Published in final edited form as:

Methods Mol Biol. 2018 ; 1731: 307–324. doi:10.1007/978-1-4939-7595-2_26.

Generation of Highly Selective MMP Antibody Inhibitors

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Abstract

Inhibiting individual MMPs of biomedical importance with high selectivity is critical for both fundamental research and therapeutic development. Here we describe the methods for discovery of inhibitory monoclonal antibodies from synthetic human antibody phage display libraries carrying convex paratopes encoded by long complementarity-determining region (CDR)-H3 segments. We demonstrate the application of this technique for isolation of highly specific and potent antibody inhibitors of human MMP-14.

Keywords

inhibitory antibody; long CDR; synthetic library; convex paratope

1 Introduction

Inhibition of MMPs using zinc-chelating compounds (*e.g.* hydroxamates) has been extensively studied as a therapeutic strategy to treat cancer [1]. Although pre-clinic results were encouraging, all these small molecule inhibitors toward broad-spectrum MMPs failed in clinical trials due to severe side effects such as musculoskeletal pain and inflammation caused by the poor selectivity [2, 3]. It is now known that metalloproteinases play more complex and paradoxical roles in tumor progression beyond simple ECM degradation [4]. While many facets of proteolytic action are pro-tumorigenic, some metalloproteinases indeed exhibit tumor-suppressing effects in certain circumstances [5]. For example, MMP-8 favors host defense instead of stimulating tumor proliferation, suggesting its protective role in cancer processes [6]. In addition, metalloproteinases exert different roles at different steps of cancer progression, *e.g.* the opposing roles of MMP-9 at different microenvironments [7]. For these reasons, selectively blocking individual tumorigenesis-promoting metalloproteinase(s) at appropriate timeframe is highly desired for a successful therapy.

However, achieving target specificity and selectivity in small-molecule MMP inhibitors is remarkably challenging [8, 9]. Because the catalytic mechanism and catalytic domain fold are conserved among the MMP/ADAM/ADAMTS superfamily members, the available small-molecule inhibitors target multiple proteinases, resulting in off-target side effects [2, 3, 9, 10]. In this respect, antibody-based metalloproteinase inhibitors are emerging as both

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research tools and potential therapeutic agents [11–16] because of: (i) high affinity and high specificity due to the large antigen-antibody interactions area provided by multiple complementarity-determining regions (CDRs); (ii) long half-life and the well-known mechanisms of antibody action; (iii) low immunogenicity and low toxicity; (iv) multiple MMPs potentially targetable by antibodies [17].

Natural protease inhibitors exhibit a convex-shaped conformation that inserts into the enzyme active site and blocks the substrate access and/or catalytic function [18]. However, there is a low probability of generating antibodies with the convex antigen-binding sites (paratopes) from naive or immunized human or murine antibody libraries. The proteolytic pocket is often buried inside a major cleft or concave enzyme structure, and, as such, it is normally inaccessible by the cave-like, grooved, or flat antigen-binding surface in human and murine antibodies [19]. In contrast, dromedary antibodies are enriched in the long CDR-H3s encoding the extended convex-shaped paratopes and, intriguingly, a large proportion of antibodies isolated from camels and llamas, compared with human and murine antibodies, bind the active-site pockets and inhibit enzymatic reactions [20–22]. However, the camelid antibodies would evoke an immune response in humans, and the availability of these animals is limited.

Here we describe the design and construction of human Fab libraries in which the long, convex-shaped, camelid-like paratopes are incorporated into the human antibody scaffold [23, 24] (Fig. 1). We demonstrate the application of these libraries to screen for inhibitors of MMP-14, a proinvasive and prometastatic human proteinase [25, 26] by phage panning. As results of screen, a panel of selective Fabs with a high inhibitory potency against MMP-14 was isolated. The methods described here should be generally applicable to other MMPs and proteases of biomedical importance.

