Methods xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Methods

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

Review Article

Use of micro-emulsion technology for the directed evolution of antibodies

Diane L. Buhr [a], Felicity E. Acca [a], Erika G. Holland [a], Katie Johnson [a], Gail M. Maksymiuk [a], Ada Vaill [a], Brian K. Kay [b], David A. Weitz [c], Michael P. Weiner [a], Margaret M. Kiss [a,*]

[a] AxioMx, Inc., 688 E. Main St., Branford, CT 06405, United States
[b] University of Illinois at Chicago, 845 West Taylor Street, Chicago, IL 60607, United States
[c] Harvard University, 29 Oxford St., Cambridge, MA 02138, United States

ARTICLE INFO

Article history:
Available online xxxx
Communicated by Brian Kay

Keywords:
Phage display
Phage ESCape
Emulsions
Antibody engineering
Affinity maturation
Antibody evolution

ABSTRACT

Affinity reagents, such as antibodies, are needed to study protein expression patterns, sub-cellular localization, and post-translational modifications in complex mixtures and tissues. Phage Emulsion, Secretion, and Capture (ESCape) is a novel micro-emulsion technology that utilizes water-in-oil (W/O) emulsions for the identification and isolation of cells secreting phage particles that display desirable antibodies. Using this method, a large library of antibody-displaying phage will bind to beads in individual compartments. Rather than using biopanning on a large mixed population, phage micro-emulsion technology allows us to individually query clonal populations of amplified phage against the antigen. The use of emulsions to generate microdroplets has the promise of accelerating phage selection experiments by permitting fine discrimination of kinetic parameters for binding to targets. In this study, we demonstrate the ability of phage micro-emulsion technology to distinguish two scFvs with a 300-fold difference in binding affinities (100 nM and 300 pM, respectively). In addition, we describe the application of phage micro-emulsion technology for the selection of scFvs that are resistant to elevated temperatures.

1. Introduction

Affinity reagents, such as antibodies, are commonly used to study protein expression patterns, sub-cellular localization, and post-translational modifications in complex mixtures and tissues. Often the expression or localization of a particular protein changes with genetic mutations or disease making affinity reagents crucial to many research projects. Previously, production of an antibody to a particular protein required immunization of animals (i.e., mice, rabbits) and recovery of antibodies from their sera. This is in contrast to newer methods that involve the generation of large libraries of recombinant antibodies and the in vitro screening of these libraries for the desired binding properties.

Recombinant affinity reagents have many attractive attributes compared to polyclonal antisera and monoclonal antibodies derived from hybridomas. They are renewable through overexpression in the appropriate heterologous host, they are easily stored and transferred as DNA, and they can be genetically engineered as fusions to various enzymes, fluorescent proteins, epitope tags, or modified to have single dyes or biotin at one position in 100% of the molecules. Through the use of appropriate counter-selection steps, it is possible to guide recognition of new epitopes or discriminate between certain molecular features of a target (i.e., conformation, post-translational modification). Thus, there has been a great deal of recent interest in tapping the potential of recombinant affinity reagents for characterizing the human proteome [1–5], and the early comparisons to traditional antibodies are promising [6,7]. Furthermore, it takes less time to identify a recombinant affinity reagent to a target than to generate a rabbit polyclonal or mouse monoclonal antibody [8], and one can do some experiments that are impossible with traditional antibodies, such as evolve higher affinities [9,10], express inside cells and perturb targets [11,12], mislocalize targets in embryos [13], create biosensors [14], and incorporate unnatural amino acids for chemical derivatization [15], which allows new labeling, capturing and immobilization approaches.

Phage display is the most widely used method for selecting binding molecules from recombinant antibody libraries [16–19]. In this technique, large numbers ($>10^{10}$) of different antibodies are displayed on the surface of filamentous phage and are physically linked to their coding DNA which resides within the phage virion. Specific binders are selected by enrichment of binding phage during cycles of biopanning and propagation. Commonly, a library of single-chain antibody (scFv) fragments is displayed on the surface of M13 bacteriophage as a genetic fusion to the gpIII protein and used in 'biopanning' procedures against a protein of interest [20–22]. Since no immunization steps are required, comprehensive phage-antibody libraries permit targeting of antigens in vitro which are known to be toxic or possess low antigenicity

* Corresponding author. Fax: +1 203 506 6865.
E-mail addresses: kiss.margaret@gmail.com, mkiss@illumina.com (M.M. Kiss).

1046-2023/$ - see front matter © 2012 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.ymeth.2012.07.007

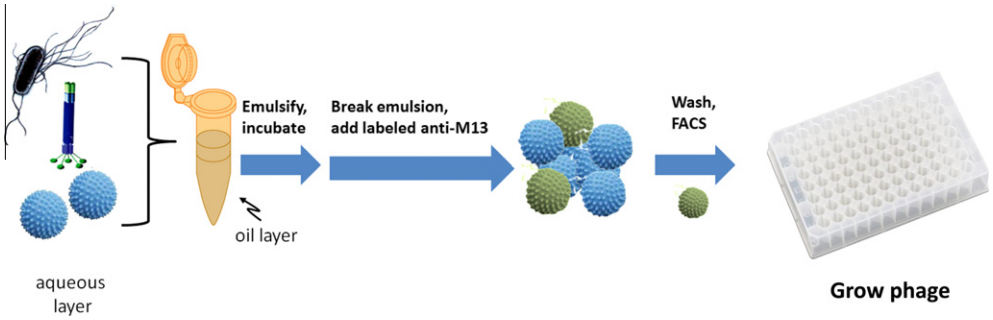


Fig. 1. Workflow for phage micro-emulsion technology. Prepared target (peptide or protein) is coupled to magnetic beads, and an emulsion is made of the beads and a phage display library transfected into *E. coli*. After overnight incubation, the emulsion is broken, the beads washed, and a FITC-labeled anti-M13 antibody added. The beads are then sorted by FACS.

in vivo. In addition, the DNA encoding the antibody is accessible in phage display, providing not only the antibody sequence, but facilitating cloning and engineering of the protein in many ways. High affinity phage antibodies have been selected against numerous cellular proteins and small molecules [23].

