Analytical Biochemistry 470 (2015) 7–13

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

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

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ARTICLE INFO

Article history:
Received 15 August 2014
Received in revised form 6 October 2014
Accepted 10 October 2014
Available online 22 October 2014

Keywords:
ELISA
Bacteriophage
Biosensor

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.

http://dx.doi.org/10.1016/j.ab.2014.10.006
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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

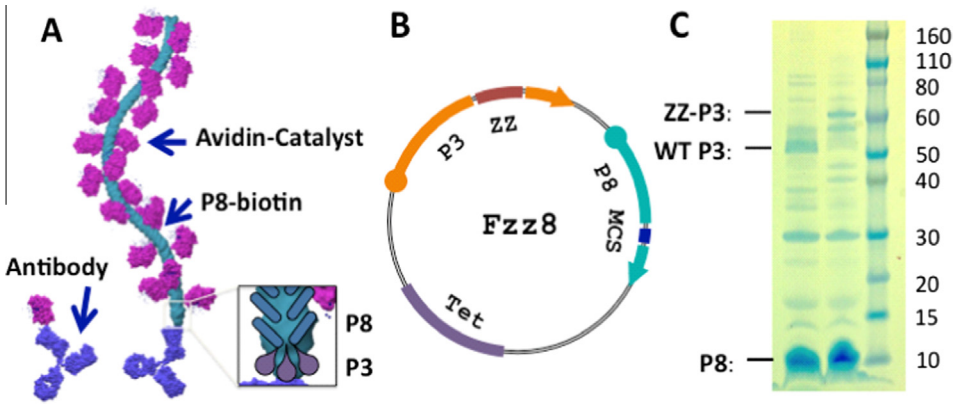

Fig.1. (A) Schematic showing the use of dually modified phage for generating signal enhancement over antibodies alone. (B) Schematic of the Fzz8 phage genome showing p3 coding sequence (orange), ZZ domain antibody binding fusion (red), p8 coding sequence (teal), and multiple cloning site (blue). (C) 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.)

phage four times. The resultant pellet was first resuspended in 200 µl of 10 mM PBS (pH 7.4), then in 1 ml of water twice, and finally in 200 µl of water. PLP-modified phage were diluted to a concentration of approximately 20 nM with 100 mM PBS (pH 4.0) prepared immediately beforehand and reacted with various concentrations of alkoxyamine–PEG4–biotin in the presence of 1 mM aniline.

MALDI and SDS–PAGE analysis

Matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) spectra were taken on a Voyager DE-STR system (Applied Biosystems) after denaturing phage in a methanol/water/formic acid mixture and using tetrahydroxyacetophenone as a matrix. Phage were denatured for SDS–PAGE analysis by dissolving in lithium dodecyl sulfate (LDS) loading buffer (Life Technologies) and heating at 80 °C for 10 min. PAGE gels were run for 35 min at 200 V and visualized by staining with Coomassie SafeStain (Life Technologies).

SPR studies

BiOptix 404pi (BiOptix) and carboxymethyl dextran-coated gold surface sensing chips (BiOptix) were used for the surface plasmon resonance (SPR) assays. The protein-coated gold chips were prepared by immobilizing human rTNFα (PeproTech) to the dextran-coated surfaces using carbodiimide chemistry. For this, 400 mM EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) and 100 mM NHS (*N*-hydroxysuccinimide) were injected simultaneously across the sensing spots for 5 min. Next, 3 µg/ml rTNFα solutions in sodium acetate buffer (pH 4.5) were injected into all of the channels. The surfaces were then blocked with 1 M ethanolamine (pH 8.5) for 10 min. For determining binding constants, 5 nM anti-human rTNFα antibody (Affymetrix eBioscience) or 5 nM anti-human TNFα antibody-conjugated Fzz phage in 0.05% (v/v) PBST (PBS with Tween 20) was injected into the experimental channels, whereas 0.05% (v/v) PBST was injected as control channels. The resulting spectra were analyzed by Scrubber 2 program (BioLogic Software).

