements. In a sense, antibody libraries actually entail a protein scaffold where specific regions responsible for the antigen binding have been randomized.

**Antibody Libraries.** Although the first use of phage display focused on peptides, antibody libraries have been arguably the most successful use of this technology and have led to the discovery of antibodies with affinities comparable to those obtained by hybridoma technology.[9,27] In fact, alongside with the development of monoclonal antibodies using hybridoma technology[28] and the isolation and cloning of antibody genes to enable expression of antibody fragments in bacteria,[29,30] the generation of antibody libraries by phage display can be considered as one of the groundbreaking methods for the antibody discovery.

Antibody libraries can be constructed from natural sources by isolating the B cells of a source animal that has been immunized with the target antigen and using the isolated antibody genes to create the antibody library.[31] These kind of libraries have a strong bias toward the antigen, and even a modest-sized library can be sufficient to isolate specific binders. However, construction of a separate immunized library is needed for each antigen, although immunization with several antigens simultaneously is also possible.[32] Most of these libraries are produced from mouse,[33−37] but also other animals, such as chicken,[38,39] rabbit,[32,40] sheep,[41] monkey,[42] camel,[43] and shark,[44] have been used. Additionally, immunized human libraries have been made using blood from patients naturally infected with viruses or parasites.[5,45]

On the other hand, naïve antibody libraries can be constructed completely in vitro resulting in nonimmunized,[46] synthetic,[47] or semisynthetic libraries.[48] The use of synthetic repertoires bypasses the need to isolate antibody genes and allows generating sequences with predefined properties, or using optimal framework sequences.[49] Nonimmunized libraries are a result of rearranged antibody genes isolated from B cells of healthy individuals that have not been intentionally immunized. Semisynthetic and combinatorial libraries combine natural and synthetic sequences. Such libraries are usually constructed by introducing synthetic diversity into a naïve library by polymerase chain reaction (PCR) assembly of germline genes, or by recombination of in vivo formed complementarity-determining regions (CDRs).[9] Synthetic libraries are constructed entirely in vitro using oligonucleotides to introduce diversity into the CDRs. While antibodies with mediocre affinities are readily screened from relatively small naïve or synthetic libraries, in general, for isolating high-affinity binders, the diversity of the library becomes significant.[9] In fact, studies have shown a linear correlation between the library size in terms of diversity and the highest affinity that has been isolated, meaning that, in particular, in the case of naïve or synthetic libraries, the size of the library must be large enough to increase the probability of finding a given antibody and enhance the quality of the antibody.[50]

**Other Functional Domains and Scaffolds.** Phage display technology also offers a means to explore other nonantibody protein binders. Functional protein domains refer to short polypeptide chains that are capable of creating a three-dimensional structure required for a defined functional activity.[5] For example, affibodies are engineered single-domain proteins, in which a functional scaffold has been derived from protein A.[51] Affibody molecule libraries have been constructed by combinatorial randomization of the amino acids in the scaffold and used to select binders by phage display.[52,53] Other binding scaffolds include, for example, anticalins,[54,55] DARPins,[56] knottins,[57] atrimers, and fynomers,[58] which all can be selected using phage display, although these binders have been less used in biosensing applications.

## IN VITRO AFFINITY SELECTIONS

Individual clones from phage-displayed libraries theoretically can be directly screened for target binding, for example, by immunoassays, in a manner resembling screening of synthetic or chemical libraries that must be screened compound by compound. However, screening of libraries consisting of millions to billions of different clones is limited by the number of clones that can be examined.[9] To efficiently isolate specific binders, the library needs to be coupled to a technology that provides the means to carry out selections from these repertoires. In vitro display technologies, which basically mimic the natural in vivo process of antibody production, have enabled such proficient enrichment and selection of binders in a rapid and controllable manner. The essence of the display technologies resides in the physical linkage between the phenotype of the phage displaying the protein or peptide on the surface and the genotype encoding for that entity packaged as the genetic information within the same phage particle. This link enables selection of phage-displayed libraries and powerful enrichment by selective propagation of the individuals with the desired properties.[9] Thus, a single displayed entity, be it a peptide, protein, or antibody fragment, of the desired trait, can be captured from a pool of billions of variants and its gene can be amplified and used for another selection round or whatever downstream purpose might be desired.[8]

Although phage display is the most renowned technique for screening libraries,[13,59] also other display methods, including cell surface display using bacteria,[60] yeast,[61] or mammalian cells,[62] as well as cell-free systems, such as ribosome display,[63]



