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1. AGENCY USE ONLY (Leave Blank)		2. REPORT DATE 04/19/00	3. REPORT TYPE AND DATES COVERED Final Report 07/01/98 - 01/31/00	
4. TITLE AND SUBTITLE Mosaic Landscape Phage as a New Class of Detector		5. FUNDING NUMBERS DAAG55-98-1-0258		
6. AUTHOR(S) Valery A. Petrenko, George P. Smith		8. PERFORMING ORGANIZATION REPORT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Curators of the University of Missouri, Office of Sponsored Program Administration, 310 Jesse Hall, Columbia, MO 65211		10. SPONSORING / MONITORING AGENCY REPORT NUMBER ARO 37715.2-LS		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U. S. Army Research Office P.O. Box 12211 Research Triangle Park, NC 27709-2211		11. SUPPLEMENTARY NOTES The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.		
12 a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited.		12 b. DISTRIBUTION CODE		
13. ABSTRACT (Maximum 200 words) Detection of single molecules or particles of biological or organic agents requires a ligand, which could be immobilized to a chip of analytical platform and should provide strong specific binding of the agent and generation of a detectable signal. We suggested new ligands--landscape phages selected from landscape libraries or mosaic landscape libraries. By testing landscape libraries with a panel of representative antigens (streptavidin from the bacterium Streptomyces avidinii, avidin from chicken egg white, and b-galactosidase from Escherichia coli) we obtained a proof of conception of using landscape phage as a new type of ligand for detector platforms. A library of organic landscapes is a rich source of substitute antibodies—filaments that can bind protein and glycoprotein antigens with nanomolar affinities and high specificity. In many ways these substitute antibodies are more convenient than their natural immunoglobulin counterparts.				
14. SUBJECT TERMS Phage display, landscape libraries, affinity selection, detectors		15. NUMBER OF PAGES 8		16. PRICE CODE
17. SECURITY CLASSIFICATION OR REPORT UNCLASSIFIED	18. SECURITY CLASSIFICATION ON THIS PAGE UNCLASSIFIED	19. SECURITY CLASSIFICATION OF ABSTRACT UNCLASSIFIED	20. LIMITATION OF ABSTRACT UL	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

20000707 148

DTIC QUALITY INSPECTED 4

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Foreword

Detection of single molecules or particles of biological or organic agents requires a *ligand*, which could be immobilized to a chip of analytical *platform* and should provide strong specific binding of the agent and generation of a detectable signal. Most platforms rely on the use of monoclonal antibodies as ligands. However, their application is hindered by high cost, low specificity, less than optimal affinity and sensitivity to unfavorable environmental conditions. We suggest a new class of ligands--landscape phages selected from landscape libraries or mosaic landscape libraries. In landscape phages, as in traditional phage-display constructs, foreign peptides or proteins are fused to coat proteins on the surface of the virus particle. Unlike conventional constructs, however, they display thousands of copies of the peptide in a repeating pattern, subtending a major fraction of the viral surface. The phage body serves as an interacting scaffold to constrain the peptide into a particular conformation, creating a defined organic surface structure (landscape) that varies from one phage clone to the next. A landscape library is a huge population of such phages, encompassing billions of clones with different surface structures and biophysical properties.

Our specific goal was to select phage that act as artificial antibodies specific for a panel of test antigens. Initially we planned a broad study of landscape phages including mosaic phages coated with two or more different random peptides interacting with each other to form complex antigen-binding sites. Since the term of the project was unexpectedly decreased from three to one year with no-cost extension to 0.5 year, some specific goals of the project were to be corrected. We focused our study on a proof of our general conception that landscape libraries can serve as a source of substitute antibodies for analytical devices. In particular we were interested to answer the questions:

- a) are peptides introduced into all copies of major coat proteins constrained and
- b) is their binding to antigens influenced by interaction with the phage body?

By testing landscape libraries with a panel of representative antigens, we obtained a proof of conception of using landscape phage as a new type of ligand for detector platforms. This library of organic landscapes is a rich source of substitute antibodies—filaments that can bind protein and glycoprotein antigens with nanomolar affinities and high specificity. In many ways these substitute antibodies are more convenient than their natural immunoglobulin counterparts.