We have previously described a novel approach that utilizes water-in-oil (W/O) emulsions for the identification and isolation of cells secreting phage particles that display desirable scFvs [24]. Emulsions are heterogeneous systems of two immiscible liquid phases with one of the phases dispersed in the other as droplets of microscopic or colloidal size. The water-in-oil emulsion has been applied to the in vitro compartmentalization of biochemical reactions, including selecting catalysts of DNA methyltransferases [25] and for the directed evolution of Taq DNA polymerase [26]. Using the method we developed [24], a large library of antibody-displaying phage will bind to beads in individual compartments (Fig. 1). Rather than using biopanning on a large mixed population, this method allows us to individually query clonal populations of amplified phage against the antigen. Depending on droplet size, a 100 μl reaction mix dispersed in 0.5 ml oil can form 2×10^{10} aqueous compartments suitable for cell growth. The use of emulsions to generate microdroplets has the promise of accelerating phage selection experiments by permitting fine discrimination of kinetic parameters for binding to targets. Affinity discrimination by flow cytometry has already been demonstrated for antibodies displayed on the surface of yeast [27]. In this study, we demonstrate the application of phage micro-emulsion technology for directed evolution experiments.

2. Materials and methods

2.1. Error-prone PCR Library Construction

The synthetic anti-MS2 single-chain variable fragment (scFv), AFX704, was generated by CDR-grafting of an antibody provided by James Carney at Aberdeen Proving Ground and amplified by error prone PCR. The PCR reaction contained 7 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3), 0.01% gelatin, 0.2 mM dGTP, 0.2 mM dATP, 1 mM dCTP, 1 mM dTTP, 0.5 mM $MnCl_2$, 30 pmol of each primer, 20 femtomol of input DNA, and 5 units of Taq polymerase. The reactions were incubated for 30 cycles of 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 1 min. No hot start procedure or prolonged extension time at 72 °C was used. The PCR product was digested with Hind III and Sal I and ligated into the pAP-III_6 vector for phage display [28]. The frequency of mutations in the scFv was approximately 2% and the library size was estimated to be approximately 1.7×10^7. Phage displaying the scFvs were prepared by growth of aliquots of bacteria from the library and superinfection with a helper phage, KM13 [29].

2.2. Phage biopanning

Phage displaying the scFvs were prepared by growth of aliquots of bacteria from the error prone PCR library and superinfection with helper phage. Immunotubes (Nunc Maxisorp) were coated with MS2 coat protein (10 μg/ml) overnight at 4 °C. After washing with phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g $NaHPO_4$, and 0.24 g $KHPO_4$ per liter), tubes were blocked with 2% nonfat dry milk in PBS (MPBS). Phage (around 1×10^{13} transducing units) from the library was added to the tubes in 4 ml MPBS and incubated for 2 h at room temperature. After rigorous washing with PBS containing 0.1% (v/v) Tween 20, bound phage were eluted by addition of 0.5 ml of trypsin (1 mg/ml; Sigma–Aldrich, St. Louis, MO) in PBS. One half of the eluate was used for infection of 1.75 ml aliquots of exponentially growing *Escherichia* coli TG1. Infection was carried out for 30 min. at 37 °C without shaking. Serial dilutions of bacteria were then plated to 2xYT (16 g tryptone, 10 g yeast extract, 5 g NaCl per liter) agar containing ampicillin for overnight growth at 30 °C to determine the number of phage recovered from selection. The remaining infected bacteria were centrifuged briefly, resuspended in 250 μl of 2×YT media and spread onto selective 2×YT plates containing ampicillin. The following day, colonies were scraped into 7 ml of 2×YT containing ampicillin. Glycerol was added to a final concentration of 15% (v/v) and the suspension stored at −80 °C.

2.3. Isolation of scFvs with improved thermal stability by phage micro-emulsion technology

Tosyl-activated magnetic beads (Dynabeads M-280; Invitrogen, Carlsbad, CA) were coated with the recombinant coat protein of the MS2 bacteriophage [30] to saturation. A secondary glycerol stock (500 μl) of the enriched library was added to 50 ml 2×YT containing ampicillin and grown at 37 °C until absorbance at 600 nm reached 0.4. A 2 ml sample was withdrawn and 1×10^{10} pfu of KM13 helper phage were added. The samples were held at 37 °C without shaking for 30 min to allow phage infection and then centrifuged at 3000× *g* for 10 min. The bacteria were resuspended in 20 ml (and subsequently diluted 1:10) of 2×YT containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml). MS2-coated beads (30 μl; 7×10^8 beads/ml) were mixed with 2 ml cultures, and 6 ml encapsulation oil (perfluorocarbon oil containing fluorinated PEG surfactant) [31] was added. The mixture was vortexed at top speed for 20 s to generate an emulsion. Average droplet size was estimated to be 100 μm using a hemocytometer. The emulsions were incubated in a 30 °C shaker overnight for phage production and capture on the beads. After overnight incubation, the emulsions were broken, the top (aqueous) layer containing beads was removed to a new tube, and the beads were captured on a magnetic separator.