ELISA

To target phage to rTNFα, 3 nM biotin-conjugated Fzz8 phage were mixed at a 3:1 molar ratio with anti-rTNFα antibody clone Mab11 in 10 mM PBS (pH 7.4). Unbound antibodies were removed by PEG precipitating the phage. Phage were resuspended in blocking buffer (10 mg/ml BSA in 10 mM PBS, pH 7.4) at a concentration of 3.3 nM. The biotinylated version of the same targeting antibody (anti-TNFα antibody clone Mab11) was also made to be 3.3 nM in blocking buffer. rTNFα (Affymetrix eBioscience) was diluted in 10 mM PBS (pH 6.0), and 50 µl of each dilution was coated onto high-binding capacity polystyrene wells for at least 16 h at 4 °C. The wells were then further blocked by adding blocking buffer and shaking at 170 rpm for 1 h at room temperature. Then, 100 µl of either the biotinylated antibody or phage solution was added to each well for 1.5 h. Wells were washed three times by pipetting in 0.01% PBST solution for 5 min and tapping out gently on a paper towel. Then, 100 µl of avidin–HRP diluted in blocking buffer to the recommended concentration was added to each well for 1 h and wells were washed as before. Signals from bound HRP were developed with ABTS for 10 min and measured at 418 nm using a Beckman Coulter DU730 spectrophotometer.

Results and discussion

To build highly sensitive protein sensors from filamentous bacteriophage, we chose to generate a virus that was capable of binding biologically relevant antigens via the p3 proteins located at the tip of the phage while conjugating multiple handles to the approximately 4000 p8 proteins to bind catalysts. For this, we first produced phage whose p3 proteins were genetically modified to display a repeated synthetic version of domain B from staphylococcus protein A ("ZZ" domain) that binds tightly with the Fc portion of IgG-type antibodies [31]. The ZZ-expressing phage (Fzz) could then be targeted to an antigen by conjugating it with an antibody specific to that antigen. For this, phage expressing two repeats of the IgG-binding Z domain fused to each of the p3 coat proteins (fUSE5-ZZ phage) were first generously provided by Itai Benhar [30]. Next, to fuse specific peptides to each of the p8 proteins, the fUSE5-ZZ genome was further modified via site-directed mutagenesis such that a *Bam*HI and *Pst*I site was located on either side of the coding sequence for the N terminus of the mature p8 protein (Fig. 1B) [27,32,33]. A *Bam*HI site was also deleted within the p3 coding sequence such that the newly introduced restriction enzyme sites were unique. The introduction of the new *Bam*HI site caused the third amino acid from the N terminus of the mature p8 coat protein to change from aspartic acid to glutamic acid, but all other mutations were silent. The new restriction enzyme sites would allow peptide sequences to be expressed as N-terminal fusions to all p8 coat proteins. The newly engineered genome termed Fzz8 was next transformed into chemically competent K91BlueKan cells and plated on LB plates containing 40 µg/ml tetracycline. Specific colonies were then grown overnight in K91BlueKan cells in LB with 40 µg/ml tetracycline at 37 °C. After centrifuging the cells, the Fzz8 phage were precipitated out of solution by using 20 wt% PEG (MW = 8000) and 2.5 M NaCl. The resulting phage pellets were resuspended in 1 ml of 10 mM PBS (pH 7.2) and centrifuged for 5 min to remove additional cell debris, followed by a second PEG precipitation step. The final phage concentration in solution was estimated by measuring the absorbance at 269 and 320 nm [26].

To confirm the presence of the ZZ domain on the p3 proteins, SDS–PAGE was run. As a control, M13KE phage that carries a gene very similar to the wild-type p3 protein was run as a negative control [34]. Both the Fzz8 and M13KE were first concentrated via a PEG precipitation and resuspended in water at a concentration of approximately 1e14 particles per milliliter (166 nM) and then denatured by mixing with LDS buffer (Life Technologies) and heating in a thermocycler at 80 °C for 10 min. As shown in Fig. 1C, the Fzz8 lane clearly contains a band corresponding to a p3 protein conjugated with a ZZ domain, whereas the M13KE lane contains only a band corresponding to the molecular weight of an unmodified p3 protein.