Statement of the problem studied

The repertoire of antibodies in a vertebrate animal can be considered as a huge library of molecular landscapes, containing binding sites for almost any antigen. Each binding site comprises a set of highly variable peptide loops—the complementarity determining regions (CDRs)—contained within, and conformationally constrained by, a structurally constant framework. The CDRs vary widely in sequence and conformation from one antibody to another, and largely determine the antigen-binding specificity of the antibodies. The immune system has evolved cellular machinery for selecting those binding specificities that are needed to meet the

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challenge of an invading antigen, specifically amplifying those antibodies until they become prevalent in the total antibody population.

Antibodies have proven to be extremely adaptable and effective biomaterials in a great variety of biomedical and technical applications, but there are limits to their use. Polyclonal antibodies are relatively cheap to produce but heterogeneous, while monoclonal antibodies are homogeneous but expensive. Some target ligands, such as metals and toxic substances, cannot be used to elicit specific antibodies. Both polyclonal and monoclonal antibodies easily lose their binding properties under unfavorable environmental conditions.

To some extent, these limitations have been addressed by phage antibodies, an artificial, *in vitro* immune system in which antibodies (more accurately, antigen-binding domains of antibodies) are displayed on the surface of filamentous phage carriers whose genomes contain the antibody coding sequence (Hoogenboom, de Bruine et al. 1998). Phage-displayed antibodies can be propagated and cloned simply by infecting the phage into fresh bacterial host cells, and can be selected for binding to a particular target antigen using simple microbiological procedures. Despite its promise, however, phage antibody technology is not without difficulties. The last step in particular—expressing the selected antibody genes to make usable quantities of antibody—has proven troublesome, differing idiosyncratically from one antibody to another. These considerations and others have led researchers to cast about for non-immunoglobulin frameworks that might serve as the scaffold for artificial antibodies that would be easier to work with (Ku and Schultz 1995; McConnell and Hoess 1995; Nord, Nilsson et al. 1995; Martin, Toniatti et al. 1996; Nygren and Uhlen 1997; Gao, Mao et al. 1999). This project focuses on a particularly simple type of substitute antibody, in which the phage filament itself serves as the framework for random-peptide “CDRs” fused to the N terminus of every copy of the major phage coat protein. We call such particles “landscape” phage, since the random peptide subtends a major fraction of the organic landscape on the phage surface; a large mixture of such phage, displaying up to billion of different guest peptides, is called a “landscape library.” In some landscape phage, the guest peptide is conformationally constrained by interaction with the surrounding phage body, resulting in a well-defined, repeating surface structure (Kishchenko, Batliwala et al. 1994); in others, the peptide is not so constrained (Malik, Terry et al. 1996). We reported previously that a small organic hapten can select hapten-binding phage from a very large landscape library (Petrenko, Smith et al. 1996). Here we report that three different protein antigens likewise select antigen-binding phage from the same landscape library. Taken together, this work validates the concept of landscape phage as substitute antibodies.

Summary of the most important results

Selection and characterization of antigen-binding landscape phage.

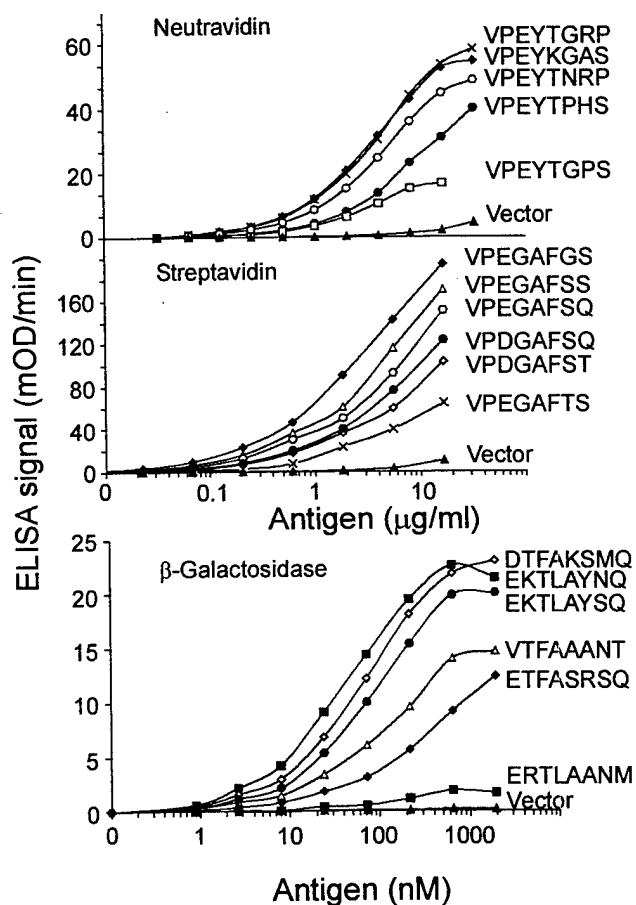
The model protein antigens in this work were streptavidin (from the bacterium *Streptomyces avidinii*), avidin from chicken egg white, and β -galactosidase from *Escherichia coli*. Streptavidin is a slightly acid tetrameric protein composed of four identical chains, each of 159 amino acid residues (Green 1990); it binds biotin with exceedingly high affinity. Avidin—another biotin-binder—is a tetrameric glycoprotein with 128-residue subunits having 33% structural homology with streptavidin (Green 1990). The architecture of the biotin-binding pockets of streptavidin and avidin are almost identical (Livnah, Bayer et al. 1993). The form of avidin used