The beads were washed 3× with 1 ml PBS containing 0.1% (v/v) Tween 20. To isolate scFvs with high thermal stability, the phage-coated beads were heated to 70 °C for 15 min, then cooled on ice for 10 min in the presence of a 10-fold molar excess of MS2 coat protein. The beads were again washed 5 × with 1 ml PBS-Tween and FITC-conjugated anti-M13 antibody (Acris Antibodies, San Diego, CA) was added at a 1:200 dilution in PBS containing 3% BSA. After a 1 h incubation with FITC-conjugated anti-M13 antibody, the beads were again washed 3× with 1 ml PBS containing 0.1% (v/v) Tween 20 and resuspended in 500 µl PBS. The beads were analyzed and sorted using a FACS Aria III (BD Biosciences) into individual wells of a 96-well plate containing 50 µl trypsin (1 mg/ml) in PBS. The phage were recovered by infection of early log-phase *E. coli* TG1 cells (OD_{600} = 0.4; 250 µl/well) for 30 min at 37 °C without shaking. Media, 2×YT containing ampicillin, was added (1 ml/well) to select for infected cells, and the cultures were grown overnight at 37 °C with shaking. 2 µl samples were transferred to 200 µl of medium in fresh deep-well blocks and phage were produced for monoclonal phage enzyme-linked immunosorbent assay (ELISA) as below.

2.4. Monoclonal phage enzyme-linked immunosorbent assay (ELISA)

Bacteria were grown overnight at 37 °C and 2 µl samples transferred to 200 µl of medium in fresh blocks. Once the OD_{600} reached 0.4, 25 µl aliquots of KM13 helper phage, each containing approximately 1×10^9 virus particles, were added to each well to initiate superinfection. After a further 1 h growth, the plates were centrifuged at 1800× *g* for 10 min and the supernatant aspirated from each well. Individual cell pellets were resuspended in 200 µl of 2×YT containing ampicillin and kanamycin and the plates incubated at 30 °C with shaking overnight to allow viral replication. The plates were then centrifuged at 1800× *g* for 10 min and supernatants were transferred directly to an ELISA plate that had been coated with the antigen. For coating of the ELISA plates, MS2 coat protein or neutravidin (2 µg/ml in PBS; 100 µl/well) was added and plates were incubated overnight at 4 °C. The wells were washed 3× with PBS (250 µl/well) and blocked for 1 h with 2% nonfat dry milk in PBS (MPBS). The wells were again washed 3× with PBS, and phage-containing supernatants were then added for 1 h at room temperature. For the thermal stability experiments, phage-containing supernatants were heated to 70 °C for 30 min and quenched on ice prior to incubation with the antigen-coated wells. Binding of the phage was detected using a monoclonal anti-M13 Ab, conjugated to horseradish peroxidase (HRP; GE Healthcare, Piscataway, NJ). Unique clones could be identified by DNA sequencing or genotyping using a BioPlex Instrument (BioRad, Hercules, CA).

2.5. Protein expression

The scFvs were expressed from the AP-scFv vector [28] in Mach1 cells (Invitrogen, Carlsbad, CA) in low phosphate media (per liter: 3.57 g ammonium sulfate, 0.71 g sodium citrate dehydrate, 1.07 g potassium chloride, 5.36 g yeast extract, 5.36 g Sigma Hy-Case SF casein hydrolysate, 7 ml 1 M $MgSO_4$, 14 ml 1 M glucose, pH 7.3 adjusted with KOH) supplemented with ampicillin (100 µg/ml) for 20 h at 30 °C. Baffled flasks at a maximum 20% of flask volume were used to ensure good aeration (necessary to achieve phosphate depletion). The cultures were centrifuged and the pellets frozen at −80 °C.

2.6. Protein purification

Cell pellets were resuspended in BugBuster (EMD Chemicals, Gibbstown, NJ) supplemented with 5 µg/ml Benzonase (EMD Chemicals, Gibbstown, NJ), 1 mM PMSF, and protease inhibitor cocktail. The resuspended pellets were incubated with gentle shaking at room temperature for 20 min and then sonicated 3× for 30 s each (with a 1 min pause between each pulse). Lysates were clarified by centrifugation at 15,000 rpm in a Sorvall SS34 rotor for 30 min at 4 °C. The scFvs were purified from the cleared lysates on HisPurTM Cobalt resin (Thermo Scientific, Rockford, IL). After binding the scFv proteins, the columns were washed with 50 mM Tris buffer pH 7.4 containing 250 mM sodium chloride, 10 mM imidazole, and 1 mM PMSF. The scFvs were eluted with 25 mM Tris, pH 7.4 containing 250 mM sodium chloride, 500 mM imidazole, and 1 mM PMSF. Eluted protein was collected and analyzed by SDS–PAGE. For purification of AFX719, the eluted protein from the cobalt column was subjected to ion exchange chromatography using a HiTrap SP HP column (GE Healthcare, Piscataway, NJ). The column was equilibrated with 25 mM sodium phosphate, pH 7.0. The protein was bound to the column and eluted with a gradient of 0 to 300 mM NaCl in 25 mM sodium phosphate, pH 7.0. HiPrep 26/10 Desalting columns (GE Healthcare, Piscataway, NJ) were used for buffer exchange to PBS.