2 Materials

2.1 Library Construction

2.1.1 Long CDR-H3 Assembly

1. Oligonucleotides (Table 1, Integrated DNA Technologies)
2. T4 DNA polymerase and dNTP mix (New England Biolabs)
3. T4 DNA ligase and buffer (New England Biolabs)
4. 10× annealing buffer: 10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10 mM EDTA
5. TAE buffer: 40 mM Tris-acetate, 1.0 mM EDTA, pH 8.0
6. TAE/agarose gel: TAE buffer, 1.0% (w/v) agarose, 1:5000 (v/v) 10% ethidium bromide
7. DNA Clean & Concentrator-5 kit (Zymo Research)

2.1.2 Preparation of Electrocompetent *E. coli*

1. *E. coli* XL-Blue (Stratagene)

2. *E. coli* Jude-I (DH10B F' [proAB lacI^Q lacZ M15 Tn10(Tet^R)] [27])
3. LB/Tet agar: LB (BD Difco), 1.5 g/L agar, 10 µg/ml tetracycline
4. SOB/Tet: SOB (BD Difco), 10 µg/ml tetracycline

2.1.3 In-frame Selection of CDR-H3 Fragments

1. Plasmid pVH-bla [28]
2. AflIII and HindIII with buffers (New England Biolabs)
3. Plasmid DNA miniprep kit
4. DNA gel purify kit (Zymo Research)
5. 0.2-mm gap electroporation cuvette (Fisher Scientific)
6. 2×YT/Amp/IPTG agar: 2×YT (BD Difco), 1.5 g/L agar, 50 µg/ml ampicillin, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG)
7. 2×YT/Chlor: 2×YT, 34 µg/ml chloramphenicol
8. 245 mm square bioassay dishes (Corning)
9. 80% glycerol, autoclaved
10. Cell scraper (BD Falcon)

2.1.4 Cloning Functional CDR-H3 into Fab Phagemids

1. Plasmid pFab-PIII [29]
2. Fab phage F library [29]
3. BsmBI with buffer (New England Biolabs)
4. 2×YT/Amp: 2×YT, 100 µg/ml ampicillin
5. 2×YT/Amp agar: 2×YT, 1.5 g/L agar, 100 µg/ml ampicillin

2.2 Isolation of MMP-14 Specific Fab Clones by Phage Panning

2.2.1 Preparation of Fab Phage Libraries

1. 250-mL sterile polypropylene centrifuge bottles (*see* Note 1)
2. 2-L culture flasks (*see* Note 1)
3. M13KO7 helper phage (New England Biolabs)
4. 35 mg/ml kanamycin stock
5. 5× PEG/NaCl: 20% PEG-8000 (w/v), 2.5 M NaCl
6. Phage resuspension buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl₂, 0.1 mM ZnCl₂

¹To avoid cross-contamination, keep a dedicated set of centrifuge bottles, culture flasks, pipettes for phage experiments. Clean used bottles and flasks with 10% bleach solution. Discard used pipette tips directly to bleach solution.

7. 0.22 μ m sterile syringe filters (EMD Millipore)

2.2.2 Phage Panning on Immobilized MMP-14

1. Human MMP-14 catalytic domain [30, 31] (also see **Chapter 7**)
2. Human TIMP-2 N-terminal domain [32]
3. Phosphate-buffered saline (PBS): 137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄. Adjust pH to 7.2 with HCl and autoclave
4. Streptavidin, 1 mg/ml (New England Biolabs)
5. Maxisorp 96-well immunoplates (Thermo Scientific)
6. Assay buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl₂, 0.1 mM ZnCl₂
7. Blocking buffer: assay buffer, 0.5 % (w/v) gelatin from porcine skin (Sigma Aldrich). Incubate on 45 °C in water bath with occasional shaking until completely dissolved.
8. Washing buffer: assay buffer, 0.05 % (v/v) Tween 20
9. 100 mM trimethylamine (Sigma Aldrich)
10. 1 M Tris-HCl, pH 8.0

2.2.3 Monoclonal Phage ELISA

1. 2 $\times$ YT/Amp/KO7: 2 $\times$ YT, 100 μ g/ml ampicillin, 10¹⁰ phage/ml of M13KO7
2. Polystyrene 96 well round-bottom microplates (Corning)
3. Horseradish peroxidase/anti-M13 antibody conjugate (Amersham Pharmacia)
4. TMB (3,3',5,5'-Tetramethylbenzidine) ELISA substrate solution (Thermo Scientific)
5. 2 M H₂SO₄

2.3 Antibody Characterizations

2.3.1 Fab Cloning, Expression and Purification

1. Plasmid pHX-EX [33]
2. BglII and SalI-HF with buffers (New England Biolabs)
3. Ultrafiltration centrifugal units, 10K MWCO (EMD Millipore)
4. 15% SDS-PAGE gels

