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Notes & Tips

Dual genetically encoded phage-displayed ligands

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ABSTRACT

M13 bacteriophage display presents polypeptides as fusions to phage coat proteins. Such phage-displayed ligands offer useful reagents for biosensors. Here, we report a modified phage propagation protocol for the consistent and robust display of two different genetically encoded ligands on the major coat protein, P8. The results demonstrate that the phage surface reaches a saturation point for maximum peptide display.

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M13 bacteriophage or "phage" have a readily customizable protein coat allowing the display of peptide or protein ligands on their surfaces. Such molecular display links the phenotype of the displayed ligand with its encoding DNA encapsulated by the phage. The display of one peptide in single or multiple copies on the phage surface, termed individual display here, is used extensively for therapeutic and ligand discovery [1,2]. However, some experiments require two different ligands displayed on the same phage surface, termed dual display. For example, we previously attached to the phage surface two different ligands: one chemically synthesized and one genetically encoded. Such viruses with two ligands presented were then incorporated into a bioaffinity matrix for the sensitive detection of prostate-specific membrane antigen (PSMA)[1] [3–5].

M13 viruses infect only bacteria and consist of a single-stranded DNA genome encapsulated by a protein coat. Approximately 2700 copies of the major coat protein, P8, appear along the length of the virus, and 5 copies each of the four minor coat proteins cap the ends of the virus [6–9]. Manipulating the viral genome allows the display of individual ligands as fusions to the phage coat proteins. Multi-copy ligand display can increase the affinity for the target due to avidity effects. Here, we report a method to simultaneously introduce two different genetically encoded ligands on the phage surface. Fusion of each ligand to P8 can best allow the two different ligands to simultaneously bind to the target because both binding sites appear along the length of the virus.

Double transformations can result from bacteria having two plasmids simultaneously or consecutively inserted. To achieve such double transformations, bacteria can be infected by two phage; however, infection of the same *Escherichia coli* cell by two phage is generally an inefficient process [10]. Despite this caveat, double infection remains a staple procedure in phage display because helper phage typically infect phage-infected cells after each round of selection. As an additional caveat, the growth advantages of one plasmid can result in the loss of the other plasmid after double transformation [11]. As reported here, the phage propagation protocol can be modified to provide a consistent combination of two peptides presented on the phage surface. Furthermore, the modified procedure prevents loss of the slower propagating plasmid, as confirmed by enzyme-linked immunosorbent assay (ELISA) and other assays described below.

Two previously reported peptide ligands specific for PSMA (provided by Molecular Express), binding ligand 1 (LDCVEVFQNSCDW) and ligand 2 (SECVEVFQNSCDW) [12], were simultaneously displayed using a modified phage propagation protocol. First, *E. coli* cells were transformed with the phagemid DNA encoding the first ligand. Next, the bacterial cell cultures were infected with bacteriophage displaying the second ligand. The resultant phage were designated either phage-12 or phage-21. This nomenclature identifies the ligand introduced through genetic transformation as the

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[1] *Abbreviations used:* PSMA, prostate-specific membrane antigen; ELISA, enzyme-linked immunosorbent assay; ORF, open reading frame; MOI, multiplicity of infection; PCR, polymerase chain reaction; BSA, bovine serum albumin; HRP, horseradish peroxidase.

first number, with the second number indicating the second ligand introduced by super-infection. Reversing the order of ligands used during the two steps generates two combinations of the dual-ligand system. The phage resulting from each combination could have potentially the same or different copy numbers of each ligand.

Phage propagation was performed as reported previously [3]. Chemicals and reagents were purchased from Sigma–Aldrich and used as received unless otherwise noted. Phage-1 consistently propagates 20% more efficiently than phage-2 (yields of 412 and 342 pmol per 150-ml culture, respectively). Briefly, the M13 phage display vectors (phagemids) with an open reading frame (ORF) encoding either peptide 1 or 2 fused to P8 were used to transform $CaCl_2$ competent *E. coli* XL-1 Blue cells before plating onto LB agar plates supplemented with carbenicillin. For example, phage-12 resulted from a single colony of cells transformed with phagemid-1 and then grown at 37 °C in 2 ml of 2YT medium supplemented with carbenicillin and tetracycline until the culture reached log-phase growth. The culture was then infected with phage-2 at a multiplicity of infection (MOI) of 200:1 and shaken at 250 rpm for 45 min at 37 °C. Next, M13-KO7 (GE Healthcare) phage (MOI of 3:1) was added. The starting culture was then transferred to 75 ml of 2YT medium and grown overnight. Phage were isolated from the cells by polyethylene glycol (PEG)–NaCl precipitation. Phage concentration was determined by ultraviolet (UV) absorbance at 268 nm (OD_{268} = 8.31 nM).