2.7. Heat-resistance ELISA

ELISA was conducted on control and heated scFvs to determine the amount of binding following heating. The scFvs at 1.0 mg/ml in PBS were exposed to 70 °C for increasing amounts of time up to 1 h and then allowed to re-equilibrate to 4 °C on ice. ELISA plates were coated with 2 µg/ml MS2 coat protein followed by incubation for 1 h with a dilution series of the scFv. Binding was detected using anti-FLAG-HRP (Abcam). To test the stability of MS2, MS2-coated beads (7×10^8 beads/ml) were heated in PBS-Tween (0.1%) to 70 °C for 15 min and then allowed to re-equilibrate to 4 °C on ice. The beads were incubated with AFX704 (5 µg/ml) for 1 h. Binding was detected using anti-FLAG-HRP (Abcam).

2.8. Surface plasmon resonance (SPR) analysis

For kinetic analysis, MS2 coat protein was coupled to a GLC sensor chip in a ProteOn system (Bio Rad, Hercules, CA) [32]. The scFvs were flowed as the analyte at five concentration points, one buffer blank, and repeated 5×. Contact time, flow rate, and dissociation time of the analyte was adjusted as needed to achieve minimum residuals of fit and analyte binding at equilibrium. Data was analyzed by on board software or exported to Scrubber-Pro for analysis. The standard method of analysis utilized a 1:1 Langmuir model.

2.9. Affinity discrimination by fluorescence activated cell sorting (FACS)

The scFvs specific for the MS2 coat protein were generated by phage display affinity maturation of an antibody provided by James Carney at Aberdeen Proving Ground. Cells expressing phage displaying the scFvs were incubated with MS2-coated magnetic beads (5 µl; 7×10^8 beads/ml) and emulsified with oil as above. After overnight incubation with shaking at 30 °C, the emulsions were broken and the beads were washed 3× with PBS-Tween (0.1%) followed by once with PBS-Tween containing a 10-fold molar excess of free MS2 coat protein (20 µg) for 15 min at room temperature. The beads were again washed 5× with PBS-Tween and incubated with anti-MS2-FITC (1:200 dilution) in PBS-Tween containing 3% BSA. The beads were washed 3× with PBS-Tween, resuspended in PBS, and analyzed by FACS as above.

3. Results

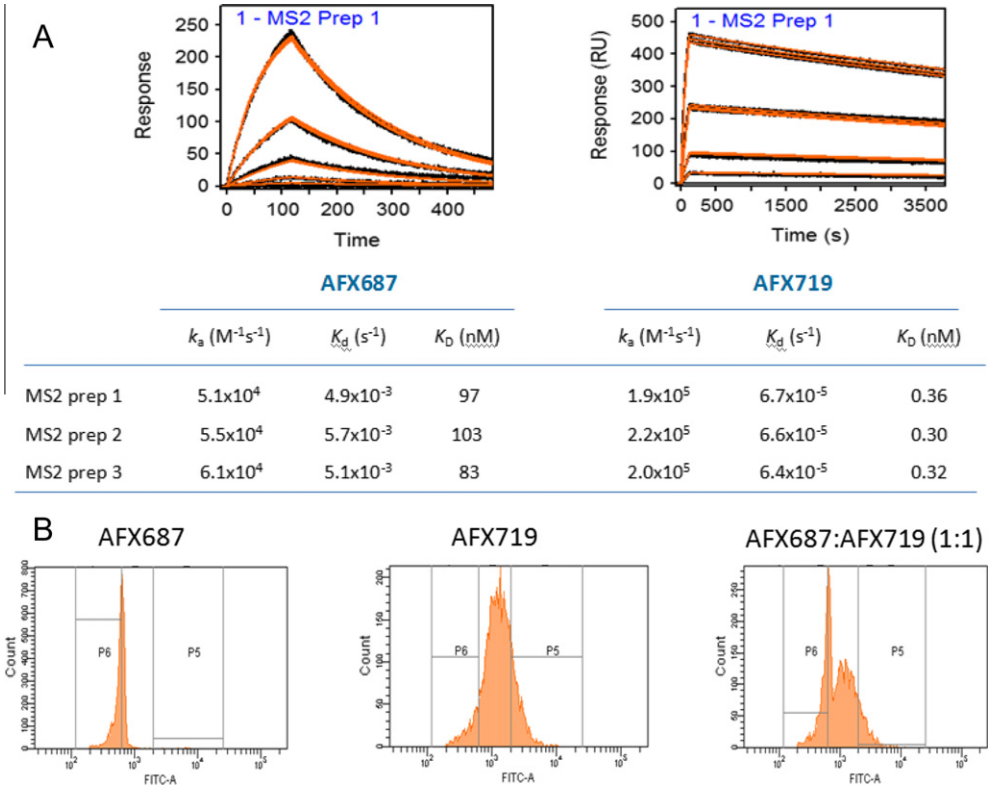
3.1. Phage micro-emulsion technology

We have previously described a novel approach that utilizes water-in-oil (W/O) emulsions for the identification and isolation

of cells secreting phage particles that display desirable scFvs [24]. *E. coli* cells, infected with a library of phage M13 encoding a population of scFvs, are compartmentalized with antigen-coated beads in a water-in-oil emulsion to give, on average, 1–10 beads and 1–10 M13 infected-bacteria per compartment. Consequently, in each compartment, multiple copies of the recombinant phage are produced, some of which may bind to the target-coated bead. As *E. coli* does not secrete proteases, we find that the target generally remains intact on the beads after an overnight incubation in LB at 30 °C. The emulsion is broken and the beads, along with any bound phage, are isolated. The beads are washed and incubated with FITC-conjugated anti-M13 antibody, and FACS enriches the fluorescing beads, together with the phage particles attached to them. Beads with fluorescence above a threshold value are then sorted in individual wells of a microtiter plate for downstream analysis. The process flow of phage micro-emulsion technology is outlined in Fig. 1. In the present study, we explore the potential of this technology for directed evolution experiments, including selection for tighter binding as well as resistance to elevated temperatures (i.e., thermal stability).