2.3.2 Binding Affinity Measurements and Selectivity Tests

1. Catalytic domains of human MMP-2 [30, 31] and MMP-9 (*see Chapter 7*)
2. Goat anti-human IgG (Fab specific)-peroxidase (Sigma-Aldrich)

2.3.3 Inhibition Potency Measurement and Inhibition Mode Determination

1. Peptide M-2359: Mca-Lys-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Arg-NH₂ (Bachem)
2. Black flat-bottom polystyrene NBS 96-well microplate (Corning)
3. 20% DMSO

3 Methods

3.1 Library Construction

The construction of Fab library incorporated with long CDR-H3 segments consists of three steps. Step 1, assembly of long CDR-H3 fragments. Step 2, selection of in-frame and full-length CDR-H3 fragments by cloning to the N-terminal of β -lactamase to remove truncated and reading-frame shifted segments introduced by degenerated codons or mismatched assembly. Step 3, cloning of functional CDR-H3 fragments into Fab library phagemids (Fig. 2).

3.1.1 Long CDR-H3 Assembly

1. Dissolve oligonucleotides (Table 1) with DDW to be 100 μ M.
2. For oligo annealing, in PCR tubes add 84.5 μ l DDW, 10 μ l of 10 $\times$ annealing buffer, 1.5 μ l (150 pmol) VH1, 1.5 μ l VH3, 1.5 μ l VH4, and 1 μ l (100 pmol) VH2_23 or VH2_25 or VH2_27 for different CDR-H3 length respectively (*see* Note 2).
3. Place the tubes in a thermal cycler for annealing reaction: 95 $^{\circ}$ C 2 min; cooling from 95 $^{\circ}$ C to 25 $^{\circ}$ C over a period of 45 min; 4 $^{\circ}$ C hold. After reaction, briefly spin the tubes and store on ice or 4 $^{\circ}$ C until use.
4. For gap fill-in reaction, to 30 μ l DDW add 50 μ l annealed oligoes, 5 μ l 10 mM dNTP mixture, 10 μ l T4 DNA ligase buffer, 2.5 μ l (1000 units) T4 DNA ligase, and 2.5 μ l (7.5 units) T4 DNA polymerase into PCR tubes.
5. Place the tubes in a thermal cycler for gap fill-in reaction: 37 $^{\circ}$ C 1 hr; 75 $^{\circ}$ C 25 min to inactivate enzymes; hold at 4 $^{\circ}$ C until use.
6. Purify assembled DNA samples by electrophoresis using 1% TAE/agarose gel with ethidium bromide for DNA visualization (typical results shown as Fig. 2 inset). Cut the target bands and recover the DNAs. These fragments are now ready for cloning into in-frame selection vector described in **Subheading 3.1.3**.

3.1.2 Preparation of Electrocompetent *E. coli*

1. Streak Jude-I or XL1-Blue stock on LB/Tet agar plates to grow fresh colonies overnight at 37 $^{\circ}$ C.

²Initial trials of annealing and gap fill-in reaction with equimolar of oligoes resulted in a smear band observed on DNA gels. Because oligoes VH1, VH3, and VH4 (60 mers) are not PAGE purified, truncated products are present due to DNA synthesis coupling efficiency. By increasing their usages to be 1.5 times of VH2 (PAGE purified 94-106 mers) and reducing the reaction volume to 100 μ l, clear assembled fragments are obtained.

2. Seed a single colony in 5 ml SOB/Tet and grow overnight at 37 °C with shaking at 250 rpm.
3. Inoculate 2.5 ml overnight culture to 250 ml pre-warmed SOB/Tet and culture at 37 °C with shaking at 250 rpm until OD₆₀₀ reaches to 0.8–1.0 (typically 2–3 hrs for Jude-I and 3–4 hrs for XL-1Blue).
4. Incubate the culture flasks on ice for 30 min.
5. Collect cells by centrifugation at 8,500×g 4 °C for 5 min using two autoclaved pre-chilled 250 mL centrifuge bottles and decant the supernatants.
6. Add 5 ml autoclaved cold DDW to each centrifuge bottle and shake the bottles in their standing position at 90 rpm 4 °C until cells are fully resuspended. Add 125 mL DDW to each bottle and slowly rotate to mix.
7. Centrifuge the cells at 8,500×g 4 °C for 5 min and decant supernatant.
8. Repeat **steps 5–7** twice to completely remove culture medium. Reduce DDW usages to 125 ml (62.5 ml each bottle) and 25 ml (12.5 ml each bottle) in the second and third washes.
9. Suspend cells in 1 ml DDW by shaking at 90 rpm 4 °C. Competent cells are ready for electroporation (**Subheadings 3.1.3** and **3.1.4**) and should be used in the same day to avoid loss of competency (*see* Note 3).