Next, to use the phage as detection platforms to produce highly enhanced ELISA signals, we decided to develop methods to conjugate multiple catalysts to each virus body. By doing so, we could increase the signal generated many-fold for each antigen-binding event over what can be achieved using antibodies alone, which at most can have only a few enzymes attached. To achieve this, we decided to attach multiple biotin groups to the p8 proteins, which could then be reacted with avidin-conjugated enzymes such as HRP. Although it is possible to detect the presence of phage by using an HRP-conjugated anti-M13 antibody, this would require the need for an additional antibody that only increases the complexity and cost of the sensing assay. To chemically bind biotin groups to the p8 proteins, we employed a conjugation strategy developed by Francis and coworkers in which N-terminal amines are first converted to ketones by a transamination reaction with

PLP and are then reacted with alkoxyamine-functionalized PEG4–biotin to form the corresponding Schiff base [35]. By targeting only the N termini of the coat proteins, we minimized the chance of conjugating biotin groups to the p3 proteins and disrupting the phage binding to the targeting antibody. After reaction, the biotinylated Fzz8 phage was characterized by MALDI–TOF mass spectrometry. As shown in Fig. 2A, the MALDI data contained two main peaks, one of which corresponded to the mass of the unmodified p8 coat protein (5253 Da) and the other of which matched the expected molecular weight of a p8 protein fused to a PEG4–biotin group (5669 Da). A smaller peak can also be seen in between these two peaks and corresponds to the mass of the p8 protein modified with a PLP adduct. Higher amounts of biotin groups could also be attached per phage simply by increasing the amount of alkoxyamine PEG4–biotin added during the reaction (Fig. 2A). However, as the number of PEG4-biotin groups per phage increased, this also caused the phage to become more difficult to precipitate out of the reaction mixture, thereby lowering the yields of biotinylated phage obtained. Using a molar ratio of 2800:1 alkoxyamine–PEG4–biotin to phage during the reactions produced biotinylated viruses that could easily be recovered from solution by PEG/NaCl precipitation. Next, to determine the number of enzymes that could be attached to each virus, 1 μM avidin–HRP was added to 13 nM biotinylated Fzz8 in PBS and incubated at 4 °C for 1 h. The solutions were then centrifuged through 0.22-μm filters to determine how much of the HRP had bound to each phage by measuring the flow-through by UV–Vis (ultraviolet–visible) analysis. To make sure that the enzymes alone could pass through the filters, solutions of avidin–HRP were also filtered through 0.22-μm filters. Through such measurements, we were able to determine that approximately 70 avidin–HRP bound to each phage (see Fig. S1 in online Supplementary material).

In addition to using Fzz8 phage that contain unmodified p8 proteins, the amino acid sequence alanine-lysine-threonine (AKT) was also genetically expressed at the N terminus of the Fzz8 p8 proteins. The reason the AKT sequence was chosen was due to the observation that these peptides showed a higher oxidation rate of the N terminus by PLP [36,37], which could further prevent any modification of the p3 proteins. For this, the lysine-threonine DNA sequence was inserted between the N-terminal alanine and the adjacent glutamic acid present in the wild-type protein. As shown in Fig. 2B, MALDI analysis showed the expected molecular weight of a p8 protein fused to an additional lysine-threonine sequence (5669 Da). However because the AKT-expressing phage showed very limited solubility at neutral pH, presumably due to the extra lysine group leading to an increased isoelectric point, we were unable to move forward in reacting the AKT-modified viruses with PLP and alkoxyamine biotin.

To see whether the Fzz8 phage could next be targeted to specific protein antigens, the biotinylated Fzz8 phage were conjugated with anti-rTNFα antibody by using a 1:3 molar ratio of phage to antibody in PBS for 1 h at 4 °C, followed by PEG precipitation. The resulting phage pellet was resuspended in 10 mM PBS (pH 7.2) with 8.6 mg/ml BSA at a concentration of 3.3 nM. The antibody-conjugated phage were next reacted with high-binding polystyrene wells bound with rTNFα. Any bound phage were reacted with HRP-conjugated anti-M13 antibodies, followed by thorough washing. ABTS and H_2O_2 were then added to react with bound HRP. Control wells bound with no recombinant rTNFα and blocked with BSA were also tested simultaneously. As shown in Fig. 3A, only the rTNFα-bound well showed any colorimetric signal, indicating that (i) the Fzz8 phage could successfully bind the anti-rTNFα antibody and (ii) the antibody-conjugated phage could recognize the antigen rTNFα.