3.2. Affinity discrimination of anti-MS2 scFvs

To test the hypothesis that phage micro-emulsion technology could be used to discriminate phage displaying scFvs with different binding affinities, we produced phage displaying either of two scFvs that each recognize the recombinant coat protein of the RNA bacteriophage, MS2. Kinetic analysis of AFX687 by SPR indicates that it has a binding constant (K_D) of approximately 100 nM. An affinity-matured variant (AFX719) with single amino acid changes in two of the light chain CDRs has a binding affinity of 300 pM (Fig. 2A). The approximately 300-fold difference in affinities between AFX687 and AFX719 is largely due to differences in their off-rates (Fig. 2A). Cells expressing phage displaying AFX687 or AFX719 were emulsified with MS2-coated beads and grown separately or mixed at a 1:1 ratio. After overnight incubation at 30 °C for phage production and capture on the beads, the emulsions were broken and beads were washed. Washes were performed in the presence of an excess of free MS2 coat protein so that any displaced phage would not be re-captured on the beads. FITC-conjugated anti-M13 antibody was then added to label the phage-bound beads, and the beads were analyzed by FACS. The majority of the beads showed fluorescence above the background level, indicating that phage had bound, but the mean fluorescence intensity of the beads coated with AFX719-displaying phage was higher than the mean fluorescence intensity of the beads coated with AFX687-displaying phage. When the two populations were mixed at a 1:1 ratio, the beads with bound phage displaying the higher affinity scFv, AFX719, could be sorted from the beads with bound phage displaying AFX687. This data suggests that phage micro-emulsion technology is sensitive enough to discriminate high affinity binders from weaker binders. By varying the stringency of the wash conditions and using a less sensitive detection antibody, such as anti-FLAG, it may be possible to expand the dynamic range to discriminate antibodies with even smaller differences in affinity.



	AFX687			AFX719		
	k_a ($M^{-1}s^{-1}$)	K_d (s^{-1})	K_D (nM)	k_a ($M^{-1}s^{-1}$)	K_d (s^{-1})	K_D (nM)
MS2 prep 1	$5.1x10^4$	$4.9x10^{-3}$	97	$1.9x10^5$	$6.7x10^{-5}$	0.36
MS2 prep 2	$5.5x10^4$	$5.7x10^{-3}$	103	$2.2x10^5$	$6.6x10^{-5}$	0.30
MS2 prep 3	$6.1x10^4$	$5.1x10^{-3}$	83	$2.0x10^5$	$6.4x10^{-5}$	0.32

Fig. 2. Discrimination of scFv binding affinities by phage micro-emulsion technology. (A) Kinetic analysis by SPR. MS2 coat protein was coupled to a GLC sensor chip in a ProteOn system. The scFvs were flowed as the analyte at five concentration points, one buffer blank, and repeated 5×. Contact time, flow rate, and dissociation time of the analyte was adjusted as needed to achieve minimum residuals of fit and analyte binding at equilibrium. Data was analyzed by on board software or exported to Scrubber-Pro for analysis. The standard method of analysis utilized a 1:1 Langmuir model. (B) Phage micro-emulsion technology FACS profiles. Two different phage clones were grown in microdroplets, either separately (left, middle) or together (right), with MS2-coated beads. AFX719 differs by single amino acid substitutions in two of the light chain CDRs and has a higher affinity for MS2 coat protein than AFX687. The beads were washed 3× quickly with PBST (0.1% Tween), followed by one wash with PBST and a ten-fold molar excess free of target for 15 min at room temperature. Beads were again washed five x with PBST and incubated with anti-MS2-FITC (1:200 dilution) in PBST containing 3% BSA. The beads were washed 3× with PBST, resuspended in PBS, and analyzed by FACS.

Please cite this article in press as: D.L. Buhr et al., Methods (2012), http://dx.doi.org/10.1016/j.ymeth.2012.07.007

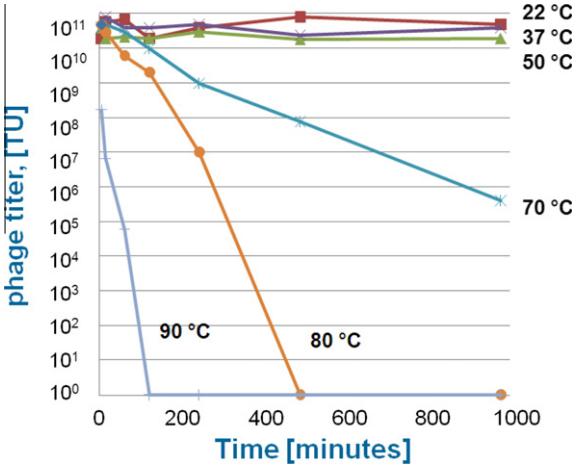


Fig. 3. Test of thermostability of M13 vector. Phage (100 µl of scFv 32H08 phage stock at 7.2×10^{10} transducing units per ml) were tested for transducing ability after incubation in a 0.4 ml plastic tube at the following temperature points: 5, 22, 37, 50, 70, 80, and 90 °C (50–90 °C points done in a thermo cycler with heated lid) and at the following time points 5, 15, 60, 120, 240, 480, and 960 min.

