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Creating highly amplified enzyme-linked immunosorbent assay signals from genetically engineered bacteriophage

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ABSTRACT

For early detection of many diseases, it is critical to be able to diagnose small amounts of biomarkers in blood or serum. One of the most widely used sensing assays is the enzyme-linked immunosorbent assay (ELISA), which typically uses detection monoclonal antibodies conjugated to enzymes to produce colorimetric signals. To increase the overall sensitivities of these sensors, we demonstrate the use of a dually modified version of filamentous bacteriophage Fd that produces significantly higher colorimetric signals in ELISAs than what can be achieved using antibodies alone. Because only a few proteins at the tip of the micron-long bacteriophage are involved in antigen binding, the approximately 4000 other coat proteins can be augmented—by either chemical functionalization or genetic engineering—with hundreds to thousands of functional groups. In this article, we demonstrate the use of bacteriophage that bear a large genomic fusion that allows them to bind specific antibodies on coat protein 3 (p3) and multiple biotin groups on coat protein 8 (p8) to bind to avidin-conjugated enzymes. In direct ELISAs, the anti-rTNFα (recombinant human tumor necrosis factor alpha)-conjugated bacteriophage show approximately 3- to 4-fold gains in signal over that of anti-rTNFα, demonstrating their use as a platform for highly sensitive protein detection.

During past years, extensive methods have been developed to both sense and identify different proteins in solution through enzymatic assays in which a detection antibody binds to both an antigen and a signaling enzyme that produces a colorimetric signal [1–3]. A typical enzyme-linked immunosorbent assay (ELISA)[1] requires three steps. First, the antigen is sequestered to a surface or surface-bound antibody. This is followed by the addition of a different antibody that binds the antigen as well as an enzyme capable of generating amplified signals such as horseradish peroxidase (HRP). This enzyme then generates a colorimetric reaction by reacting with a substrate molecule [4]. Although ELISAs are now used routinely and extensively in medical facilities and laboratories, because the detection antibodies can possess only a finite number of attached enzymes (~1–4 HRPs per antibody), the signals generated for sensing are often limited to the nanomolar range [5,6], especially in the case where the dissociation constant between antibody and antigen is high [4]. Although the binding strength of the antibody to an antigen can be limited, gains in sensitivity can be obtained by using a detection platform that possesses large surface areas for binding multiple enzymes but still binds to a single antigen [7–9].

During recent years, filamentous bacteriophage such as M13, Fd, and their derivatives have been investigated as biological tools for material science and biotechnology. The virus coat proteins may be genetically engineered to display different peptides, and panning may be used to isolate unique binding motifs for a wide variety of substrates. Filamentous phage have been used as biosensors [10–17], templating agents for energy-relevant materials [18–20], and drug delivery vehicles [21–25]. Of the five different proteins that comprise the filamentous phage capsid, two are commonly used as fusion partners to display peptides or small proteins. These are the 406-residue p3 proteins, which are present at three to five copies at one end of the virus, and the 50-residue p8 proteins, which are arranged in a helix around the length of the phage [26]. To enable the display of larger proteins on protein 3,

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[1] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); rTNFα, recombinant human tumor necrosis factor alpha; PCR, polymerase chain reaction; PLP, pyridoxal phosphate; PEG, polyethylene glycol; PBS, phosphate-buffered saline; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; LDS, lithium dodecyl sulfate; SPR, surface plasmon resonance; PBST, PBS with Tween 20.

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such as antibody fragments that bind with greater specificity and affinity to antigens than short peptides, the genome of the Fd phage can be modified with a tetracycline resistance gene inserted into the origin of replication to create a modified phage known as Fd-tet [27,28]. In wild-type phage, protein fusions to p3 larger than approximately 20 amino acids typically prevent the p3 proteins from binding to the f-pilus of male bacteria and initiating the infection process, thereby preventing propagation. However, Fd-tet may be replicated without the need for infectivity because it can be maintained within a host culture through tetracycline selection. Because of this, Fd-tet phage have been used to clone libraries of large fusions such as antibody fragments [29] and IgG-binding domains [30] onto the p3 proteins. Furthermore, the increased length of the Fd-tet genome causes the resulting phage to possess approximately 4000 p8 proteins as opposed to the typical 2700 p8 proteins seen in wild-type Fd.

For biosensing applications, the p3 proteins at the end of the phage are primarily responsible for binding a single antigen, whereas the thousands of p8 proteins are modified to bind other materials. For example, in earlier work we showed that thiol groups or DNA strands could be attached to the viruses to bind metal nanoparticles to generate unique optical or spectroscopic signals [10,11]. In this article, we first show that genetically modified Fd-tet phage can bind the Fc portions of monoclonal antibodies and that these can be used to target specific antigens with high affinity and selectivity. Furthermore, we show methods to modify the p8 proteins with biotin groups without inhibiting the phage from binding to the detection antibody or the targeted antigen. These biotin groups were then used to attach approximately 70 avidin-conjugated HRP enzymes to each virus to yield large gains in signal in direct ELISAs over what can be achieved using antibodies alone (Fig. 1A).