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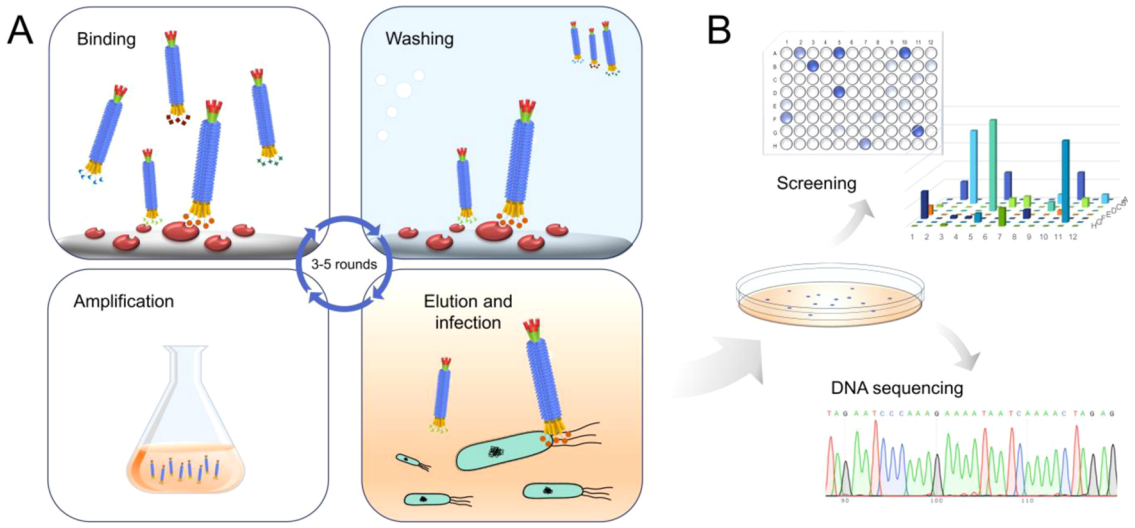


**Figure 2.** Selection of affinity binders from a phage-displayed library. (A) Selection process consists of binding, washing, elution, and amplification steps, which are usually repeated to three to five times to enrich target-specific binders. (B) Target specificity of individual clones can then be determined by screening the monoclonal clones from single colonies in enzyme-linked immunosorbent assay (ELISA), and the positive clones can be identified by DNA sequencing.

have been successfully applied to screen antibody libraries. Although less popular than phage display technology, these methods present some advantages, such as the ability of yeast cells to express complex proteins that require post-translational modifications or the possibility to create larger libraries using cell-free methods that are not restricted by the bacterial transformation efficiency.[64]

The process of in vitro affinity selection, whether it is based on phage or other display systems, commonly includes (I) the generation of genotypic diversity by constructing protein or peptide libraries that consist of millions or billions of different variants; (II) the display method that creates a physical link between the expressed protein variant and the gene coding for it; (III) the application of selective pressure to screen for target-specific binders, and, finally, (IV) the amplification of the selected variants.[9] Remarkable features of in vitro selection technologies include the possibility of defining the selection conditions carefully, the potential for high-throughput applications, and further improvement of selected binders by various protein engineering methods, for example, to achieve better affinity or stability, to reduce unwanted cross-reactivities, or to add tags for further purification or immobilization.[65−67] These systems provide immediately the genes and corresponding DNA sequences of the clones selected against the specific target. Simple subcloning can allow presenting modifications or adding functionalities, such as purification tags or fusions to enzymes or fluorescent proteins.[65] Moreover, also conventionally challenging targets, such as toxic or nonimmunogenic molecules, have been successfully used.[68,69]

The screening process of phage-displayed libraries, commonly known as panning, includes introducing the phage-displayed library to the target captured to a solid surface, washing to remove the unbound and nonspecifically bound phages, elution of the bound phages, and amplification of the eluted phages through bacterial infection (Figure 2A). Ideally, only one round of selection is required, but as nonspecific binding limits the enrichment in one selection round, in most cases, three to five iterative rounds of selection and amplification are performed to select individual binders.

