

THE PROMISE OF PHAGE DISPLAY: Customized Affinity and Specificity


Phage display offers readily tailored binding affinity for a wide range of analytes, resulting in a capability that can be increasingly exploited.

Before a biosensor can detect a molecule, it must concentrate this molecule within a bioaffinity matrix consisting of a support (often porous) decorated with receptors. These receptors recognize and bind the target molecules of interest. For biosensors, monoclonal antibodies have been the gold-standard receptors in terms of affinity and selectivity for 30 years (1). Researchers favor antibodies for several reasons, including their wide availability and robustness.

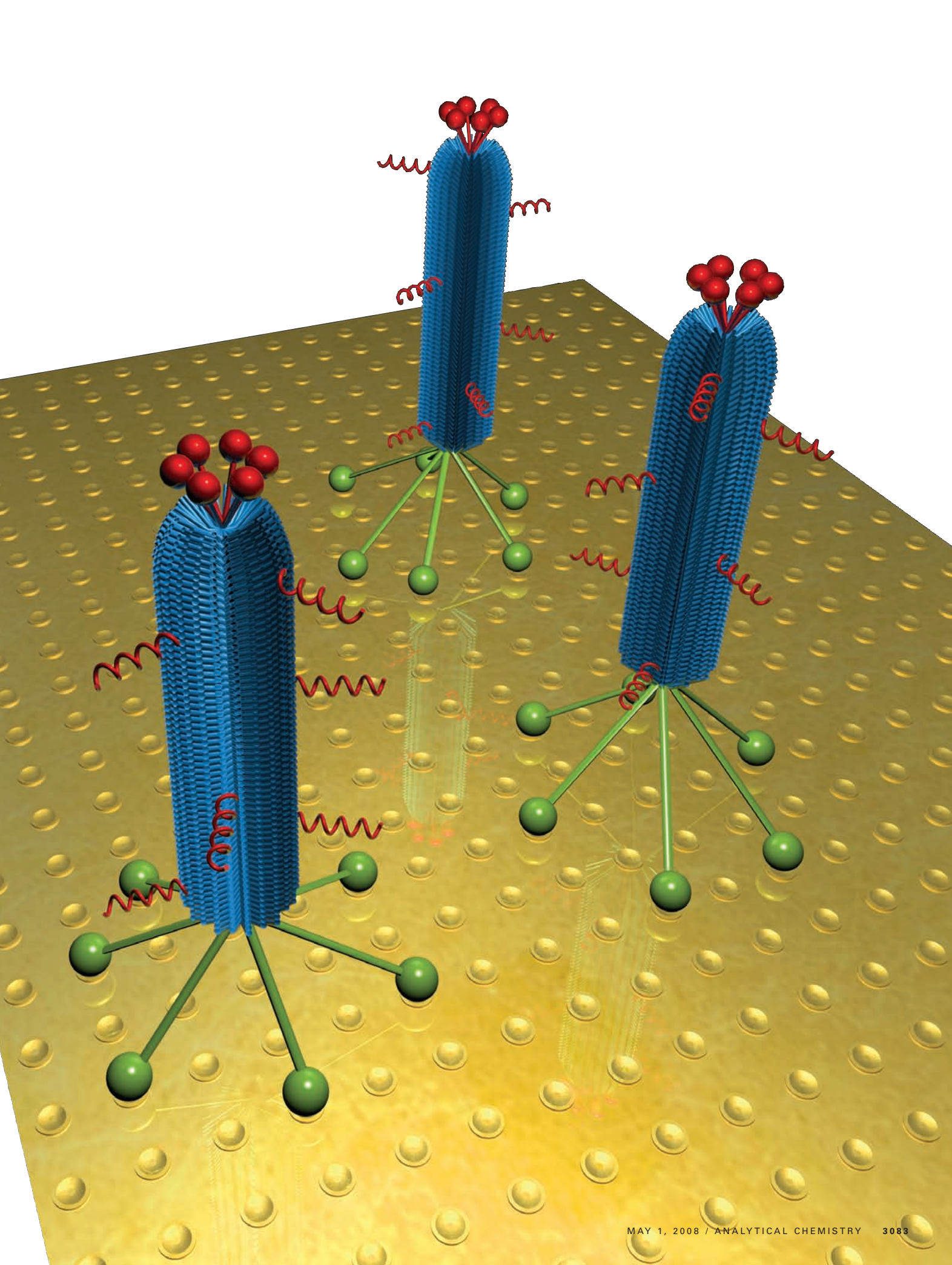
In addition, antibodies perform quite well in two critical metrics that characterize a receptor's performance. The first is the equilibrium constant for the dissociation of the receptor–target complex, $K_D = k_d/k_a$, where k_d is the dissociation rate of the complex and k_a is the association rate, also known as the “on rate”. K_D determines the minimum target concentration at which receptors will be occupied by target molecules, and it is therefore a factor in determining the sensor's detection limit (2). The on rate, k_a , is the second key metric. It is the biomolecular rate constant for the reaction of the binding site on the receptor with the target molecule of interest. This parameter determines the activation-limited response time and, therefore, whether a particular analysis is possible and practical in terms of time (3). The on rate is related to the equilibrium constant for the binding reaction, K_A , as $K_A = k_a/k_d$.