In addition to using HRP-conjugated anti-M13 antibodies for testing phage–antibody binding to the antigen, we also enlisted the use of SPR to determine the dissociation constant (K_D) values of phage–antibody versus antibody alone [38–40]. In the SPR assay, a carboxymethyl dextran-coated gold surface was used to inhibit nonspecific binding of bacteriophage to the gold chip surface. Next, rTNFα was immobilized onto the dextran-coated surfaces by carbodiimide chemistry. As shown in Fig. 3B, the SPR sensorgram showed that even though antibody-conjugated Fzz8 phage had a higher response unit (RU) due to having a mass 110 times that of the antibody, the K_D value of the complex to rTNFα was 270 ± 33 pM ($n = 3$), which was very close to the measured K_D value of the antibody alone (K_D = 298 ± 99 pM, $n = 3$). These results supported the continuing study of using phage–antibodies as biosensing platforms.

Next, to see whether the biotin-modified antibody–phage could enhance ELISA signals compared with biotinylated detection antibodies alone, we ran direct ELISAs using either reagent. Because of the noncovalent interactions between the ZZ domain on the phage and the antibodies, running indirect ELISAs caused nonspecific interactions between the phage–antibodies and the capture antibody surfaces, leading to large background signals. Current work is under way to overcome these challenges. In the direct ELISAs tested, the amounts of antibody, avidin–HRP, ABTS, and H_2O_2 were kept constant among all wells, whereas the concentrations of rTNFα absorbed to the polystyrene wells were varied. First, 50-μl solutions containing varying concentrations of rTNFα ranging from 40 to 0 nM in 10 mM PBS (pH 6.0) and BSA were absorbed for at least 16 h to 96-well high-binding polystyrene plates at 4 °C. After removing the rTNFα solutions and washing, the wells were blocked further by adding 300 μl of 10 mg/ml BSA in 10 mM PBS (pH 7.2) and shaking gently at room temperature. After 1 h, the

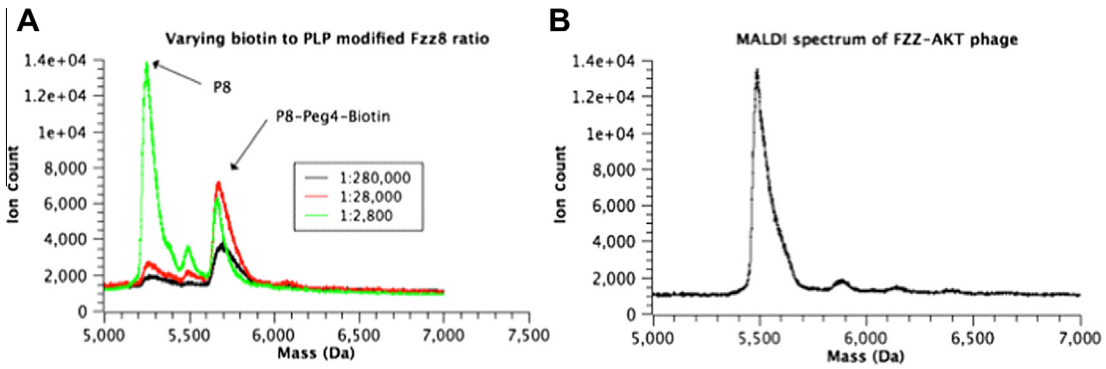

Fig.2. (A) MALDI–TOF spectrum of Fzz8 phage reacted first with 100 mM PLP and then different molar phage:alkoxyamine PEG–biotin ratios. (B) MALDI–TOF spectrum of Fzz–AKT phage.