A variety of modifications in the panning protocol and details for improved selections have been described for the identification of high-affinity binders from phage-displayed libraries. Generally, the experiment can be designed to maximize either phage capture or affinity discrimination. While effective capture is best used for the first selection round, the conditions can be modified in the later rounds to produce better affinity discrimination.[5] The early rounds of selection can be considered as the most important ones because any bias or loss of diversity during the first round will be amplified in the subsequent rounds. Since the number of potential binders is low among the highly diverse library, a common practice is to maximize the capture of all interesting clones. Later, when enrichment of selective clones is seen, and the diversity is reduced in the subsequent rounds, stringency can be increased by decreasing the target concentration or increasing the number or length of the washes.[6]

The selections are commonly carried out in solution or at a solid phase. Most frequently, the target is directly immobilized on a solid support, such as an immunotube, a bead, or a microtiter plate. This enables the separation of bound and unbound phages simply by washing the support.[6] Small molecules, which cannot be directly immobilized, are often conjugated to a carrier protein, or chemically coupled to a linker like biotin. For binding in solution, a biotinylated target is incubated with the phages in solution, after which the target bound phages are captured by streptavidin, for example, using magnetic beads.[5]

Because binding is an equilibrium reaction where the amount of the binder−target complex formed is determined by the affinity and the concentrations of the binder and the target, the amount of the target used for the selections is one of the critical factors in the process. Theoretical models suggest that high-affinity binders should be selected with a target concentration lower than the dissociation constant ($K_D$), but often target excess is used, in particular, in the first rounds, to allow capture of a higher fraction of the phage population and decrease the risk of losing the rare high-affinity binders.[70] However, by using limited and decreasing amounts of the target in the later rounds, the selection favors high-affinity



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binders.[9] Additionally, if multivalent display is used, avidity will play a significant role in the binding and seemingly good binders, with actually low affinity, might be selected due to avidity effects. When selection of high-affinity binders is desired, monovalent display is thus preferred,[6] although the use of a controlled density of the target during the selections can reduce avidity effects, favoring one-to-one binding interactions even with multivalent displays.[24]

On the other hand, also, the washing step is of great importance. Typically, affinity, in terms of the $K_D$ that is the ratio of the association ($k_{on}$) rate to the dissociation rate ($k_{off}$), can be improved by removing phages with fast dissociation rates in the washing step. The number and length of the washings can be thus optimized to select high-affinity binders. Short incubation times, short washes, low target concentration, and a decreased number of input phage might favor the selection of clones with fast binding.[9,71] Finally, in the elution step, bound phages are typically eluted by addition of an acid or a base to break the binding interactions. If such treatment is not efficient enough, it will result in the loss of high-affinity binders. On the contrary, the application of too harsh conditions might affect the infectivity of the phage and have consequences in the amplification step. Alternatively, elution can be done by competition, i.e., by adding an excess of the free target to the selections or by cleavage of phages that include a specific protease cleavage site between the phage and the displayed binder.[5]

One of the remarkable features of phage display is the possibility to carefully determine the selection conditions favoring the selection of binders, for example, with fast kinetics or improved biophysical properties. For instance, thermodynamically or otherwise stable variants have been selected by subjecting the library to elevated temperature,[72] low pH, or protease treatment.[73] Heat denaturation can be also used to select aggregation-resistant proteins or identify features that promote or prevent protein aggregation.[74,75] Finally, it should be also noted that the amplification step is susceptible to artifacts such as biased production of clones that are capable of growing faster than an average clone.[6] Thus, it is not only the affinity of the binder that determines which clones enrich but also the toxicity of the specific clones to the bacterial host, the solubility, folding efficiency, and stability of the expressed variant may contribute to the outcome of phage display.

The outcome of any selection process is a mixture of clones with different target-binding properties. Screening of individual clones, typically by phage-based immunoassays (for example, enzyme-linked immunosorbent assay, ELISA), is needed to evaluate their target-binging capabilities. Finally, DNA sequencing of selected clones can be used as a guideline to define at what stage to screen the library and identify the individual protein or peptide variants (Figure 2B).[9] Recent developments in the automation of the selection process have enabled rapid screening, for example, for several targets simultaneously. Moreover, screening robots allow testing of thousands of different binders[9] and protein microarrays can prove useful for high-throughput analysis of the specificity and affinity.[76]

In continuation of mimicking the natural selection process, similarly to affinity maturation in vivo, in which the immune response is capable of producing antibodies with an increased affinity,[77] a variety of in vitro techniques can be used to improve the affinity and specificity of the binders. The process, known as in vitro affinity maturation, comprises a variety of mutagenesis strategies that have been used to construct second-generation libraries, based on desired characteristics of the parental peptide or antibody, with some fixed or biased sequences. Various techniques used include random mutagenesis using DNA modifying enzymes or error-prone PCR, introducing mutations to certain areas or residues of the antibody, usually in the variable region and the hypervariable loops, for example, by oligonucleotide-directed mutagenesis. Also, DNA recombination, including using natural evolution or recombination of the target gene, can be used to recombine homologous segments to emphasize the desired property or to shuffle heterologous genes to create new diversity.[6,78]