The best monoclonal antibodies exhibit K_A values of 10^{10} M^{-1} and k_a values of 10^6 M^{-1} (2). These metrics are essentially unsurpassed by other types of artificial receptors, but there is a catch—the production of an antibody that targets a particular antigen harnesses the immune systems of mice and requires 2–12 months, depending on the target, to yield the desired result (4).

Recently, a handful of laboratories have begun using ensembles of whole virus particles as the bioaffinity matrix within biosensors. In this case, the virus particles perform two functions—they serve



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as high-surface-area supports for receptors, and they manufacture and display the receptors on their surfaces. In this article, we describe the “phage display” machinery that makes this adaptation of virus particles possible and summarize recent key advances and research results from several laboratories.

The phage

Phage display, invented by George Smith, manipulates polypeptides displayed on the surfaces of virus particles, known as bacteriophage because they “eat” or infect bacteria (5). Filamentous phage, such as M13 (Figure 1) and fd, play the lead role in phage display, and their attributes are critical in the applications described here. Filamentous phage are preferred over other types of bacteriophage because fusing peptides of interest to their viral surfaces typically has little effect on the infectivity

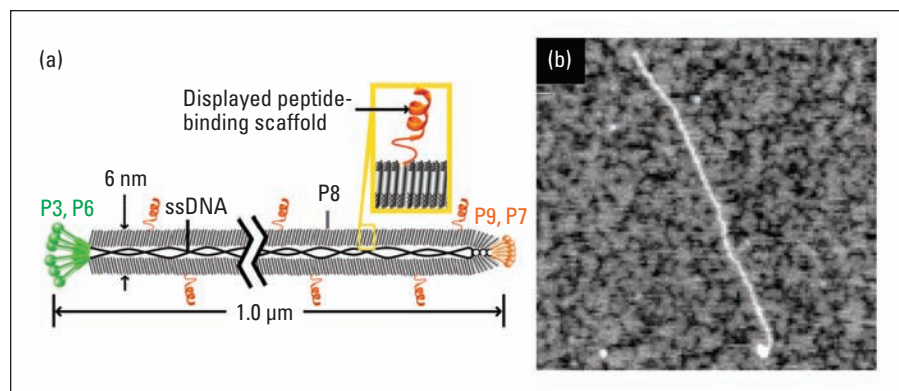


FIGURE 1. (a) Schematic and (b) AFM image of a single M13 virion on $1 \times 1 \mu\text{m}$ mica. (ssDNA, single-stranded DNA; image courtesy of Theresa M. McIntire.)

of the virus or on its life cycle. Filamentous virions also release progeny without lysing their bacterial hosts—a sometimes underappreciated attribute that simplifies growth and containment in the laboratory.

These phage (members of the Ff family) share a common architecture (Figure 1a; 6). The viral capsid, a tube for the viral DNA with an outer diameter of 6 nm, is composed principally of 2700 copies of P8, the major coat protein. The N-terminus of the 50 amino acid P8 is exposed to the outside of the virus. Its C-terminus is buried within the interior of the capsid, where the P8 sequence provides several lysine residues for packaging the negatively charged viral DNA (6). The cylindrical virus, 1.0 μm long, is capped at one end by five copies of each of the minor coat proteins P3 and P6 and at the other end by the coat proteins P7 and P9 (7).