Materials and methods

fUSE-5 genomic DNA was kindly provided by Itai Benhar (Tel-Aviv University). K91BlueKan cells were kindly provided by George P. Smith (University of Missouri). Oligonucleotides were ordered from integrated DNA technologies. The highly competent DH5α cells, high-fidelity polymerase, *Dpn*I, *Bam*HI, *Pst*I, and ligase enzymes were purchased from New England Biolabs. Miniprep kits were purchased from Qiagen. Gel extraction kits and 4–12% Bis–Tris SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) gels were purchased from Life Technologies. Tetracycline hydrochloride, pyridoxal-5′-phosphate hydrate, bovine serum albumin (BSA), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and Tween 20 were purchased from Sigma–Aldrich. Alkoxyamine–PEG4–biotin and high-binding polystyrene 96-well plates were purchased from Thermo Scientific Pierce. Recombinant human tumor necrosis factor alpha (rTNFα), avidin-conjugated HRP, and biotinylated and unmodified versions of the anti- TNFα antibody clone Mab11 were purchased from Affymetrix eBioscience. Anti-M13–HRP antibodies were purchased from GE Healthcare.

Creating Fzz8 and Fzz8–AKT

The fUSE-5 genome was modified with restriction sites for p8 cloning by site-directed mutagenic polymerase chain reaction (PCR). Two rounds of mutagenic PCR were used to first remove the *Bam*HI site located within the p3 coding sequence and then introduce a *Bam*HI and *Pst*I site at the N terminus of p8. Each time, the PCR products were digested with *Dpn*I to remove template DNA and then transformed into DH5α cells, and resulting colonies were screened for the correct mutation by digesting with the correct enzyme and analyzing on a 0.8% agarose gel. The resulting mutated fUSE-5 was named Fzz8 and was used to create Fzz8–AKT by cloning in the AKT sequence into the N terminus of p8 via restriction ligation cloning. The Fzz8 genome was cut with *Bam*HI and *Pst*I, and the larger fragment was purified by gel extraction. Oligonucleotides coding for the AKT sequence and compatible restriction sites were annealed together and then ligated into the Fzz8 fragment to recircularize it as Fzz8–AKT. Phage were grown by transforming K91BlueKan cells with Fzz8 or Fzz8–AKT phage genomes and plating on LB plates with 40 μg/ml tetracycline. Colonies were pricked from these plates and grown for 20 to 24 h at 37 °C with vigorous shaking in 80 to 160 ml of LB with 40 μg/ml tetracycline.

PLP reaction and biotin conjugation

Phage were reacted with pyridoxal phosphate (PLP) by polyethylene glycol (PEG) precipitating a phage preparation and resuspending the pellet in freshly prepared 25 mM phosphate-buffered saline (PBS, pH 6.5) at a concentration of approximately 141 nM. PLP was dissolved in the same buffer from frozen stock at a concentration of 200 mM by bringing the pH to approximately 6.5 with NaOH. Then, 150 μl of the phage and PLP solutions was mixed and reacted for 16 h at room temperature with mild shaking. Excess PLP was removed by PEG precipitating the

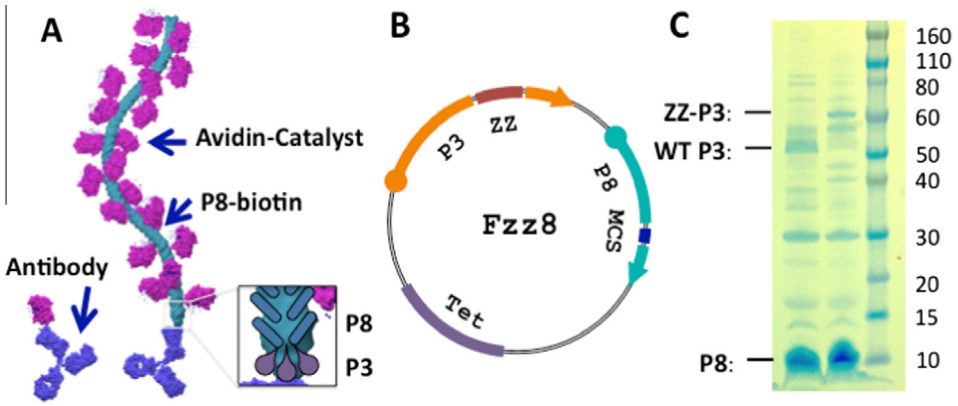

Fig.1. (A) Schematic showing the use of dually modified phage for generating signal enhancement over antibodies alone. (B) Schematic of the Fzz8 phage genome showing p3 coding sequence (orange), ZZ domain antibody binding fusion (red), p8 coding sequence (teal), and multiple cloning site (blue). (C) SDS–PAGE gel showing capsid proteins from M13KE control phage (left), Fzz8 phage (center), and a labeled protein ladder in kDa (right). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)