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in our work was "neutravidin," a chemically modified form of the protein with greatly reduced positive charge. The β -galactosidase protein is a tetramer of identical 1,023-residue polypeptides, with molecular dimensions of $17.5 \times 13.5 \times 9$ nm (Jacobson, Zhang et al. 1994). Each antigen was absorbed to the surface of a 35-mm polystyrene petri dish and the dish reacted with the landscape library. Unbound phage were washed away, and bound phage eluted with acid buffer and amplified by infecting fresh bacterial host cells. After three rounds of selection individual phage clones were propagated and sequenced partly to determine the amino acid sequence of the displayed peptide.

Binding of the selected phage to their respective antigens was characterized by enzyme linked immuno sorbent assay (ELISA) in which the phage were immobilized on the plastic surface of the ELISA wells and reacted with their antigens in solution phase (Goldberg and Djavadi-Ohanian 1993; Yu and Smith 1996). Fig. 1 graphs ELISA signals as a function of antigen concentration, and demonstrates specific dose-dependent binding with nominal dissociation equilibrium constants (K_{DS}) in the nanomolar range. ELISA data are summarized in the Table (see below), the phage-display peptides being listed in order of decreasing ELISA signal.

Figure 1. Binding of antigens to immobilized phage as measured by direct ELISA. Phage displaying the indicated peptides were immobilized in the wells of microtiter dishes and reacted with graded concentrations of antigen— β -galactosidase, streptavidin labeled with alkaline phosphatase (AP-SA) or neutravidin labeled with alkaline phosphatase (AP-NA). Antigen remaining bound after washing was quantified using chromogenic substrates of β -galactosidase or alkaline phosphatase (*o*-nitrophenyl-b-D-galactoside or *p*-nitrophenyl-phosphate) and measuring the difference between the optical density (OD) at 405 and 490 nm at 3-min intervals to obtain a slope (mOD/min)



The antigen-binding power of a phage might reside in the peptide alone; alternatively, it may be an emergent property of the peptide interacting with the surrounding phage body. To address this question, we compared binding of native phage, partially denatured phage, and (in some cases) synthetic peptide. Partial denaturation was achieved by shaking a solution of phage

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with chloroform, which transforms the particle from an infectious filament to a non-infective hollow sphere (Griffith, Manning et al. 1981; Lopez and Webster 1982); the surface architecture of the phage is radically altered by this treatment, α -helix content of pVIII decreasing from 90% to 50–60% (Griffith, Manning et al. 1981; Roberts and Dunker 1993) and the particle becoming sensitive to detergents, 5M urea, proteases, heating, 40% formamide and salt (Griffith, Manning et al. 1981). Each of these structures was used at various concentrations to competitively inhibit the binding of soluble antigen to immobilized phage. The results of these inhibition ELISAs will be discussed in the context of individual antigens below.

Streptavidin

The streptavidin-binding phage displayed peptides with the sequence motif VP(E/D)(G/S)AFX, where 60% of X is S or T (Table, see below). This peptide motif has no noticeable similarity with the HPQ, GDWVFI or PWPWLG streptavidin-binding peptides selected in other phage-display experiments (reviewed in (Smith and Petrenko 1997)). Nevertheless, our streptavidin-binding phage resembles the previously reported ones in that biotin inhibits the binding (data not shown), indicating that in all cases binding site on streptavidin is located close to its biotin-binding pocket. Partially denatured phage is as potent as native phage in inhibition ELISA (data not shown); the synthetic peptide AVPEGAFSSDPAKAC-NH₂, corresponding to the N-terminal part of one of the streptavidin-binding phage, also inhibits binding, though with 10 times lower affinity than native and denatured phage (data not shown). Binding of the synthetic peptide to streptavidin could also be demonstrated by direct ELISA (data not shown). Taken together, these results show that the phage body serves only as a carrier for the streptavidin-binding peptides.

