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Nanofabrication of Bioselective Materials Using Diverse Nanolandscapes Displayed on Live Viruses

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ABSTRACT

Filamentous phages, such as fd used in this study, are thread-shaped bacterial viruses. Their outer coat is a tube formed by thousands of equal copies of the major coat protein pVIII. We constructed libraries of random peptides fused to all pVIII domains and selected phages that act as probes specific for test antigens and biological threat agents. Because the viral carrier is infective, phage-borne bio-selective probes can be cloned individually and propagated indefinitely without any need for chemical synthesis or reconstruction. We demonstrated that biorecognition layers fabricated from phage-derived probes can bind biological agents, and as a part of an analytical platform generate detectable signals. Phage are superior to antibodies: they are inexpensive, highly specific, strong binders resistant to high temperatures and environmental stresses, and thus may be suitable as antibody substitutes for field-use detectors.

Keywords: landscape phage, phage evolution, detection, threat agents, biosensor

1 FILAMENTOUS PHAGE AS A SCAFFOLD FOR NANOMANIPULATIONS

The Ff class of filamentous phage includes strains fl, M13, and fd. These phages are flexible, thread-like particles approximately 1 μm long and 6-7 nm in diameter (Fig. 1, left). The bulk of their tubular capsid consists of 2,700 copies of the 50-residue major coat protein pVIII arranged in a helical array possessing five-fold rotational axis and a coincident two-fold screw axis with a pitch of 3.2 nm. The major coat protein constitutes 87% of total virion mass. Each pVIII subunit is largely α -helical and rod-shaped; its axis lying at a shallow angle to the long axis of the virion. About half of its 50 amino acids are exposed to the solvent, the other half being buried in the capsid. At one tip of the particle, the outer tube is capped with five copies each of minor coat proteins pVII and pIX; five copies each of minor coat proteins pIII and pVI cap the other end. It is assumed that the minor proteins form rings that match the five-fold rotational symmetry of the pVIII array. The capsid encloses single-stranded DNA — the viral or plus strand. Longer or shorter plus strands, including recombinant

genomes with foreign DNA inserts, can be accommodated in the capsid whose length matches the length of the enclosed DNA by including proportionally fewer or more pVIII subunits. Phage chimeras with foreign polypeptides fused to the coat proteins can be engineered by splicing the peptide-encoding DNA's into the structural genes of the phage [1]. The grafted peptides arranged regularly on the body of the phage (Fig. 1, center) can act as binding sites for targeted bioorganic receptors and ligands, crystallization origins of mineral compounds, anchors for immobilization, or perform other predetermined roles [2,3]. Multifunctional phage particles with different peptides engrafted into the predetermined regions of the capsid can be also obtained by fusing corresponding DNA fragments to different structural genes of the phage. We call this genetically-driven nanomanipulation technique "phage landscaping." It allows the creation of an infinite variety of organic landscapes composed of 20 natural amino acids arranged in designed or random patterns.



Figure 1: Left: Electron micrograph of filamentous phage fd. Center: The molecular model of landscape phage clone (~1% segment of the viral sheath), with foreign octamer peptides shown in black against the dark gray background of wild-type phage amino acids. Right: The molecular model of a mosaic phage clone with foreign N-terminal peptides shown in dark and mutated amino acids 12-19 shown in white against the gray background of wild-type phage amino acids.

2 DIRECTED PHAGE EVOLUTION

We conceived and justified a new route to mosaic phage clones through Directed Phage Evolution (DPE). We propose that the performance of phage as probes may be enhanced by inducing mutations in the areas neighboring phage-borne binding peptides. This may increase affinity, selectivity and stability of phage-derived probes. The

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principle of DPE is illustrated in Fig. 1, right, in which black areas show primary N-terminal binding peptides selected in the first round of affinity selection, and white areas show amino acids which are mutated to form a new sublibrary, which is used for selection of improved phage probes. These areas correspond to the segments of pVIII shown in the structure below.

Black Grey White Grey ----Buried----
 AXXXXXXXXDPAKAAFXXXXXXXXXXEYIGYAWAMVVVIVGAT...