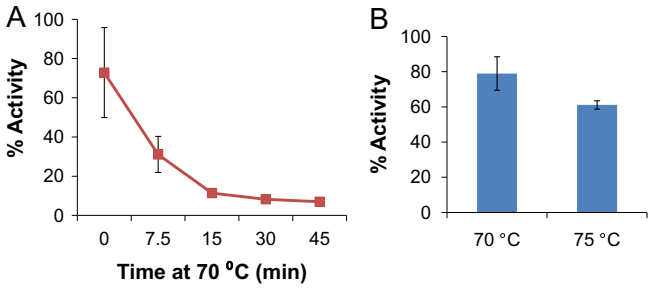


Fig. 4. Thermal resistance of AFX704 and MS2 coat protein. (A) Heat-resistance ELISA. ELISA was conducted on the control (unheated) and heated scFv, AFX704, to determine the amount of binding following heat exposure. AFX704 at 1.0 mg/ml in PBS was exposed to 70 °C for increasing amounts of time up to 1 h and then allowed to re-equilibrate to 4 °C on ice. ELISA plates were coated with 2 µg/ml MS2 coat protein followed by incubation for 1 h with a dilution series of AFX704. Binding was detected using anti-FLAG-HRP. Percent activity relative to the unheated scFv is plotted versus heat exposure time. (B) ELISA was conducted against MS2-coated beads before and after heat exposure. MS2-coated beads were exposed to 70 °C or 75 °C for 15 min and then allowed to re-equilibrate to 4 °C on ice. The beads were incubated with AFX704 (5 µg/ml) for 1 h. Binding was detected using anti-FLAG-HRP. Percent activity relative to the unheated scFv is plotted versus temperature.

3.3. Phage resistance to high temperature

To determine whether phage micro-emulsion technology could also be useful for selecting phage displaying scFvs with higher thermal stability, we first investigated the resistance of the M13 bacteriophage to elevated temperatures. A phage stock was exposed to temperatures ranging from 22 to 90 °C for increasing amounts of time up to 16 h. After heating, the phage were tested for transducing ability by selection of infected cells on ampicillin-containing plates. The number of transducing phage at each temperature is plotted versus time in Fig. 3. The phage are completely resistant to heating at 50 °C, and no significant loss in phage infectivity is observed by heating to 70 °C for an hour (Fig. 3). In fact, our data indicates that the phage remains infective after 10 h at 70 °C, and after several hours post incubation at 80 °C.

3.4. Identification of scFvs with improved thermal stability by phage micro-emulsion technology

To derive antibodies with resistance to elevated temperature, we generated a phage display library by error-prone PCR mutagenesis of an anti-MS2 scFv. The starting scFv (AFX704) has a melting temperature of 72 °C (data not shown) but shows substantial loss of activity in an ELISA against MS2 coat protein after exposure to 70 °C for 15 min or more (Fig. 4A). MS2 coat protein, however, retains functionality on the beads after heating to 70 °C for at least 15 min (Fig. 4B). The AFX704 mutagenized library was pre-enriched by one round of biopanning against immobilized MS2 coat protein to eliminate clones that had lost the ability to recognize MS2. The enriched library was subsequently screened by phage micro-emulsion technology using magnetic beads coated with MS2 coat protein. The emulsions were estimated to contain approximately 1–10 cells and 5 beads per droplet in a droplet volume of approximately 520 pl.

After overnight incubation of the emulsions at 30 °C for phage production and capture on the beads, the emulsions were broken and the beads were washed. The phage-bound beads were then heated to 70 °C for 15 min and cooled on ice. An excess of free MS2 coat protein was immediately added to the beads on ice in order to prevent re-binding to the beads of phage displaying scFvs that became denatured and then refolded upon cooling. The beads were again washed, incubated with FITC-labeled anti-M13 antibody, and analyzed by FACS. The FACS profile from the heated library beads is shown in Fig. 5A. The beads with fluorescence above background were sorted and collected in individual wells of a microtiter plate. The sorted phage were propagated and analyzed by ELISA against MS2 coat protein or the control protein, neutravidin, both before and after heating at 70 °C for 30 min. 73% of the clones selected by phage micro-emulsion technology showed greater than 50% activity in the phage ELISA after heat exposure (Fig. 5C), whereas phage displaying the original scFv, AFX704, showed only 30% activity (Fig. 5B). Four phage clones showed no loss of activity after 30 min of heat exposure.

4. Discussion

We believe that phage micro-emulsion technology provides numerous, significant advantages over current phage-display methodologies. For one, by keeping all of the phage copies in a single droplet, the signal of each clone is amplified. Individual, infected bacterial cells secrete phage particles into the microdroplets, which in turn have an opportunity to bind to the target-coated beads. These phage particles are then readily detected with a fluorescent antibody against the major coat protein (i.e., pVIII), of which there are 2,700 copies per virus particle. Second, the numbers of rounds of screening can be reduced to only one or two. Reducing the number of rounds of screening not only saves time, but it also eliminates the loss of diversity when one clone is amplified at the expense of others. In a typical phage display method, it is difficult to recover rare clones due to absorptive losses, and there is always clonal bias (and subsequent amplification) toward the strongest interactors. Because phage micro-emulsion technology amplifies the signals within a compartment, strong and weak interactors are no longer in competition for binding the target. For directed evolution of proteins, specifically in the affinity maturation of antibodies, reliable recovery of all improved rare mutants from a library is critical to rapid improvement.