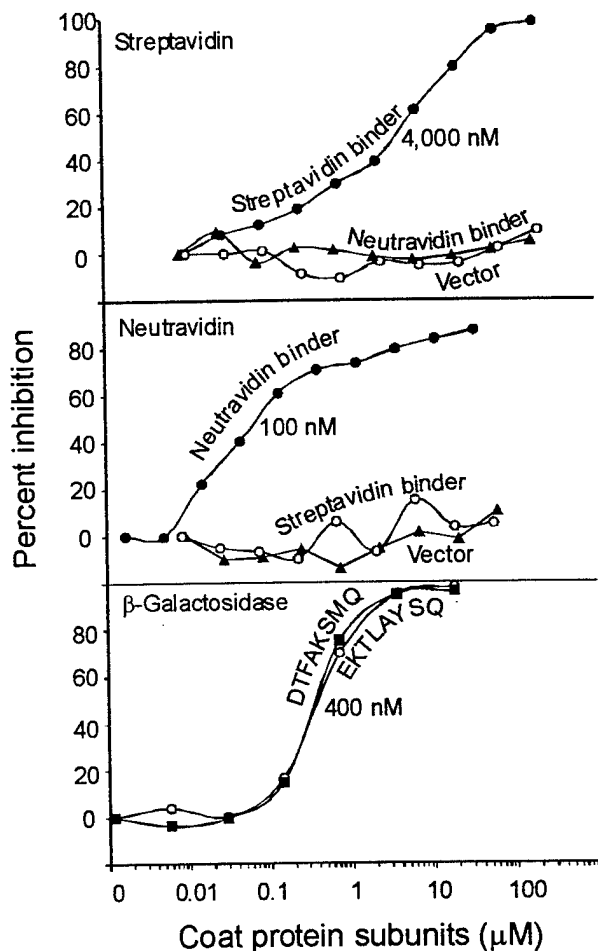
Table. Antigen-binding phage. Peptides displayed on phage selected by each of the three antigens are listed in order of decreasing ELISA signal (see Fig.1). Dominant motifs are indicated by highlighting. A “?” means that the corresponding codon was unreadable in the nucleotide sequence. When a peptide is displayed by more than one phage clone, the number of clones is shown in parentheses.

β -Galactosidase	Neutravidin	Streptavidin
D TF AKMAQ	VPEYSRPS	VPEGAFGS
E KT L AY N Q	VPEYKGAS	VPEGAFSS (7)
D TF AKSMQ (3)	VPEYTGRP (3)	VPEGAFSQ
E TFAKMSQ	VPEYVNTK	VPDGAFSQ (2)
E KT L AYSQ (2)	VPEYTNRP	VPEGAFST
E TFAKMTQ	VPEYTRT	VPE S AFAQ
V T F A AANT (2)	VPEYTPHS	VPDGA F ST
E TFARMNA	VPEYVPDR	VPEGAF T S (3)
D TF AKSAS (2)	VPEYISPQ	VPE S AFAQ
E TFARQS?	VPEYTPNT	VPD S A F NT
E TF A ASRR	VPEYTGPS	
E TF A ASNR	VPE F HSAQ	
E TF A SRSS	VPEYVKDT	
D TF AKSNA	VPEYNAHT	
E TF A RSQS	VPEYTSQP	
E TF A SRSQ	VPEYTSSQ	
D TF ARQNA	VPEYTSSS	
E TF A KSNA	V F DDTRT	
D TF AKSGQ	VPEYSSPS	
E TF A STRS	VPD Y OGPQ	
D TF ARTQS	VPD Y EPAR	
E TF A A?ST	VPEYNPAA	
E TF A RSNS	VPD D YSRQ	
D TF ARSSS	VPEYDRSS	
E Q T F A SSR		
E RT L AANM		

Neutravidin

The peptides displayed on neutravidin-binding phage have the sequence motif VPE(F/Y)XXXX, where the positions marked X are occupied with an unusually high portion (34%) of S and T. This motif is strikingly similar to the motif on the streptavidin-binding phage discussed above, and in this case too biotin blocks binding to the antigen (data not shown). However, it is evident that the phage-borne peptides are not true mimetics of biotin, since their binding is species specific: phage selected with streptavidin don't bind neutravidin and *vice versa* (Figure 2).