DPE strategy was developed using the model phage 1G40 binding β -Galactosidase (β -Gal) selected from landscape library f8/8. The phage displays peptide DTFKSMQ as shown in black in Fig. 1, right. A diverse mosaic landscape library was constructed by cloning a synthetic oligonucleotide duplex encoding the β -galactosidase-binding peptide DTFKSMQ into existent f8/6 α -library with random amino acids in positions 12, 13, 15-17 and 19 [4]. This library was used to determine if the mutations surrounding the binding peptides on the phage landscape can affect their binding efficiency. We found that some mutants were able to bind β -Gal with the same ability as original 1G40 phage, some were able to bind β -Gal even better than 1G40, while some bound β -Gal less effectively than the parent phage or even lost the binding ability. This data demonstrates the dramatic effect that phage amino acids neighboring a foreign peptide have on the binding properties of the peptide and probably on its specific conformation controlled by an integral phage organic landscape. The results of this study strongly confirm our conception of phage surface landscapes as materials with emergent properties and suggest a method of enhancing the performance of phage-derived probes by phage evolution.

3 LANDSCAPE PHAGES AS DETECTION PROBES

3.1. Phage Probes for *B. anthracis* and *S. typhimurium*

Phage landscaping methods were applied for development of phage probes against biological threat agents, such as *Bacillus anthracis* spores and *Salmonella typhimurium* [5,6,7,8]. We constructed libraries of random foreign peptides fused to all pVIII domains ("landscape libraries," Fig. 1, center), and demonstrated that the libraries contain many potential probes for surface markers of pathogenic spores and bacteria. Phage probes were isolated in a nonbiased multistage selection procedure using immobilized spores or bacteria as a selector. The performance of the probes in detection of these threats was illustrated by a precipitation test, enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting, magneto-restrictive sensors, and fluorescent, optical and electron microscopy. We have characterized landscape phage clones that bind to *B. anthracis* spores at a

higher level than other species of *Bacillus* spores. Selectivity of the best phage candidate for *S. typhimurium* was studied in comparison with nine other gram-negative bacteria, predominately *Enterobacteriaceae*. A small amount of cross reactivity of this phage was noted with *Yersinia enterocolitica* and *Citrobacter freundii*. The complex of phage with bacteria was visualized by fluorescence microscopy (not shown) and transmission electron microscopy (TEM) (Fig. 2), demonstrating the multivalent character of phage-bacteria binding.

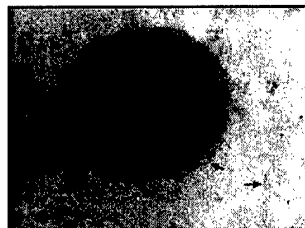


Figure 2: TEM micrograph of bacteria-phage complex. Phage is labeled with gold nanoparticles (arrows).

3.2. Phage as the Bioselective Element of Biosensors

We developed three methods of immobilization of phage-derived probes onto the sensor surfaces:

- Phage self-assembly on Langmuir-Blodgett (LB) phospholipid and bovine serum albumin (BSA) films by biotin/streptavidin coupling;
- Direct physical adsorption of phage to the sensor surface;
- "Phage skinning" — coating of the sensor with phage-derived peptide probes.

Self-assembly. Monolayers containing biotinylated phospholipids were transferred onto the gold surface of acoustic wave sensors (Maxtek, Sante Fe Springs, CA) using the LB method and treated with streptavidin and biotinylated phage [9]. Experiments were carried using a Maxtek TM-400 thickness monitor. Fig. 3A demonstrates specific dose-dependent binding of β -galactosidase to the phage immobilized to the acoustic wave sensor in comparison with phage immobilized onto an ELISA plate. It was observed that the affinity of the complex depends on the mode of phage immobilization and type of analytical platform: 0.6 nM by acoustic wave sensor versus 30 nM by ELISA. The difference in affinities were attributed to the

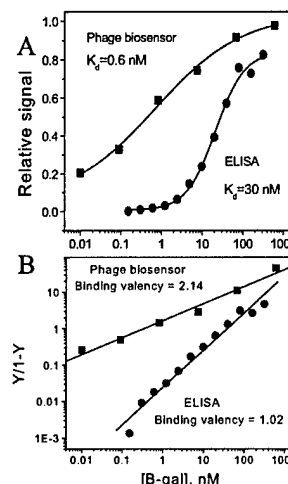


Figure 3.

monovalent (ELISA) and divalent (sensor) interaction of the phage with β -galactosidase, as is indicated by the Hill presentation of binding curves (Fig. 3B). One or another mode of interaction probably depends on the conformational freedom of the phage immobilized to the solid surface. Binding of the phage is quite specific because the response is reduced by 85% if β -galactosidase is preincubated with 4 nM phage. Binding of the phage to β -galactosidase is very selective: presence of 1000-fold excess of bovine serum albumin in mixture with β -galactosidase does not considerably change the ELISA signal and reduces the biosensor signal only by 4%.