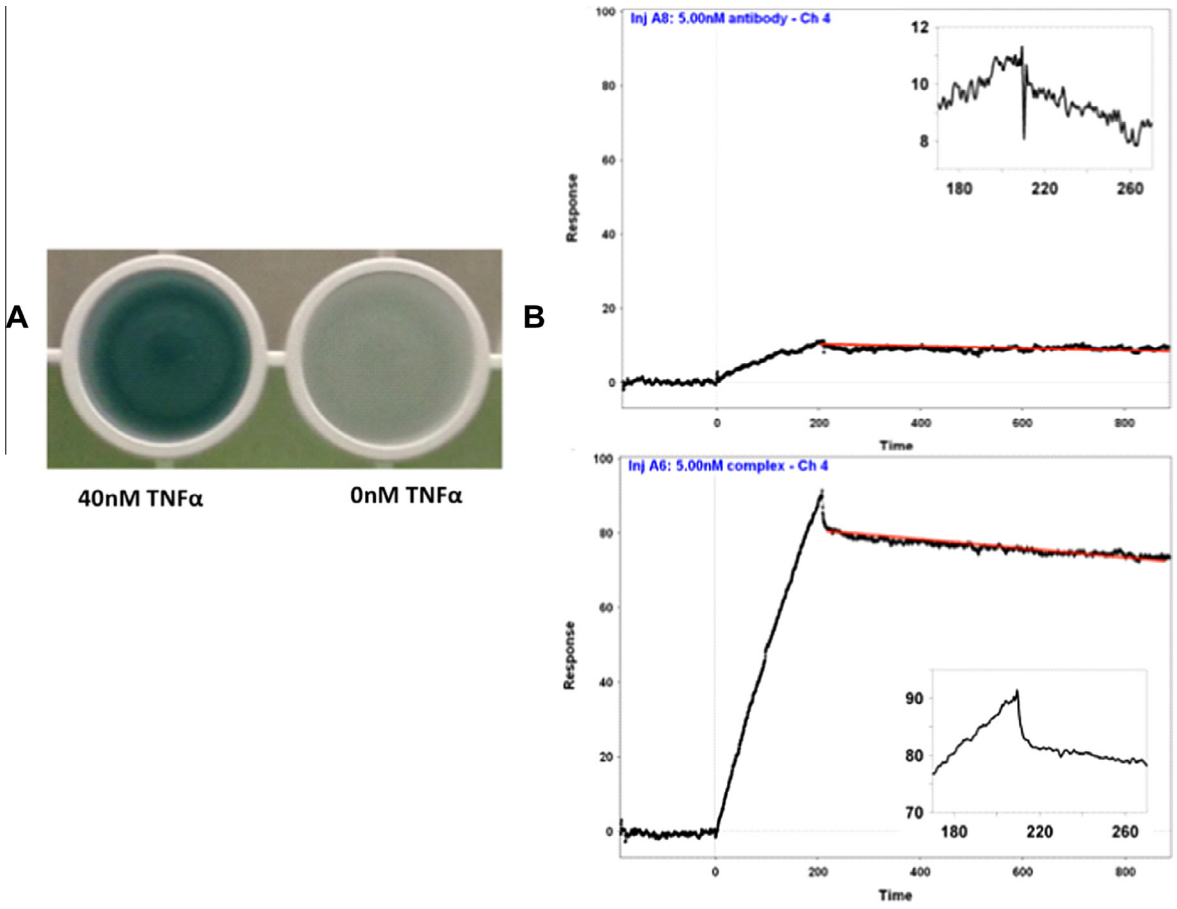

Fig.3. (A) Polystyrene wells coated with 40 or 0 nM TNFα and incubated with biotin-conjugated Fzz8 phage targeted with anti-TNFα antibody and detected with an HRP-conjugated anti-M13 antibody. Absorbance readings for the 40- and 0-nM TNFα wells were 1.776 and 0.156, respectively. (B) SPR sensorgram spectra comparing antibody–phage versus antibody alone. Association and dissociation curves of TNFα-immobilized chip to anti-human TNFα antibody (top) and anti-human TNFα antibody-conjugated Fzz8 phage (bottom) are shown. The insets show an expanded time–response curve at approximately 200 min.

blocking solutions were removed and 100 µl of either the biotinylated phage–antibody or biotinylated antibody was added to each well for 1.5 h, after which the wells were washed three times by adding 300 µl of 0.01% PBST to each for 5 min. Next, 100 µl of avidin–HRP (Affymetrix eBioscience) in 10 mg/ml BSA was added to react with any bound biotin groups for 1 h, followed by washing as above and adding 200 µl of an ABTS/H_2O_2 mixture in 50 mM citrate at pH 4.0. After 10 min, the solutions were removed and the absorbance was measured at 418 nm. The average signals and standard deviations of three assay replicates are shown in Fig. 4. The use of the biotinylated phage antibodies clearly showed a marked increase in ELISA signal over that using biotinylated antibodies alone. In these direct ELISA studies, as the concentration of TNFα decreased from 40 nM to 625 pM, the anti-rTNFα antibody-based assay gave a limit of detection of 10 nM, whereas the phage–antibody-based assay showed a limit of detection at 2.5 nM. From this result, it was observed that using phage–antibodies enabled a significantly lower detection limit than the antibody-based ELISA. To increase the overall sensitivities of the assays, future studies will work both on using phage that genetically express the variable regions of antibodies as fusions to the p3 proteins [41–43] and on introducing covalent bonds between the antibodies and ZZ domains. Further gains in sensitivity may be achieved by expressing avidin-binding peptides (histidine-proline-glutamine) [44–46] on the p8 proteins to alleviate the need for chemical conjugation that can in principle modify the p3 proteins and also requires purchasing alkoxyamine biotin compounds.