Another clear advantage of phage micro-emulsion technology is that the technique can permit discrimination between kinetic properties of binding a target in solution (i.e., on-rates, off-rates, dissociation constants, etc.). Generally, this is not the case in the simplest phage-display screening format, where the target is immobilized on the bottom of a microtiter plate well, and one isolates weak and strong binders together. Using micro-emulsion technology we can apply quantitative flow cytometric analysis to

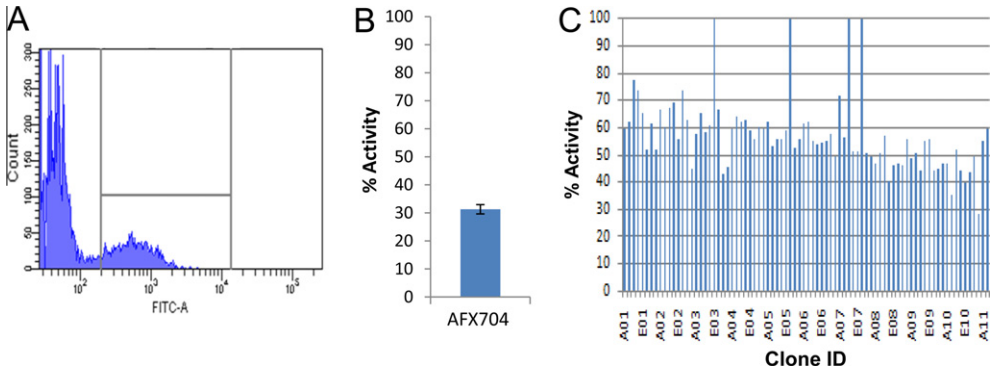


Fig. 5. Selection for improved thermal stability by phage micro-emulsion technology. (A) FACS profile. The AFX704 randomized library, enriched by a single round of biopanning against immobilized MS2 coat protein, was emulsified with MS2-coated beads and grown overnight for phage production and capture on the beads. The emulsions were subsequently broken, and the phage-coated beads were washed and then heated to 70 °C for 15 min. After heat exposure, the beads were cooled on ice in the presence of an excess of free MS2 coat protein. The beads were washed again, anti-M13-FITC was added, and the beads were analyzed by FACS. (B) ELISA was conducted on control (unheated) and heated phage displaying AFX704 to determine the amount of binding following heating. Phage-containing culture supernatant was exposed to 70 °C for 1 h and then allowed to re-equilibrate to 4 °C on ice. ELISA plates were coated with 2 μg/ml MS2 coat protein followed by incubation for 1 h with the unheated or heated phage. Binding was detected using HRP-conjugated anti-M13 (gp VIII). Percent activity relative to the unheated scFv is plotted. (C) ELISA was conducted on control and heated phage clones as in (B) above. Percent activity relative to the unheated scFv is plotted for each of the selected clones.

phage display, enabling the same kind of fine discrimination of kinetic parameters as has been achieved with yeast-display [27]. We show here that we can use phage micro-emulsion technology to distinguish two scFvs with a 300-fold difference in binding affinities (100 nM and 300 pM, respectively). By varying the stringency of the wash conditions and using a less sensitive detection antibody, such as anti-FLAG, it may be possible to expand the dynamic range to discriminate antibodies with even smaller differences in affinity. We can also incorporate a droplet generation device to control the uniformity of the droplets and the corresponding phage titers, since differences in phage expression could influence the signal on beads.

In addition, we show that phage micro-emulsion technology can be used to select scFvs that are resistant to elevated temperatures. This application relies on the thermal resistance of the target protein or peptide. Our starting antibody had a melting temperature of 72 °C, which required us to heat the phage-coated beads to 70 °C. For the majority of scFvs, which have melting temperatures between 50 and 60 °C, less extreme temperature exposure may be required to obtain improved stability. We have shown that the phage are completely resistant to heating at 50 °C for at least 16 h.

Acknowledgments

We acknowledge Geoff Lyon at the Yale University Cell Sorter Facility for working with us to carry out FACS analysis of our samples. This work was supported in part by NIH SBIR grant #1R43RR028160 and DARPA contract #N10PC20116. The views expressed are those of the author and do not reflect the official policy or position of the Department of Defense or the U.S. Government.