Physical adsorption. Phage can adsorb directly onto gold surfaces [5]. In these experiments, the acoustic wave sensor (Maxtek) with gold electrodes was exposed to phage in suspension. Following an incubation period, the sensor was rinsed in water and tested with analytes. A sensor for β -galactosidase showed the value of EC_{50} of approximately 5 nM, what is comparable with results obtained by the above described self-assembling LB method. Biosensors for *S. typhimurium* demonstrated a linear dose-response relationship ($R = -0.98$, $P < 0.001$) over six decades of bacterial concentration (Fig. 4). Scanning electron

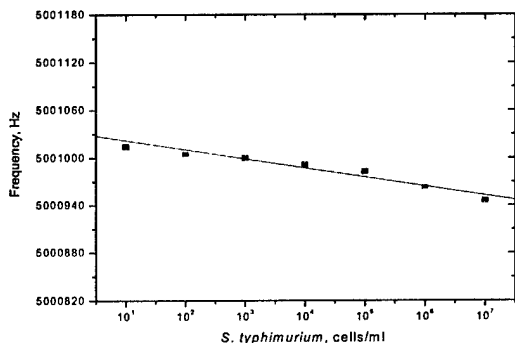


Figure 4. Dose-response relation of the mean output sensor frequencies as a function of *S. typhimurium* concentration. Curve is linear least squares fit to experimental data ($R = -0.98$, slope = -10.9 Hz, $p < 0.001$).

microscopy (SEM) (Fig. 5) confirmed bacterial binding to the sensor. The sensitivity of the biosensor (-10.9 Hz) was vastly greater than the established background. The lower limit of detection based on the dose-response curve was estimated at 100 cells/ml.

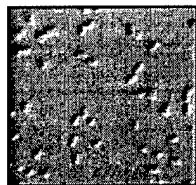


Figure 5.

Phage-skinning. As invented in this project, this method is based on three successive steps. Phages were first converted into spherical forms that resemble vesicles (Fig. 6), transformed into monolayers of the major coat protein

pVIII, and then deposited onto the sensor surface by LB method. Spheroids were prepared by treatment of filamentous phage with chloroform. The conversion of phage to spheroids was confirmed by gel electrophoresis of whole phage and spheroid particles [2].

Monolayers of phage coat proteins were prepared by allowing the spheroid suspension to run down a vertical glass rod partially submerged into the subphase. When spheroids reach the subphase surface, they are ruptured from the surface tension to create a monolayer of phage coat proteins. The formed monolayer was compressed and transferred onto the sensor surface [10].

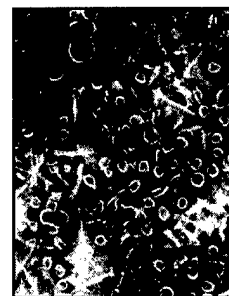


Figure 6.

We have shown that monolayers prepared by the phage skinning method produce a functional biospecific coating. For example, Fig. 7 (top) demonstrates signals generated by acoustic wave sensors coated with monolayers formed from phage specific for streptavidin. For each streptavidin-coated bead ($\sim 1 \mu m$ $d.$) concentration (10^4 - 10^8 particles/ml) reacted with the sensors, the signal approaches a steady-state response within 500 s. In Fig. 7 (center), the mean values of steady-state output sensor voltages are plotted as a function of bead concentration (upper curve - squares). The interaction of the beads with the peptide is specific since the signal is significantly lower for beads coated with BSA (lower line - circles). Binding of the beads to the sensor was confirmed by SEM (Fig. 7 - bottom; bar = $10 \mu m$).

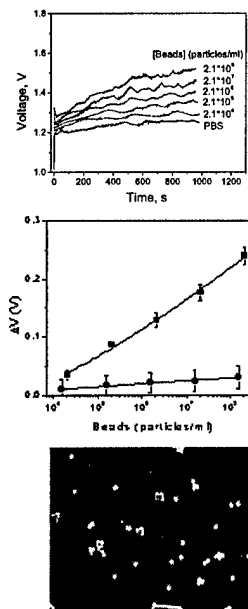


Figure 7.