3.1.3 In-frame Selection of CDR-H3 Fragments

1. Prepare 2×YT/agar with 50 µg/ml ampicillin and 0.5 mM IPTG (*see* Note 4).
2. Digest 15 µg plasmid pVH-bla and 1.5 µg assembled CDR-H3 fragments with AflII and HindIII, Gel purify the 4.8 kb and ~220 bp fragments respectively.
3. Ligate 5 µg digested pVH-Bla with 0.5 µg digested CDR-H3 fragments (at ratio of vector to insert = 1:2) at room temperature for 6 hr using T4 DNA ligase.
4. Desalt ligation product using DNA Clean & Concentrator-5 kit. Typically, 3 µg ligated DNA in 100 µl is obtained.
5. Mix 1.0 ml prepared electrocompetent cells with 3 µg desalted ligation product, and incubate on ice for 2 min.
6. Place ten 0.2 mm gap electroporation cuvettes on ice, and transfer 100 µl cell-DNA mixture to each cuvette. (*see* Note 5)
7. Electroporate using MicroPulser (Bio-Rad) with voltage set at 2.5 kV. (*see* Note 6)

³To validate the competency, mix 100 µl (~20 OD) prepared electrocompetent cells with 300 ng plasmid DNA. Electroporate as details described in **Subheading 3.1.3**. 5×10⁸ or more transformants indicate an acceptable competency for library construction.

⁴The ampicillin and IPTG concentrations should be optimized [28].

⁵To construct large libraries, enough amounts of cells, e.g. >200 OD, are needed. Each 0.2 mm gap electroporation cuvette can hold approximately 100 µl (~20 OD cells) and 300 ng ligated DNA.

⁶The time of electroporation should read at 4.8–5.6 ms as an indicator of good transformation.

8. Immediately add 1 ml SOB to cuvette, and mix with electroporated cells by gentle pipetting. Transfer the liquid to a 50-ml conical tube. Wash the cuvette twice with 1 ml SOB. Collect all electroporated cells.
9. Repeat electroporations for all cuvettes. Culture collected cells (~30 mL) at 37 °C for 1 hr with shaking at 250 rpm.
10. Spread cultured cells on ten square dishes of 2×YT/Amp/IPTG agar. Allow the moisture completely adsorbed then incubate at 30 °C overnight. Save 30 µl transformed cell culture for library size determination by titrating (see Note 7).
11. In the following day, collect cells from the dishes with cell scrapers. Suspend library cells in 80% glycerol for storage at –80 °C.
12. When necessary, repeat **steps 2–11** until achieving desired library size.
13. To prepare pVH-bla library carrying in-frame long CDR-H3s, inoculate appropriate OD of library cells (>10-fold coverage of diversity) in LB/Amp and culture at 37 °C for 6 hr. Extract plasmid DNA by miniprep.

3.1.4 Cloning Functional CDR-H3 into Fab Phagemids

1. Add $> 5 \times 10^{10}$ phage particles of F library [29] to 200 ml exponentially growing *E. coli* XL1-Blue (OD₆₀₀ = 0.4–0.5) in 2×YT/Tet (see Note 8).
2. Incubate 20 min at 37 °C without shaking.
3. Add ampicillin to be 100 µg/ml. Culture at 37 °C for 5–6 hr with shaking at 250 rpm.
4. Extract F library plasmids pFab-pIII from 20 ml cell culture by miniprep.
5. Digest 15 µg prepared F library pFab-pIII and 40 µg in-frame selected pVH-bla with AflIII and BsmBI. Gel purify the 4.9 kb and 129–141 bp bands respectively (see Note 9).
6. Ligate 5 µg purified pFab-pIII fragments with 0.5 µg prepared long CDR-H3 segments (at a ratio of vector to insert = 1:2) with T4 DNA ligase at room temperature for 6 hr.
7. Desalt ligated mixture using DNA Clean & Concentrator-5 kit.
8. Transform 3 µg ligated DNA to 1.0 ml freshly prepared XL1-Blue component cells by electroporation as details described in **Subheading 3.1.3**. Incubate the cells at 37 °C for 1 hr.