## RECOGNITION ELEMENTS FOR BIOSENSORS

The importance of monitoring and regulating various biologically significant molecules in many different disciplines, such as clinical chemistry, environmental protection, drug discovery, forensics, and food industry, has incited the development of reliable sensing devices capable of accurate analysis.[79−84] Biosensors can provide rapid, sensitive, low-cost, real-time, and on-site analysis with compact and low-power devices. Owing to their appealing advantages, they have emerged as one of most interesting approaches to overcome the limitations of currently applied conventional methods, for example, for environmental monitoring, food analysis, and point-of-care medical applications.[85] The high specificity and sensitivity of biomolecular recognition, which is typically driven by a concert of many weak interactions, is the cornerstone of biosensor development, as the binding event has a direct effect on the accuracy of the method.[1,86] The recognition element of choice must be able to detect the target analyte even at low concentrations, and moreover, it ought to differentiate it from other similar molecules that might be present in the sample simultaneously.

Initially, biosensors were mainly based on enzymes and polyclonal or monoclonal antibodies as recognition elements, but in recent years, phage display has become a useful tool to identify new biorecognition elements for particular targets.[1] For example, the use of recombinant antibodies has several advantageous characteristics in comparison with conventional polyclonal and monoclonal antibodies. To begin with, the small size of antibody fragments is usually accompanied by a decrease of nonspecific binding often caused by the Fc region of the intact antibody and the possibility to immobilize the antibodies at a higher density.[87] Moreover, unlike full-length antibodies, recombinant antibody fragments can be propagated in bacteria, such as *E. coli*, which significantly lower the cost of production as no specialized cell culture facilities for hybridoma cell lines are needed.[88,89] On the other hand, phage display also offers the possibility of selecting various other bioinspired recognition elements,[25] such as peptides or other binding proteins and scaffolds, which might provide further advantages compared to antibodies. In vitro selections by phage display facilitates the production of novel receptors beyond known biomolecular interactions, or against toxic or nonimmunogenic targets, or even using whole cells as the target.[1]

Wild-type or genetically modified phages, as well as individual phages displaying a particular protein or peptide of interest, can be directly used as biorecognition elements as has been reported in a myriad of phage-based biosensors.[90,91] Biosensors for pathogen detection can be based on the inherent ability of the phages to bind to their target



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Table 1. Recent Examples of Biosensors Based on Recognition Elements Discovered by Phage Display[a]

| target analyte | recognition element | phage library | transduction method | sensitivity (LOD) | ref |
|---|---|---|---|---|---|
| varcinoma cells (SW620) | phage-displayed peptide | f8/8 landscape library | EIS | 79 cells $mL^{-1}$ | 99 |
| *Staphylococcus aureus* | pVIII fusion protein | f8/8 landscape library | absorbance | 19 CFU $mL^{-1}$ | 100 |
| *Salmonella* | phage-displayed peptide | Ph.D.-12[140] | SPR | $8.0 \times 10^7$ CFU $mL^{-1}$ | 98 |
| cholera toxin (CTX-B) | synthetic peptide | Ph.D.-12 | LSPR | 1.89 ng $mL^{-1}$ (LSPR) | 106 |
| | | | SERS | 3.51 pg $mL^{-1}$ (SERS) | |
| noroviral capsid protein (rP2) | synthetic peptide | Ph.D.-12[110] | EIS | 1.44 $\mu$g $mL^{-1}$ (99.8 nM) | 109 |
| dengue fever biomarker NS1 | phage-displayed peptide | Ph.D.-12 | EIS | 0.025 $\mu$g $mL^{-1}$ | 113 |
| flame retardant (DBDE) | synthetic peptide | Ph.D.-12 | CNT-FET | 1 fM | 130 |
| prostate-specific antigen (PSA) | nanobody | immunized (alpaca) | DPV | 0.08 ng $mL^{-1}$ | 141 |
| T-2 toxin | Fab and anti-IC Fab | immunized and naive | TR-FRET | 0.38 ng $mL^{-1}$ | 121 |