Packaging of both phagemids was confirmed by polymerase chain reaction (PCR), followed by sequencing (GENEWIZ) (Fig. 1). This procedure can verify propagation of both phagemids but does not confirm dual display. The forward primers were specific for ligand 1 (CAGCCATATGGCCTATGCATTGGACT; primer-1) or ligand 2 (CAGCCATATGGCCAGCGCGTCG; primer-2). Each PCR used the same reverse primer (CAGGAAACAGCTATGACGACAACAACCATCGCCC) and was performed with iProof DNA polymerase. When subjected to PCR and sequencing, phage-12 and phage-21 confirmed the presence of both individual phagemids in each of the dual-displayed phage samples. Each dual-displayed phage sample, phage-12 or phage-21, includes both phagemid-1 and phagemid-2. Phage-1 amplified with primer-1 and phage-2 amplified with primer-2 serve as the positive controls for priming and amplification. Phage-1 amplified with primer-2 and vice-versa were used as negative controls to test for nonspecific annealing of the primers.

Next, a previously reported phage-based ELISA was used to examine binding to PSMA [3]. Briefly, specific wells of a 96-well microtiter plate (Nunc MaxiSorp) were coated with a 5.6 nM PSMA solution, followed by blocking with bovine serum albumin (BSA). The wells were then incubated with serially diluted, phage-displayed ligands. Levels of bound phage were quantified using a horseradish peroxidase (HRP)-conjugated anti-M13 antibody. The negative controls included phage-displayed ligands targeting BSA and Stop-4 phage replacing the phage-displayed ligands. Stop-4 phage consists of a phagemid with the wild-type phage coat, four

stop codons in the ORF encoding the display sequence, and thus no peptide displayed on the phage surface.

An ELISA compared binding by dual- and individual-displayed phage. This experiment also examined possible differences in affinity resulting from the new propagation protocols compared with conventional practices (phage-1 and phage-2). Phage-12 and phage-21 produced an overlapping response on binding to PSMA (Fig. 2A). In comparison, the individual-displayed phage-1 and
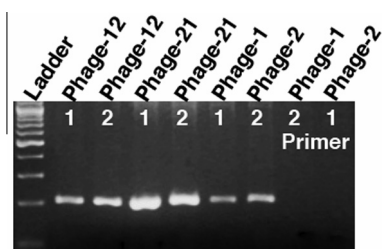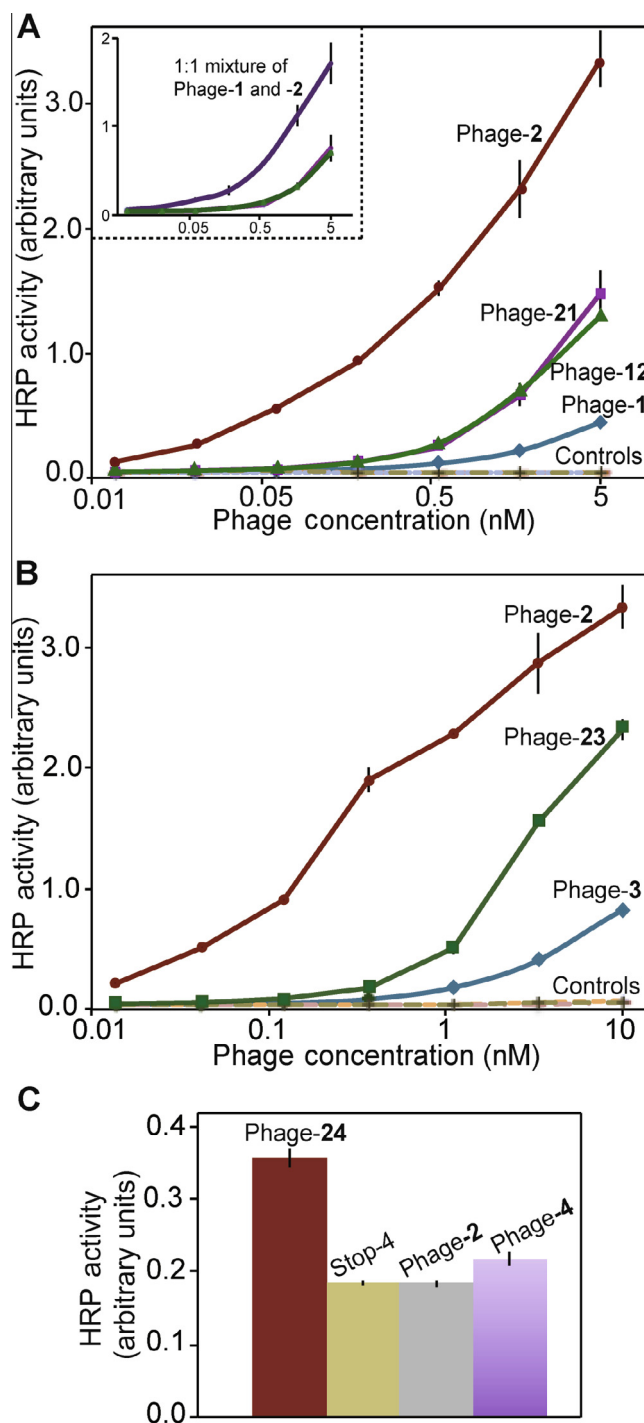
Fig.2. Phage-based ELISAs demonstrating the binding affinity of dual-displayed phage. (A) Phage generated with similar ligand sequences targeting PSMA. The inset compares a 1:1 mixture of phage-1 and phage-2 with phage-12 and phage-21. (B) Phage generated with dissimilar ligand sequences targeting PSMA. (C) Phage-based sandwich ELISA demonstrating simultaneous binding to both BSA and PSMA. Stop-4, helper phage packaging the phagemid DNA, serves as the negative control. Error bars indicate standard errors (*n* = 3).