⁷Serially dilute and culture on 2×YT/Amp/IPTG agar and 2×YT/Chlor agar to determine library size and to observe selection effects. In the following day, count colony numbers on titration plates. Randomly pick a few colonies from 2×YT/Amp/IPTG agar and inoculate in 5 ml 2×YT/chlor for growth at 37 °C overnight. Extract plasmids for DNA sequencing to assess the quality of constructed library.

⁸The design diversity of all other CDRs on the F library (approximately 2×10^8 , 29) should be covered >100-folds by enough number of phages.

⁹Because the optimal temperature for BsmBI (55 °C) is different from AflIII (37 °C), sequential digestion is needed.

9. Spread the culture on two 245 mm square dishes of 2×YT/Amp agar and incubate at 30 °C overnight. Save 30 µl transformed cell culture for library size determination by titrating and quality check by DNA sequencing (*see* Note 7).
10. In the following day, scrape the cells from the dishes. Suspend library cells in 80% glycerol for storage at −80 °C.
11. If necessary, repeat **steps 5–10** until desired library size and quality are achieved.

3.2 Selection of Specific Fab Clones by Phage Panning

3.2.1 Preparation of Fab Phage Libraries

1. Inoculate 30 OD cells carrying constructed long CDR-H3 Fab library to 600 mL 2×YT/Amp (*see* Note 10).
2. Culture at 37 °C with shaking at 250 rpm to OD₆₀₀ of 0.4–0.5 (approximately, 1.5–2.0 hr) (*see* Note 11).
3. Add helper phages at a ratio of phages to bacterial cells = 10–20:1.
4. Incubate at 37 °C without shaking for 30 min for phage infection.
5. Add kanamycin to a final concentration of 35 µg/ml and culture overnight at 30 °C with shaking (250 rpm) to produce Fab library phages.
6. Chill the culture on ice for 20 min. Clarify the media by centrifugation of *E. coli* cells at 4,000×g 4 °C for 15 min, and transfer supernatant to a 2-liter container (*see* Note 12).
7. Add cold PEG/NaCl solution as 1/5 volume of the supernatant. Incubate on ice for 1 hr to precipitate phage particles.
8. Centrifuge at 10,000×g 4 °C for 15 min to collect phages. White pellets should be seen. Discard supernatant and aspirate remaining liquid. Resuspend the phage pellets in 45 mL assay buffer (*see* Note 13).
9. Centrifuge the phage solution at 10,000×g 4 °C for 15 min to remove bacterial contaminants. Transfer the supernatant to a new tube.
10. Add cold PEG/NaCl solution (1/5 volume of phage solution) to precipitate the phage. Incubate at 4 °C for 5 min. The solution should appear clouding.
11. Centrifuge at 10,000×g 4 °C for 15 min. Decant supernatant and aspirate remaining liquid (*see* Note 14).

¹⁰For library preparation, the inoculum should be at least 10-fold covering the library diversity, and the starting OD₆₀₀ should be 0.05 or less.

¹¹Culturing at 37°C improves phage infection efficiency likely by promoting F pilus expression. And over-growth (OD₆₀₀ >0.5) will result in a lower infection rate.

¹²Pelleted cells may appear sticky due to the toxicity of phage production and Fab expression to the *E. coli* host cells. It is recommended to use serological pipettes to collect the supernatant after centrifugation. Trace amounts of cells can be removed during phage purification steps.

¹³To maintain MMPs in their native conformation, 0.1 mM Zn²⁺ and 5 mM Ca²⁺ present in assay buffer.

¹⁴To remove the residue PEG/NaCl completely, we recommend to wash phage pellets by briefly rinsing with assay buffer and quickly decanting it. Because of excess number of phages prepared, the loss during wash does not affect panning results.

12. Resuspend phage pellets with assay buffer in a volume that is 1/50 of the original culture volume. Filtrate the phage solution through 0.22 µm filters.
13. Estimate phage concentration spectrophotometrically (1 OD at 268 nm is equivalent to $\sim 5 \times 10^{12}$ phage/ml) [33]. This purified phage libraries can be used immediately for selection (**Subheading 3.2.2**) and remaining phage can be stored -80°C in 15% glycerol. Use 20 µl purified phages for titrating measurement (*see Note 15*).