Phage-displayed libraries

A library—a large collection of different phage particles—can be synthesized, and each phage can have a different peptide or protein sequence displayed (i.e., covalently attached) on a fraction of the endogenous P8 or P3 coat proteins. DNA encapsulated by the phage particles (Figure 1a) encodes the displayed polypeptide (7). Alteration of the encapsulated DNA by site-directed or stochastic mutagenesis can program a library

of proteins in which each member of the library is fused to its encoding phage. The displayed polypeptide, ranging in size from 6 to >200 amino acids, is typically fused to either the N-terminus of P8 or the C-terminal domain of P3 (7). Often, the introduced sequence is expressed in a minority of P8 or P3 proteins through dual encoding of coat proteins, one with and one without the fusion protein.

The diversities of phage-displayed libraries can exceed 10^{10} different polypeptides, which makes individual examination of each library member impractical (8). Instead, “selections” isolate members of the library that meet specified criteria. For example, to select binders to the target analyte, a solid support is coated with analyte and incubated with the library. Non-binding members of the library are washed as dictated by the application (e.g., stringent wash conditions for the highest-affinity binding). Phage selected for binding can be propagated in the *E. coli* host, and the process is repeated for multiple rounds until phage with the desirable properties are identified.

Negative selections also can be used to subtract members of the library with undesirable criteria (e.g., off-target binding to a closely related receptor). After selections, binding assays or other screens can examine the properties of individual library members. Other molecular techniques, such as mRNA display, can enable access to even greater library diversities (9). Although larger libraries could be expected to yield receptors with higher affinity and higher specificity, recent advances with phage-displayed libraries demonstrate careful design. Even libraries with limited diversity have enough to enable the discovery of high-performance antibodies (10–12).

Smith and Petrenko identified >80 targets that had been used for the affinity screening of phage libraries (7). In the past 10 years, the greatly expanded uses of phage display include receptor identification in vivo, mapping side-chain functional contributions to molecular recognition, and small-molecule binding (12–15). In general, phage display works well for targeting soluble proteins with $pI < 10$. However, some important caveats limit the technique’s applicability. First, very few examples have been reported of targeting individual membrane proteins with phage-displayed libraries. Phage libraries can readily identify binding partners to the soluble domains of membrane-associated proteins (e.g., receptors for human growth hormone and others) and can even target intact cells or living organisms, likely also through binding to the soluble portions of cell-surface receptors (16–23). High- pI targets stick nonspecifically to the high negative charge of the P8 coat, preventing selections for partners that bind to the fused peptide. The few examples of targets in this category are biotinylated for attachment to streptavidin beads, thus at least partially shielding the positive charge of the target protein (24).

Why phage?

First is the versatility and variety of the displayed receptors (3, 25, 26). A wide assortment of scaffolds has been displayed on the phage surface (Figure 2; 10). This includes a menagerie of antibody fragments and interesting single-chain antibodies from animals such as camels, sharks, and llamas (11–13, 27–29). Although some descriptions of the technique have incorrectly suggested an absolute limitation on the size of the displayed receptor, this persistent expectation is refuted by numerous examples of large proteins displayed on the surface of the phage, such as alkaline phosphatase (120 kD homodimer; 14) and amylase (86 kD heterodimer; 15).

Receptor size is not the most important criterion for successful phage display and selections. Instead, the emphasis is on expressing the recognition scaffold and targeting it to the phage assembly process. Low or inconsistent levels of receptor display are perhaps the biggest challenge to effective selections for target binding. Balky protein display levels can be improved ~100-fold by introducing into the anchoring P8 coat protein mutations that enhance its stickiness for the phage coat (16, 17). In addition, altering the signal peptide directing the recognition scaffold to the correct subcellular location for phage assembly can dramatically boost display levels (18). Other considerations, such as codon choice for effective expression in *E. coli*, also can factor into the optimization of protein display levels (30, 31).

After display of the receptor on the phage surface, mutagenesis of the encapsulated DNA can program the library of receptors. Next, selections and screens are used to isolate members of the library with the desired binding specificities and other properties (Figures 2 and 3). Although the vast majority of library selections focus on biomedical applications, the technique offers the analytical chemist the rare opportunity to specify particular attributes required for useful molecular recognition. Need recognition at low (or high) pH? The phage are stable at pH 2–12 (19). Selections for binding at one pH and dissociation at a different pH are routinely used. Phage stability in organic solvents has also been reported (20).