Conclusion

The early detection of disease has been shown to greatly increase the effectiveness of treatment and increase the overall health and well-being of patients. Although ELISA is currently the most common method for detecting biomarkers, its limited detection sensitivity caused by insufficient and widely varying K_D values between antibodies and antigens has hindered its potential for sensing low antigen concentrations in biological media. In this work, we have empowered conventional ELISA techniques by engineering and using modified filamentous bacteriophage as sensing platforms. First, the phage were genetically engineered to bind to the Fc portion of detection antibodies, allowing them to bind specific antigens with higher specificity than what can be achieved by using short peptide sequences. The genome of the viruses was also designed to enable cloning short peptide sequences onto the approximately 4000 p8 proteins. Next, multiple biotin groups were attached to the viruses by specifically modifying the N termini of the coat proteins with ketone groups, which could then be reacted with alkoxyamine-modified biotin. The chemical modification of the coat proteins with biotin groups did not seem to prevent the p3 proteins from interacting with the detection antibodies. Although previous research has shown that AKT sequences can increase the oxidation rate of the N terminus by PLP, when all p8 proteins were modified with the AKT sequences, the phage became insoluble, hindering further use. Lastly, the biotinylated phage–antibodies were tested in direct ELISAs against antibodies alone

A

TNFα conc.	40nM	20nM	10nM	5nM	2.5nM	1.25nM	625pM
Fzz8-biotin	5.561 ± 0.333	4.452 ± 0.322	2.54 ± 0.17	1.677 ± 0.452	0.679 ± 0.118	0.269 ± 0.13	0.136 ± 0.146
Biotinylated Antibody	2.596 ± 0.461	1.827 ± 0.375	1.076 ± 0.287	0.425 ± 0.162	0.129 ± 0.018	0.044 ± 0.014	0.016 ± 0.008

B

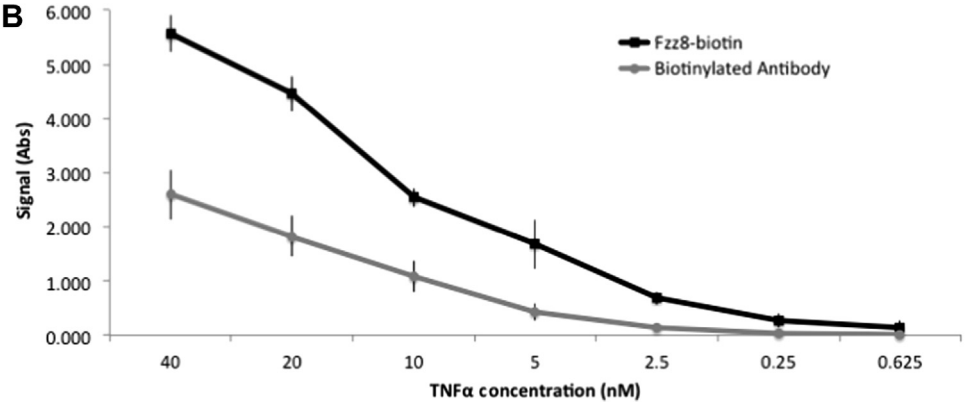

Fig.4. (A) Average ELISA signals over background and standard deviations for three replicate assays with varying concentrations of rTNFα detected with Fzz8 phage reacted with 100 mM PLP and a 1:2800 molar excess of alkoxyamine and then conjugated with anti-rTNFα antibody. Signals are compared with assays done with a biotinylated version of the same antibody clone. (B) Bar graph of the data in panel A.

to show that the phage–antibodies could yield on average a 3- to 4-fold increase in signal at all antigen concentrations. Future work will include using capture antibodies to improve antigen binding to surfaces as well as genetically expressing avidin-binding peptides on the p8 proteins to avoid the need for chemical conjugation strategies.

Acknowledgments

We thank Itai Benhar for providing us with the phage containing the fUSE5-ZZ genome. We also thank George P. Smith for providing us with K91BlueKan cells and instructions for their use. This work was supported by a National Science Foundation (NSF) Career Award (DMR-1056808). We thank Andrew Goodwin for help in editing the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2014.10.006.

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