A phage-derived biodetector for *S. typhimurium* was also prepared from spheroids combined with phospholipid - 1,2-diphytanoyl-sn-glycero-3-phosphocholine (PC). Fig. 8 depicts the experimental correlation coefficient, R , of 24 assayed spheroid-PC sensors as a function of their sensitivity. Correlation coefficients, R , were derived from the linear fit to dose-response signals for each tested sensor; sensitivities, S , were derived from the slope of the linear fit. A sigmoidal fit to experimental data points indicates a very good strength of association ($r^2 = 0.92$). The majority of the sensors (14 of 24) tend to group into one cluster at the positive end of the curve, possessing direct linear correlation with a mean $R = 0.90$ and mean sensitivity of

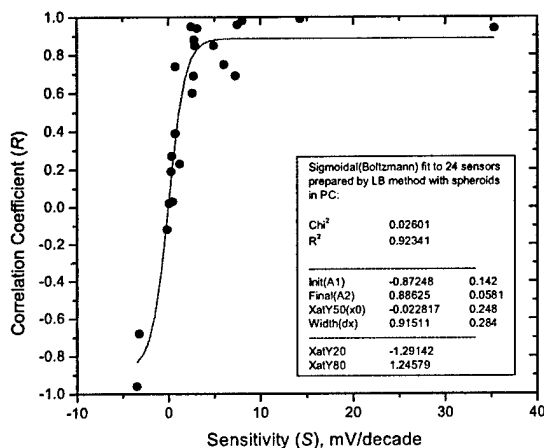


Figure 8.

8.1 mV. Six of these sensors (25%) possess goodness of fit and sensitivity greater than the acceptance criteria of $R \geq 0.90$ and sensitivity greater than 2.5 mV/decade. These results demonstrate proof in concept development of biosensors that incorporate phage as probes for the detection of threat agents such as *S. typhimurium*.

We believe our developed technique of engineered phage reconstruction represents a good prospective start for the directed nanofabrication of bioselective materials, with possible application to biosorbents, biosensors, nanoelectronics, and other areas of medicine, technology, and environmental monitoring.

4 CONCLUSIONS

We demonstrated that genetically driven "phage landscaping" allows the generation of libraries possessing diverse nanostructures accommodated on the phage's surface – a huge resource of diagnostic and detection probes. Biorecognition layers fabricated from the phage-derived probes bind biological agents, and as a part of analytical platforms, generate detectable signals. They may be suitable as antibody substitutes for field-use detectors of critical threat agents.

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REFERENCES

- [1] Smith, G.P., Petrenko, V.A., 1997. Phage Display. *Chem. Rev.* 97, 391-410.
- [2] Petrenko, V.A., Smith, G.P., Gong, X., Quinn, T., 1996. A library of organic landscapes on filamentous phage. *Protein Eng.* 9, 797-801.
- [3] Petrenko V.A., Smith, G.P., 2000. Phage from landscape libraries as substitute antibodies. *Protein Eng.* 13, 589-592.
- [4] Petrenko, V.A., Smith, G.P., Mazooji, M.M., Quinn, T., 2002. Alpha-helically constrained phage display library. *Protein Eng.* 15, 943-950.
- [5] Olsen, E., 2005. Ph.D. Dissertation, Auburn University, AL.
- [6] Brigati J., Williams, D.D., Sorokulova, I.B., Nanduri, V., Chen, I-H., Turnbough, C.L., Petrenko, V.A. Petrenko, 2004. Diagnostic probes for *Bacillus anthracis* spores selected from a landscape phage library. *Clin. Chem.* 50, 1899-1906.
- [7] Petrenko V.A., Sorokulova, I.B., 2004. Detection of biological threat agents. A challenge for combinatorial biochemistry. *J. Microbiol. Methods* 58, 147-168.
- [8] Sorokulova, I.B., Olsen, E.V., Chen, I-H., Fiebor, B., Barbaree, J.M., Vodyanoy, V.J., Chin, B.A., Petrenko, V.A., 2005. Landscape Phage Probes for *Salmonella typhimurium*. *J. Microbiol. Methods* (In Press).
- [9] Petrenko V.A., Vodyanoy, V.J., 2003. Phage display for detection of biological threat agents. *J. Microbiol. Methods* 53, 253-262.
- [10] Petrenko, V.A., Vodyanoy, V.J., Sykora, J.C. Methods of forming monolayers of phage-derived products and uses thereof. U.S. Patent 20040229215.

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