Shelf life or stability of the recognition elements before use is another important consideration. Several phage-displayed methods have been described for selecting folded and more thermodynamically stable proteins from libraries. For example, the proside technique features phage-displayed protein libraries inserted between two of the domains of P3 required for infection of the *E. coli* host. Selection for protein stability applies denaturing conditions, with either chemical denaturants or elevated temperatures, in the presence of proteases. More stable, folded proteins resist

denaturation and digestion by the protease (32, 33). For example, to build in the protease resistance necessary for long-term monitoring of analytes, the proside technique could be readily adapted for conditions identical to or slightly harsher than the measurement requirements.

Complex biological fluids (e.g., blood or urine) can present a myriad of potential binding surfaces, which complicates selections. Negative selection against off-target binding may be necessary to obtain specificity. Nonspecific binding to plastic surfaces and other hydrophobic interactions also can be removed through clever selections, often as pretreatments

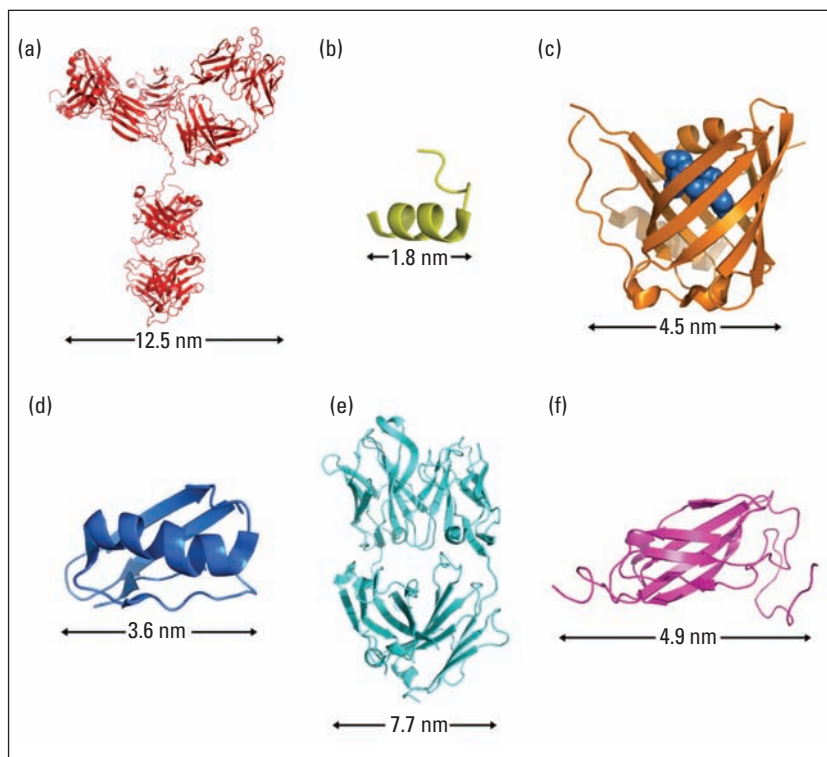


FIGURE 2. Comparison of antibody and phage-displayed binding scaffolds.

Phage display extends the range of binding scaffolds beyond (a) the immunoglobulin G (IgG; PDB entry 1HZH) domain of antibodies to include (b) the structured peptide 1LB7, (c) the anticalin or engineered lipocalin (orange) for binding to small molecules (digitoxigenin, blue; 1LNM), (d) the affibody or IgG-binding protein (1ZXH), (e) the Fab domain 1ZA3, and (f) the fibronectin domain FN3. (Adapted from Refs. 30–33, 41, and 42.)

before the positive selection. For example, hydrophobic receptors were removed from a ribosome-displayed library. In such negative selections, agarose beads modified with hydrophobic functionalities adsorbed surface-exposed hydrophobicity (21). Other selections with precipitation agents for improved solubility also could improve recognition-scaffold solubility for particular applications.