3.2.2 Selection of Fab Phage Clones on Immobilized cdMMP-14—In this protocol, cdMMP-14 is immobilized via biotinylation and streptavidin coated plates. And nTIMP-2, a natural protein inhibitor of MMP-14, is exploited as the eluent. Because nTIMP-2 binds to the active-site of native MMP-14 [18], this strategy results in enrichment of epitope specific antibodies possessing inhibition function.

1. Coat immunoplate wells with 100 µl streptavidin solution (5 µg/ml in PBS buffer) for 2 h at room temperature or overnight at 4°C . The number of wells required depends on library size (*see Note 16*).
2. Remove coating solution. Rinse with washing buffer twice. Block the wells with 250 µl blocking buffer (0.5% gelatin) for 2 hr (*see Note 17*).
3. Remove blocking solution. Wash twice. Add 100 µl biotinylated cdMMP-14 (2 µg/ml in assay buffer) to each well and incubate for 20 min at room temperature.
4. Remove cdMMP-14 solution. Wash twice.
5. To deplete streptavidin binders, add 100 µl phage solution (containing 10^{12} – 10^{13} Fab library phages in blocking buffer) to streptavidin coated wells and incubate for 1 hr at room temperature with shaking at 700 rpm.
6. After depletion, transfer phage supernatant to the wells coated with cdMMP-14. Incubate at room temperature for 1 hr with shaking at 700 rpm.
7. Remove the phage solution and wash 10 times with washing buffer and 5 times with assay buffer (*see Note 18*).
8. Add 100 µl 6 µM nTIMP-2 each well. Incubate for 1 hr at room temperature for elution of phages carrying epitope specific Fab clones.
9. To further elute all bound phages, add 100 µl 100 mM triethylamine per well and incubate 5 min at room temperature (*see Note 19*).

¹⁵To decide the number of input phage for phage panning, it is necessary to estimate the phage concentration quickly using a spectrophotometer. Based on numerous experiments, it is found that when OD₂₆₈ is less than 0.5, the UV method agrees well with the titration results.

¹⁶The phage concentration should not exceed 10^{13} phage/ml, and the total number of phage should exceed the library diversity by 1000-fold. It is also recommended to coat extra wells for polyclonal ELISA to track the progress of phage panning.

¹⁷The blocking agent should be compatible with the antigen of interest. Common choice includes BSA, gelatin, skim milk and ChemoBlocker. For biotinylated antigens, skim milk should not be used due to the presence of excessive biotin.

¹⁸Washing in the first round of selection is relatively mild (generally 10 times). As the copy numbers of positive clones increase after initial selection, in the sequential rounds of panning, more stringent washing conditions can be applied. To reduce non-specific bindings, washing with a weak acidic buffer, *e.g.* pH 5.0 adjusted by citric acid, can also be considered [35].

¹⁹Incubation longer than 10 min may reduce phage infectivity.

10. Add 50 μ l 1 M Tris-HCl (pH 8.0) per well for neutralization.
11. Incubate eluted phages with 10 volumes of exponentially growing *E. coli* XL1-Blue ($OD_{600} = 0.4\text{--}0.5$ cultured in 2 $\times$ YT/Tet) for 30 min at 37 $^{\circ}$ C without shaking for infection. Titrate input and output phages by serials dilutions.
12. Centrifuge cultured cells at 4,500 $\times g$ 4 $^{\circ}$ C for 15 min. Decant supernatant and resuspend with 1–2 ml 2 $\times$ YT/Amp depending on the titers of output phages. Plate on LB/Amp agar, and incubate at 30 $^{\circ}$ C overnight.
13. The following day, scrape cells from the plates. Suspend cells in 10% glycerol for storage –80 $^{\circ}$ C.
14. For the sequential rounds of panning, prepare phage antibody libraries as described in **Subheading 3.2.1**. Culture volume can be decreased to 50–100 ml depending on the titers of output phages.
15. Repeat the selection, **step 1–13**, for total three or four rounds. Washing stringency can be increased to 20 times with washing buffer and 5 times with assay buffer. Selection pressure can also be given by reducing cdMMP-14 usage (to 1 μ g/ml).

3.2.3 Monoclonal Phage ELISA—After multiple rounds of panning, individual Fab clones can be tested for cdMMP-14 binding by monoclonal phage ELISA.