Fig.1. Agarose (1%) gel electrophoresis confirms the presence of both phagemids from dual-displayed phage samples.

phage-2 revealed the expected extremes of weak and strong binding, respectively. Phage-2 binds with more than 100-fold higher affinity to PSMA than phage-1. Strikingly, the binding affinity of the dual-displayed phage falls between the binding affinities of each individual-displayed ligand.

The intermediate binding observed for the dual-displayed phage suggests that the number of displayed peptides per phage particle remains narrowly constant. Thus, the apparent binding failed to result from the simple addition of binding affinities of phage-1 and phage-2 that would result in a much higher apparent binding affinity by the dual-displayed phage. In addition, the overlapping responses for both phage-12 and phage-21 demonstrate that the inherent growth advantage for phagemid-1 does not eliminate phagemid-2 during propagation of dual-displayed phage. Thus, the two distinct modes of propagating phage-12 or phage-21 result in the same display levels. Comparing this result with binding by the mixture of phage-1 and phage-2 (Fig. 2A, inset) suggests that the dual-displayed phage present a mixture of peptides on their surfaces. This experiment rules out the possibility of phage-12 or phage-21 consisting of a mixture of individual-displayed phage resulting from each propagation condition; such a mixture would be expected to have different phage levels and altered apparent affinities from the two modes of propagation with two phage experiencing unequal growth conditions.

To further investigate the generality of the method, dual-displayed phage were prepared with two PSMA binding ligands having dissimilar sequences, namely ligand 2 and ligand 3 (CALCEFLG) [11]; ligands 1 and 2 differ in only two residues, but ligands 2 and 3 differ in every position. As shown above, similar binding affinities of dual-displayed phage result from a different order of propagation, and thus only phage-23 was produced. The propagation protocol and negative controls used for this experiment were as described above. The binding affinity of phage-23 falls between the binding affinities of phage-2 and phage-3, as expected (Fig. 2B). This experiment demonstrates that the generation of dual-displayed phage is independent of the ligands' sequence homology.

To further demonstrate dual display of two different peptides on the surface of the phage, a sandwich ELISA was designed for simultaneous detection of two ligands displayed on the phage surface. In this experiment, dual-displayed phage were prepared with PSMA binding ligand 2 and BSA binding ligand 4 (SSQDVCELGRWLSEECELYM). The binding affinity of phage-24 toward BSA and PSMA confirms the presence of both ligands on phage (Fig. 2C). The ELISA protocol was modified as follows. Briefly, specific wells were coated with a 5.6 nM BSA solution, followed by blocking with SuperBlock (Thermo Scientific). The wells were then incubated with phage-displayed ligands, followed by incubation with PSMA (16.8 nM). Levels of bound PSMA were quantified using anti-PSMA (YPSMA-1) antibody (Abcam) at a 1:1000 dilution, followed by HRP-conjugated anti-mouse antibody (Sigma) at a 1:1000 dilution. The negative controls included phage-displayed ligands targeting SuperBlock and Stop-4 phage replacing phage-24 in the sandwich ELISA setup. Packaging of both phagemids for phage-23 and phage-24 was verified by PCR as described above.

As reported here, the technique of super-infection provides a method for consistently generating *E. coli* cells with two different phagemids. The approach allows for the display of two genetically encoded ligands fused to P8. Such dual-displayed phage particles could potentially be used for targeting different markers and, thus, could be enormously useful for biosensing.

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