[a]Abbreviations: anti-IC, anti-immune complex; CFU, colony forming unit; CNT-FET, carbon nanotube field-effect transistor; DBDE, decabrominated diphenyl ether; DPV, differential pulse voltammetry; EIS, electrochemical impedance spectroscopy; Fab, antibody fragment antigen binding; LOD, limit of detection; LSPR, localized surface plasmon resonance; Nb, nanobody; Ph.D.-12, combinatorial library of random 12-mer peptides by New England Biolabs; scFv, single-chain fragment variable antibody fragment; SERS, surface-enhanced Raman spectroscopy; TR-FRET, time-resolved fluorescence resonance energy transfer.

pathogen,[92] or phages can be utilized as a means to display the biorecognition element, be it an antibody fragment, a peptide, or any other protein binder. Antibody fragments, mostly single-chain variable fragments (scFv), have been widely used for various targets in biosensing applications where their small size in comparison with the intact IgG can be of advantage.[87] Other common recombinant antibody fragments include the heavy-chain antibodies ($V_{HH}$), also known as nanobodies or single-domain antibodies, which have gained significant attention due to their extraordinary structure that is completely devoid of the light chains.[66,93] Alternatively, short peptides originating from phage-displayed libraries have been introduced as an interesting alternative for the development of new biosensing platforms because of their small size and cost efficiency. Peptides often suffer from a poor or mediocre affinity in comparison with antibodies, which might limit their use in certain applications. Nevertheless, although the interactions of an individual peptide with its target might be weak, stronger binding has been achieved by multivalent display on the phage particles, resulting in significant avidity effects. Various recognition elements have been applied to biosensors in their phage-displayed format, but, on the other hand, once an individual target-specific phage has been identified, the phage itself becomes expendable. After identification, the recognition element thus may also be subcloned into an expression system for large-scale production and different downstream applications. Alternatively, short peptides can also be chemically synthesized via solid-phase peptide synthesis.

Recombinant antibodies and short peptides originating from phage-displayed libraries have been reported in various biosensor applications, most notably in ELISA and surface plasmon resonance (SPR).[1] Furthermore, numerous different phage-based sensing schemes have been published recently using other transduction techniques, for example, fluorescence, Raman, quartz crystal microbalance, magnetoelastic, and electrochemical techniques. In this context, we will introduce a few interesting and recent applications for different kinds of targets (Table 1), but for a comprehensive overview of phage-based biosensors, we refer the reader to recent review articles.[7,91,92,94]

Label-free techniques, such as SPR, are often able to provide simple detection of the target binding to the recognition element immobilized on the sensor surface. Such measurements enable real-time monitoring of the binding reaction and can be used to evaluate also the kinetic and thermodynamic parameters of the molecular recognition process.[95,96] SPR biosensors for the detection of whole cells such as *Listeria monocytogenes*[97] and *Salmonella*[98] have been reported using phage-displayed antibodies or peptides, enabling detection of the cells in the range of $10^6$−$10^7$ cfu $mL^{-1}$. Another label-free biosensor based on electrochemical impedance spectroscopy and specific peptides selected from a landscape phage library-enabled[99] detection of cancer cells down to 79 cells $mL^{-1}$. Phages have also arisen as an interesting option for multifunctional structures. For example, *Staphylococcus aureus*-specific pVIII fusion protein identified by phage display was used in combination with bifunctional gold nanoprobes for label-free, rapid, and sensitive colorimetric detection of pathogens.[100] For optical detection of cancer cells, phage fusion proteins were combined with Au@Ag heterogeneous nanorods in a self-assembled biomimetic nanostructure.[101] In a different approach, phages displaying a target-specific peptide were assembled with silver nanoparticles to identify U937 cells by surface-enhanced Raman spectroscopy (SERS).[102]

Antibody fragments originating from phage-displayed libraries have been reported for various protein targets, such as scFv against dengue virus nonstructural protein[103] or single variable domain antibodies ($V_{HH}$) from camel against prostate-specific antigen (PSA) that were applied to PSA detection by SPR.[104,105] Also, various peptide binders against protein targets have been selected by phage display, for example, for the development of biosensors applied to the analysis of protein targets, such as cholera toxin,[106] troponin I,[107] prostate-specific antigen,[108] norovirus capsid proteins,[109,110] alanine aminotransferase,[111] and sepsis[112] or dengue fever biomarkers.[113] Recently, a SERS biosensor based on M13 phages that display cysteine-rich peptides on the pVIII was developed for triplex assay in sepsis diagnosis. The assays on magnetic templates with picomolar detection limits was described as a promising alternative for early clinical diagnosis of sepsis.[114]