Figure 2. Binding of antigens to soluble phage inhibitors as measured by inhibition ELISA. Phage displaying antigen-binding peptides—VPEGAFSS for streptavidin, VPEYKGAS for neutravidin, DTFKSMQ for β -galactosidase—were immobilized in the wells of ELISA dishes. Meanwhile, antigen—streptavidin labeled with alkaline phosphatase (AP-SA), neutravidin labeled with AP (AP-NA), or β -galactosidase—at fixed concentration was pre-incubated with graded concentrations of soluble competitor phage (either native or chloroform-denatured) displaying the indicated peptide. The antigen-competitor mixtures were reacted with the phage-coated wells, and the antigen remaining bound after washing was quantified as described in a legend to Fig.1. Binding of antigen to the soluble competitor decreases binding to immobilized phage, thus depressing the ELISA signal. The concentrations of competitors required to depress the ELISA signal by roughly 50% are indicated, and correspond roughly to dissociation equilibrium constants (Goldberg and Djavadi-Ohanianc 1993).



There is another very important difference in behavior of streptavidin- and neutravidin-binding phage. As was mentioned above, binding of the streptavidin-binding phage is retained in the spherical form of the phage produced by chloroform treatment. In contrast, chloroform treatment reduced the apparent affinity of neutravidin-binding about 100-fold in inhibition ELISA (data not shown). Evidently binding of these phage to their antigen is strongly abetted by interaction between the displayed peptide and neighboring amino acids on the phage body.

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β -Galactosidase

Two families of guest peptides were displayed on β -galactosidase-binding phage, with consensus motifs EKTLYXQ and (D/E)TFA(K/R/x)XXX; the position marked (K/R/x) has >50% basic K and R residues. Phages from the two families compete with each other for binding to β -galactosidase, and therefore probably bind nearby sites on the antigen (Figure 2). IPTG, a competitive inhibitor of the enzyme with a K_i of 70 μ M (Ring and Huber 1990), failed to inhibit binding of either phage, even at a concentration of 100 mM (data not shown). That suggests that neither phage interacts with the active site of the enzyme. Chloroform treatment destroys completely the antigen-binding capacity of both families of phage (data not shown), indicating that antigen binding is an emergent property of the guest peptide in complex with the surrounding phage body.

Conclusions and Prospects

In this work we show that phage selected from landscape library can functionally mimic antibodies in their ability to bind protein antigens. Because it is the affinity-selected phage themselves that serve as the "antibodies," this technology bypasses one of the most troublesome steps of phage-antibody technology: re-engineering of the selected antibody genes to express them at a high level. Indeed, a culture of cells secreting filamentous phage is an efficient, convenient protein production system. The yield of landscape phage regularly reaches 20 mg/liter, and they are secreted from the cell nearly free of intracellular components. Further purification is easily accomplished by simple, routinizable steps that do not differ from one clone to another. The surface density of the phage particle is 300-400 m^2/g , exceeding probably the best known absorbents and catalysts. The randomized amino acids that form the "active site" of a landscape phage comprise up to 25% by weight of the particle and subtend up to 50% of its surface area—an extraordinarily high fraction compared to natural proteins, including antibodies. In many applications, the extreme multivalency of landscape phage—thousands of binding sites/particles—may be a great advantage. The phage structure is extraordinarily robust, being resistant to heat (up to 70°C), many organic solvents (e.g., acetonitrile) (Olofsson, Ankarloo et al. 1998), urea (up to 6 M), acid, alkali and other stresses. Purified phage can be stored indefinitely at moderate temperatures without losing infectivity. These characteristics commend landscape phages as substitute antibodies in many applications, such as biological detectors, affinity sorbents, hemostatics etc.

List of publications

Petrenko V.A. and Smith G.P. (2000) Microfibers from phage landscape libraries as substitute antibodies. Protein Engineering (Submitted).

Petrenko V.A., Smith G.P., Mazooji M.M. and Quinn T. (2000) Alfa-helically constrained phage display library. Protein Engineering (In preparation).

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