Phage as a bioaffinity reagent

Anderson and co-workers were the first to integrate phage display with analysis (22). They selected M13 phage from a library of 12 residue peptides displayed on P3 and containing ~10⁹

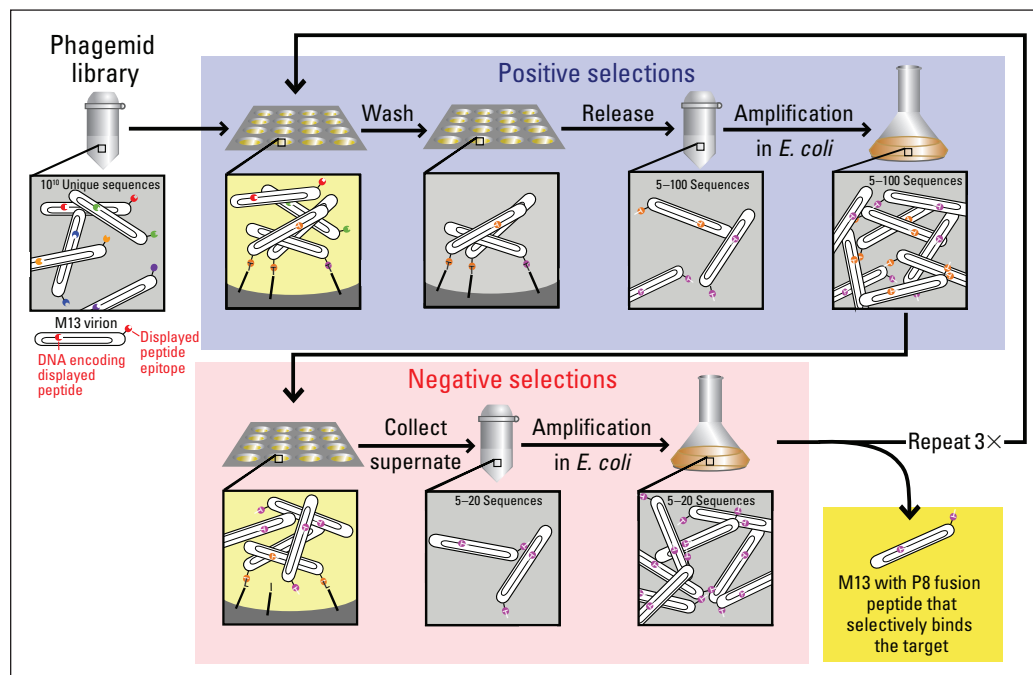


FIGURE 3. Depiction of the process for selecting phage from a large library on the basis of affinity for a particular target ligand T (positive selections), which also possesses selectivity for T relative to its affinity for potential interfering molecules I and L (negative selections). Negative selections to subtract receptors with off-target binding can precede or follow positive selections. The time required for this process is ideally 2 weeks.

unique sequences for affinity to Staphylococcal enterotoxin B (SEB). After amplification, the selected phage were labeled with a Cy5 fluorophore (300–2000 copies per phage) and used for fluoroimmuno sandwich assays at SEB-modified surfaces of a 96-well plate and of a Raptor fiber-optic-based sensor (23, 25). With SEB attached directly to the plate and fiber surfaces, exposure to fluorescently labeled phage generated signals that were reliable but somewhat lower than those for a fluorescently labeled anti-SEB antibody. However, success was mixed for a sandwich assay in which an anti-SEB antibody was immobilized at the plate and fiber surfaces, SEB was bound, and fluorescence was measured after exposure to labeled phage and antibodies (in separate experiments). No signal was observed at the optical fiber, whereas concentrations of 1.4 ng/well were detected with the microtiter plate.

Later, this group used the same library (New England Biolabs) to isolate M13 that selectively bound 2,4,6-trinitrobenzene (TNB) in a background of artificial seawater. Again, these phage were fluorescently labeled and used for a displacement assay in a continuous-flow mode. The TNB-modified chromatographic stationary phase was preloaded with TNB-binding phage (Figure 4a). TNT in seawater was introduced and displaced the fluorescently labeled phage to produce a measurable fluorescence signal at a downstream detector to achieve a detection limit of 10 mg/L. By comparison, an earlier demonstration of TNT detection relied on the same methodology but used a fluorescently labeled antibody instead of phage to

achieve a detection limit of 2.5 ppb for TNT (34). These two papers constitute proof of principle for the utility of affinity-selected phage as analytical reagents in several assay formats.