Small-molecule targets, haptens, which might be challenging targets for antibody development, have been also successfully used to develop antibodies by phage display. The selection and application of scFvs against various targets, such as morphines,[115] mycotoxins,[116−118] and environmental toxins including microcystins,[119,120] have been reported. For such applications, soluble antibody fragments are commonly expressed in *E. coli* after identifying the target-binding clones



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by phage display. For example, HT-2 mycotoxin was analyzed using antibody fragments selected from an immunized library together with anti-immune complex antibodies from a naïve phage-displayed library.[121] The noncompetitive immunoassays for HT-2 analysis enabled detection of the toxin with excellent sensitivity and specificity. In a different approach, scFv for 2,4,6-trinitrotoluene was conjugated to luminescent quantum dots to develop solution-phase nanoscale sensing assemblies based on fluorescence resonance energy transfer (FRET) for the specific detection of the explosive in aqueous environments.[122] Also, some applications of nanobodies for small-molecule detection have been reported recently, mainly for mycotoxins.[123−126] Nanobodies have some advantageous characteristics beyond their small size, most notably excellent shelf life and high stability even in harsh conditions or at elevated temperatures.[66,127]

Short peptides originating from phage-displayed libraries have also been reported for small-molecule detection. For example, for the detection of herbicide glyphosate, an oligopeptide was identified from a phage display library and the synthetic was immobilized onto the SPR gold sensor chip for the analysis.[128] Alternatively, bisphenol A was detected by differential pulse voltammetry (DPV) and a cysteine-flanked heptapeptide as the recognition element.[129] For the detection of a flame retardant polybrominated diphenyl ether, a peptide receptor was integrated with a carbon nanotube field-effect transistor for the selective detection of the target in the femtomolar range.[130] Interestingly, phage display has also been employed to select binders against inorganic materials, such as ZnS,[131] CdS,[132] ZnO,[133] and $TiO_2$,[134] widening the range of biosensor applications based on phage assemblies and phage−nanomaterial complexes.[135]

## FUTURE PERSPECTIVES

Since the first description of phage display more than 30 years ago, various applications have demonstrated the significance of the technology and it has been established as a valuable and remarkably versatile tool in many disciplines for selecting affinity binders and exploring interactions between proteins, peptides, and small-molecule ligands, making significant contributions to the study of molecular recognition.[5,6] Among the striking success stories of phage display, peptide libraries have been used for instance to develop low-nanomolar peptide agonists[136,137] and antibody libraries have opened the way for the generation of human antibodies against various targets, including more than 60 antibodies obtained from phage-displayed libraries that have been approved for human therapy.[138,139]

For biosensor development, phage display can offer a new means to discover recognition elements, even beyond natural and known biomolecular interactions. In vitro selection of novel affinity binders allows tight control over the selection conditions as well as the use of conventionally challenging targets, or even whole cells. Although immunized libraries often result in excellent affinities, synthetic and naïve phage libraries, in particular, represent an intriguing alternative as they can be used to select antibodies against virtually any target, at least theoretically, within couple of weeks without the need of animal immunization. Moreover, since the library selections are solely based on affinity, also toxins and biologically active or threatening targets can be directly used unlike when animal immunization is required.

One of the important factors when choosing a recognition element for biosensors is the shelf life and stability of the receptor. While conventional antibodies might suffer from poor stability, phage display can be used to select folded and thermodynamically stable proteins from libraries. For example, camel or shark antibodies (and especially $V_{HH}$ camel nanobodies) have gained great attention in the last years for their reduced size, high stability, improved solubility, and improved solvent compatibility, which make them of special interest for the development of biosensors.

Often after identifying the ideal recognition element, the biosensor is constructed using the recombinant or synthetic affinity element. However, also phage particles by themselves can be integrated into the biosensing devices with emerging materials and nanostructures. In this sense, phages can be magnetically or metallically combined and then bioinspired analytical nanodevices or sensing materials can be constructed with tunable sensor properties for the detection of target molecules. On the other hand, application of phages for in vivo biosensing systems (for example, for medical diagnosis, foodborne control, etc.) should be preceded by toxicity tests to avoid any safety or social concern because of their viral nature.

Although advances in this field have been impressive in the last few years, the future of phages as sensing elements will require a multidisciplinary collaboration between experts from different scientific fields to boost their applications at the level of other usual biorecognition elements. Therefore, we can expect due to phage display overall trajectory and versatility since its discovery a whole generation of phage-derived biosensing devices in the near future.

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### Notes

The authors declare no competing financial interest.

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