References

[1] O. Stoevesandt, M.J. Taussig, Proteomics 7 (2007) 2738–2750.
[2] M.J. Taussig, O. Stoevesandt, C.A. Borrebaeck, A.R. Bradbury, D. Cahill, C. Cambillau, A. de Daruvar, S. Dubel, J. Eichler, R. Frank, et al., Nat. Methods. 4 (2007) 13–17.
[3] M. Uhlen, S. Hober, Mol. Recognit. 22 (2009) 57–64.
[4] D.E. Gloriam, S. Orchard, D. Bertinetti, E. Bjorling, E. Bongcam-Rudloff, J. Bourbeillon, A.R. Bradbury, A. de Daruvar, S. Dubel, R. Frank, et al., Mol. Cell Proteomics, 2009.
[5] S. Dubel, O. Stoevesandt, M.J. Taussig, M. Hust, Trends Biotechnol. 28 (7) (2010).
[6] M. Mersmann, D. Meier, J. Mersmann, S. Graslund, S. Helmsing, P. Nilsson, M. Hust, S. Dubel, N Biotechnol, 2009.
[7] K. Pershad, J.D. Pavlovic, S. Graslund, P. Nilsson, K. Colwill, A. Karatt-Vellatt, D.J. Schofield, M.R. Dyson, T. Pawson, B.K. Kay, et al., Protein Eng. Des. Sel. 23 (2010) 279–288.
[8] B. Kotlan, M.C. Glassy, Methods Mol. Biol. 562 (2009) 1–15.
[9] E.T. Boder, K.S. Midelfort, K.D. Wittrup, Proc. Natl. Acad. Sci. USA 97 (2000) 10701–10705.
[10] J. Hanes, C. Schaffitzel, A. Knappik, A. Pluckthun, Nat. Biotechnol. 18 (2000) 1287–1292.
[11] A. Cardinale, S. Biocca, Trends Mol Med. 14 (2008) 373–380.
[12] A.S. Lo, Q. Zhu, W.A. Marasco, Handb Exp Pharmacol. (2008) 343–373.
[13] L.L. Abler, M.D. Sheets, Genesis. 35 (2003) 107–113.
[14] A. Gulyani, E. Vitriol, R. Allan, J. Wu, D. Gremyachinskiy, S. Lewis, B. Dewar, L.M. Graves, B.K. Kay, B. Kuhlman, T. Elston, K.M. Hahn, Nature Chem. Bio. 7 (2011) 437–444.
[15] C.C. Liu, A.V. Mack, M.L. Tsao, J.H. Mills, H.S. Lee, H. Choe, M. Farzan, P.G. Schultz, V.V. Smider, Proc. Natl. Acad. Sci. USA 105 (2008) 17688–17693.
[16] C.V. Lee, S.S. Sidhu, G. Fuh, J. Immunol. Methods 284 (2004) 119–132.
[17] J.D. Marks, H.R. Hoogenboom, T.P. Bonnert, J. McCafferty, A.D. Griffiths, G. Winter, J. Mol. Biol. 222 (1991) 581–597.
[18] D.J. Schofield, A.R. Pope, V. Clementel, J. Buckell, S.D.J. Chapple, K.F. Clarke, J.S. Conquer, A.M. Crofts, S.R. Crowther, M.R. Dyson, G. Flack, G.J. Griffin, Y. Hooks, W.J. Howat, A. Kolb-Kokocinski, S. Kunze, C.D. Martin, G.L. Masien, J.N. Mitchell, M. O'Sullivan, R.L. Perera, W. Roake, S.P. Shadbolt, K.J. Vincent, A. Warford, W.E. Wilson, J. Xie, J.L. Young, J. McCafferty, Genome Biol. 8 (2007) R254.
[19] M.D. Sheets, P. Amersdorfer, R. Finnern, P. Sargent, E. Lindquist, R. Schier, G. Hemingsen, C. Wong, J.C. Gerhart, J.D. Marks, Proc. Natl. Acad. Sci. USA 95 (1998) 6157–6162.
[20] C.F. Barbas 3rd, A.S. Kang, R.A. Lerner, S.J. Benkovic, Proc. Natl. Acad. Sci. USA 88 (1991) 4363–4366.
[21] A.D. Griffiths, S.C. Williams, O. Hartley, I.M. Tomlinson, P. Waterhouse, W.L. Crosby, R.E. Kontermann, P.T. Jones, N.M. Low, T.J. Allison, EMBO J. 13 (1994) 3245–3260.
[22] J. McCafferty, A.D. Griffiths, G. Winter, D.J. Chiswell, Nature 348 (1990) 552–554.
[23] H.R. Hoogenboom, A.P. de Bruine, S.E. Hufton, R.M. Hoet, J.W. Arends, R.C. Roovers, Immunotechnology 4 (1998) 1–20.
[24] M.M. Kiss, E.G. Babineau, M. Bonatsakis, D.L. Buhr, G.M. Maksymiuk, D. Wang, D. Alderman, D.M. Gelperin, M.P. Weiner, J. Immunol. Methods 367 (2011) 17–26.
[25] H.M. Cohen, D.S. Tawfik, A.D. Griffiths, Protein Eng. Des. Sel. 17 (2004) 3–11.
[26] F.J. Ghadessy, J.L. Ong, P. Holliger, Proc. Natl. Acad. Sci. USA 98 (2001) 4552–4557.
[27] K.D. Wittrup, Curr. Opin. Biotechnol. 12 (2001) 395–399.
[28] C.G. Haidaris, J. Malone, L.A. Sherrill, J.M. Bliss, A.A. Gaspari, R.A. Insel, M.A. Sullivan, J. Immunol. Methods 257 (2001) 185–202.
[29] P. Kristensen, G. Winter, Fold Des. 3 (1998) 321–328.
[30] C.-Z. Ni, R. Syed, R. Kodandapani, J. Wickersham, D.S. Peabody, K.R. Ely, Structure 3 (1995) 255–263.
[31] C. Holtze, A.C. Rowat, J.J. Agresti, J.B. Hutchinson, F.E. Angile, C.H. Schmitz, S. Koster, H. Duan, K.J. Humphry, R.A. Scanga, J.S. Johnson, D. Pisignano, D.A. Weitz, Lab Chip 8 (2008) 1632–1639.
[32] R.J. Hosse, L. Tay, M.K. Hattarki, L. Pontes-Braz, L.A. Pearce, S.D. Nuttall, O. Dolezal, Anal. Biochem. 385 (2009) 346–357.