Virus particles have also been preconcentrated onto sensor surfaces to accurately measure their concentration in solution. Klenerman and co-workers first captured phage particles (including M13) at gold-coated quartz crystal microbalance (QCM) electrodes that were functionalized with a self-assembled monolayer (SAM) of a mercaptoundecanoic acid conjugated to a monoclonal antibody for the virus (27, 29, 35). The phage coverage of the QCM surface was then assessed by using the shear forces imparted by the QCM itself to detach phage. Measurement of the recoil transient produced by the phage

detachment enabled the number of adsorbed phage particles to be measured and correlated with their concentration in solution.

Whole phage as a bioaffinity matrix

Once the positive and negative selections have been used to identify a polypeptide receptor for a biosensor application, the phage itself is expendable. A polypeptide selected from phage display can be resynthesized or expressed with an appropriate linker and used as a receptor in a biosensor (22, 26). However, retaining the phage as a display scaffold can confer important advantages. First, because of multicopy display on the surface of the phage, peptide selectants can exhibit enhanced affinities resulting from a Velcro effect when the receptors remain attached to the phage. Removal of the selected receptor from the phage surface can dramatically decrease receptor affinity (28). Second, the filamentous phage particle can serve as a high-surface-area support for the selected receptor—a chemical recognition module that obviates the additional steps required for synthesis of the free peptide, attachment of a linker, and finally conjugation of the receptor to the biosensor.

Phage display can thus reduce the challenge of immobilizing receptors to a single phage-bioconjugation step that can be generalized to every phage-displayed receptor. In principle, one could then proceed within hours from phage-display-optimized receptors to a functional biosensor incorporating the receptor. This work is in its infancy. Researchers have sought

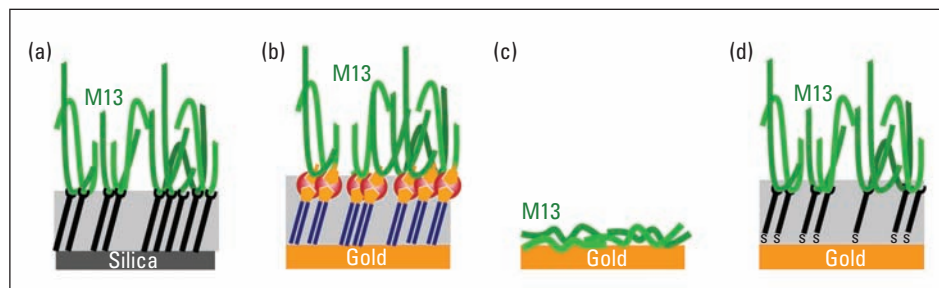


FIGURE 4. Depiction of methods for immobilizing filamentous phage on biosensor surfaces.

(a) TNB was reacted to an activated NHS-ester-terminated C_{10} phase to produce pendant TNB moieties (black rods) onto which a fluorescently labeled M13 phage with a high affinity for TNT was chromatographically loaded. The displacement of these fluorescently labeled phage particles from the affinity column by TNT-containing seawater solutions was then monitored vs time with a fluorescence detector. (b) Biotinylated LB monolayer used to bind biotinylated phage particles via streptavidin linkers to a gold surface. (c) Phage physisorbed onto clean gold surfaces. (d) M13 particles covalently anchored to an NHS-ester-terminated dithiol SAM to form a covalent virus surface.

to develop and demonstrate a robust attachment chemistry that produces a high density of immobilized phage particles on gold surfaces. Ideally, the immobilized phage will be resistant to desorption in flowing aqueous buffers with high ionic strength. In addition to robust attachment, the immobilized phage must continue to bind target molecules and resist non-specific binding interactions. In other words, attachment to the surface could compromise the properties selected under solution-phase conditions; for example, covalent modification of the receptor could interfere with binding to the target.

Can an immobilized phage layer at the surface of a transducer—such as a QCM crystal, a surface plasmon resonance (SPR) waveguide, or an electrode surface—recognize and selectively bind the target molecules of interest? The first experimental challenge is the requirement for phage immobilization. Petrenko and Vodyanoy used streptavidin linkers to bind biotinylated phage particles to biotin-modified phospholipid

monolayers that were deposited by using the Langmuir–Blodgett (LB) method (Figure 4b; 29). Before modification, the attached phage were selected for affinity to β -galactosidase. K_D was measured for phage immobilized on 96-well plates by using ELISAs and acoustic wave sensors capable of detecting changes in mass loading in the microgram range; values of 30 and 0.6 nM were obtained with these two methods, respectively (Table 1).

Vodyanoy and co-workers later showed that a much simpler approach for phage immobilization—the physisorption of the phage particles directly onto the gold surface of a transducer—can also be used with success (Figure 4c; 36, 37). Exposure of a clean gold surface of an acoustic wave mass sensor to phage for an hour produced phage coverage of 3×10^{10} particles/cm². This system was then used to detect whole cells of the bacterium *Salmonella typhimurium* and, in separate experiments, for the measurement of β -galactosidase (Table 1; 36, 37). This especially rapid mode of immobilization magnifies one of the intrinsic advantages of phage display as compared with monoclonal antibodies, which is speed. Phage physisorption onto gold was also used to prepare a bioaffinity surface for SPR-based biosensors to detect the binding of β -galactosidase (38). The apparent optical thickness of the phage layer was 3 nm, consistent with ~ 1 compact phage monolayer. A detection limit near 1 pM was reported for this 116 kDa enzyme (38).

Our own efforts have culminated in the development of a scheme for covalently attaching M13 phage particles to a gold surface via a SAM of an *N*-hydroxy succinimide (NHS)-ester-

Table 1. Phage-based biosensors.

Analyte	Detection limit	K_D (nM)	Phage attachment mode	Γ_ψ (cm ⁻²) ¹	Transducer	Refs.
TNT	55 μ M	—	None; fluorescently labeled phage used as reagents in fluoroimmuno sandwich assays	—	Raptor fiber-optic sensor	22, 23, 25, 26
SEB	1.4 ng/well	—	None; fluorescently labeled phage used as reagents in fluoroimmuno sandwich assays	—	Raptor fiber-optic sensor	22, 23, 25, 26
β -Galactosidase	0.01–0.1 nM	0.6 ± 0.4	Biotinylated phage attached to gold sensor surface with Langmuir–Blodgett layer of biotinylated phospholipids via streptavidin	—	Acoustic wave sensor	29
β -Galactosidase	1.0 nM and 1.0 nmol	1.7 ± 0.5	Physisorption onto gold surfaces	3×10^{10}	Acoustic wave sensor	36
β -Galactosidase	1.0 pM	1.3 ± 0.001	Physisorption onto gold surfaces	3×10^{10}	SPR	38
<i>S. typhimurium</i>	100 cells/mL	—	Physisorption onto gold surfaces	3×10^{10}	Acoustic wave sensor	37
PSMA	20 nM	≤ 0.5	Covalent attachment via amide bond formation to activated NHS-ester-terminated dithiol SAM on gold	$(1.1 \pm 0.2) \times 10^{11}$	QCM and gold electrode	39, 40
Antibody for P8	6.6 nM and 13 pmol	≤ 0.5	Covalent attachment via amide bond formation to activated NHS-ester-terminated dithiol SAM on gold	$(1.1 \pm 0.2) \times 10^{11}$	QCM and gold electrode	39, 40

¹ Γ_ψ is surface coverage of phage particles.

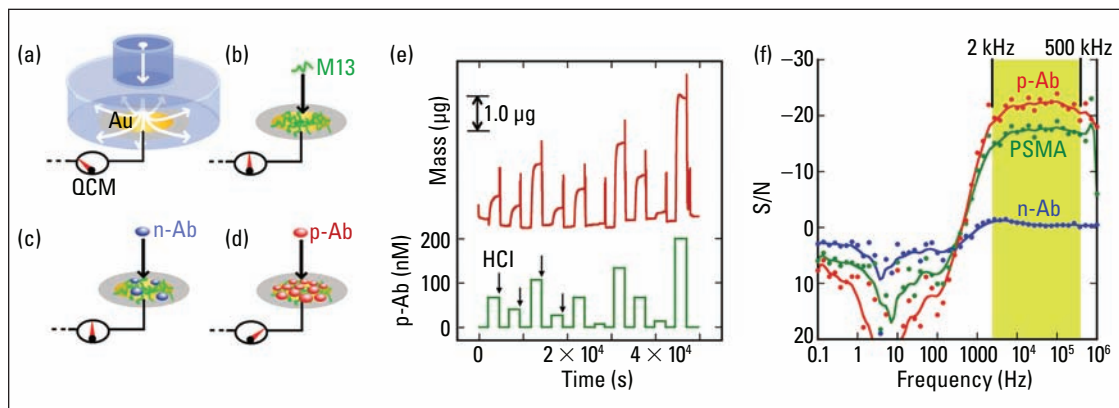


FIGURE 5. (a–d) Depiction of the mass-based detection of a “positive” antibody (p-Ab) by a CVS immobilized on a gold QCM located within a flow cell. (c) The absence of binding by a second antibody, a “negative” antibody (n-Ab). (e) Mass versus time for the detection of p-Ab. At 2000 seconds after each injection, 0.5 M HCl was injected to remove bound p-Ab and regenerate the CVS. (f) S/N as a function of the frequency for the measurement of PSMA, p-Ab, and the control n-Ab binding to a CVS. (Adapted with permission from Ref. 38.)

functionalized dithiol, resulting in phage coverage of 10^{11} particles/cm² (Figure 4d; 39, 40). This covalent virus surface (CVS) retains the affinity of the free M13 phage for the target molecule (an anti-wild-type P8 antibody) and can bind 140 target molecules per phage particle (Table 1). But the most impressive and surprising attribute of the CVS is its stability.

The CVS-modified gold surface of a QCM produced a linear calibration curve over a period of >14 hours in flowing, high-ionic-strength buffer (Figure 5). In this experiment, each of the 10 injections of antibody was followed 2000 seconds later by an injection of 0.5 M HCl that quantitatively removed the bound antibody and readied the CVS for the next injection. In addition to demonstrating the stability of the covalent phage layer for analysis in a flow-cell format, this experiment also highlights the remaining challenges, including reducing the detection limit (~6.6 nM; 39).

A long-term objective of this research is the development of cheap, disposable multichannel biosensors capable of detecting several analytes in parallel. In such a device, direct electrical transduction of analyte binding at each channel would be highly advantageous from a cost and fabrication perspective. With this objective in mind, we have explored electrochemical impedance spectroscopy as a transducer for measuring the binding of target molecules to CVS-modified macroscopic gold electrodes (40). Although electrochemical impedance has often been used for biosensor transduction, the low frequency range of <1 Hz has most often been exploited for this purpose and has obvious disadvantages in terms of the sensor response time.

We examined the effects of the phase and frequency of the measured current relative to the voltage excitation and concluded that the highest S/N (~20) was obtained by using an in-phase measurement of the current at frequencies of 2–500 kHz. Ironically, in this frequency range, the resultant signal, defined as the absolute increase in resistance of the CVS when binding occurs, is the smallest. For example, the resistance of the CVS prepared on a 0.3 cm² electrode (200–300 Ω) in-

creased by up to 14 Ω at 3 kHz when the binding sites on this surface were saturated with molecules of prostate-specific membrane antigen (PSMA), a prostate cancer marker (40). The detection limit was 120 nM, which is several orders of magnitude too large to be of clinical relevance. Can this impedance measurement be made with greater sensitivity? This is the challenge we are presently ad-

ressing in our attempts to microfabricate a conductivity cell that is optimized for phage-based biosensing.

Future improvements to the phage display platform could address the limitations highlighted here. For example, robust display of the receptor remains a challenging problem despite advances. In addition, whole-phage biosensor devices coat only a small fraction of the surface for target binding receptor, leaving mainly a wild-type coat protein surface; the technique could benefit from libraries featuring attachment of the binding receptor to every copy of P8. Improved sensitivity, specificity, and decreased detection limits will result.

Summary

Phage display can fulfill the major requirements for successful biosensing by providing a wide range of recognition scaffolds. The tailoring of recognition properties, robustness, and solubility has been explored superficially. The possibilities for creative applications of phage-displayed libraries are wide open.

Just a handful of publications report experiments in which intact phage have been used as scaffolds for phage-displayed peptides within biosensors. On the basis of the results so far, there is reason for optimism—the affinity of phage for target molecules survives attachment of the phage to biosensor surfaces, even when potentially disruptive processes, such as physisorption and covalent bonding, are involved in this immobilization. High phage loadings equivalent to multiple phage monolayers are achievable, and the immobilized phage layer does not interfere with the function of transducers. Detection limits approach the minimum expected from K_D values measured for the free-phage–target interaction by ELISAs. These early results provide ample motivation to further expand the scope and performance of phage-immobilized biosensors.

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- ▶ Applications in Life Sciences
- ▶ Applications, Other
- ▶ Other Separation Methods

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