1. Randomly pick colonies from agar plates using sterile pipette tips. Inoculate them to 96-well round-bottom microplates containing 200 μ l 2 $\times$ YT/Amp. Culture overnight at 30 $^{\circ}$ C with shaking at 250 rpm. These plates serve as the master plates (*see* Note 20).
2. In the next day, inoculate 96-well round-bottom microplates containing 200 μ l 2 $\times$ TY/Amp/KO7 with 2 μ l overnight culture from the master plates for monoclonal phage production. Add glycerol to the master plates (20% final concentration) for storage at –80 $^{\circ}$ C.
3. Coat ELISA plates with either cdMMP-14 or blocking reagent only (negative control), as described in **Subheading 3.2.2**. Add 25 μ l blocking buffer each well.
4. Centrifuge the phage culture plates at 4,000 $\times g$ for 15 min. Transfer 25 μ l supernatants to the prepared ELISA plates. Incubate at room temperature for 1 hr.
5. Wash three times with washing buffer. Add 50 μ l per well 1:5000 HRP-anti M13 conjugate in blocking buffer. Incubate at room temperature for 30 min.
6. Wash plates four times with washing buffer and once with assay buffer. Add 50 μ l per well freshly prepared TMB solution. Allow signal development for 5–10 min.

²⁰To avoid evaporation, 96-well culture plates can be placed in a closed box far away from the circulating fan. Place damp paper towel in the box.

7. Stop the reaction with 25 μ L 2 M H_2SO_4 . Read absorbance at 450 nm using a microplate reader.
8. Determine the specific bindings by comparing signals on cdMMP-14 over signals on blocking reagents (*see* Note 21).

3.3 Antibody Characterizations

3.3.1 Fab Cloning, Expression and Purification

1. Inoculate the positive clones isolated in **Subheading 3.2.3** from the master plates to 5 mL LB/Amp. Culture overnight at 37 °C with shaking at 250 rpm.
2. Extract the Fab display plasmids by miniprep. By applying standard molecular biology protocols, clone the VL-CL-VH fragments into the Fab expression plasmid [33] with BglII and SalI cutting sites.
3. Transform cloned Fab expression vectors into BL21(DE3) electrocompetent cells (**Subheading 3.1.2**, and **3.1.3**).
4. For Fab production, culture transformed cells in 500 mL 2 $\times$ YT/Amp overnight at 30 °C.
5. Harvest cells by centrifugation and prepare periplasmic fraction by osmotic shock treatment [34].
6. Purify Fabs from periplasmic preparation [34] using Ni-NTA resin (Qiagen).
7. Verify the quality of purified Fabs using 15% SDS-PAGE gels.

3.3.2 Binding Affinity Measurements by ELISA

1. Coat 96-well microplates with biotinylated cdMMP-14, and block the plates with 0.5% gelatin as details described in **Subheading 3.2.2**.
2. Starting with 1 μ M, two-fold serially dilute purified Fabs with blocking buffer in cdMMP-14 coated wells. Incubate at room temperature for 1 hr.
3. Discard Fab solutions and wash three times.
4. Incubate 50 μ L 1/5000 goat anti-human IgG (Fab specific)-HRP conjugate in blocking solution at room temperature for 1 hr.
5. Decant the second antibody solution. Wash four times with washing buffer and one time with assay buffer.
6. Incubate with 50 μ L/well TMB solution for 5–10 min. Add 25 μ L 2M H_2SO_4 solution to stop the reaction.
7. Measure absorbance at 450 nm using a microplate reader. Calculate affinity constant from a four-parameter, logistic, curve-fitting analysis to evaluate EC_{50} values of Fabs (a typical result shown in Fig 4A).

²¹In general, > 5-fold signal increases are considered as positive.

3.3.3 Selectivity Tests by Competitive ELISA

1. Coat 96-well microplates with biotinylated cdMMP-14, and block the plates with 0.5% gelatin as details described in **Subheading 3.2.2**.
2. Starting at 3 μ M, two-fold serially dilute purified cdMMP-2, cdMMP-9 and cdMMP-14 in the cdMMP-14 coated wells.
3. Incubate MMP solutions with Fabs of interest at their EC₅₀ concentrations (measured in **Subheading 3.3.2**) at room temperature for 2 hr.
4. Add antibody/antigen mixtures to 96-well microplate coated with biotinylated cdMMP-14. Incubate at room temperature for 15 min.
5. Develop the signals and measure the absorbance at described in **step 3–7 of Subheading 3.3.2**. Typical results are shown in Fig 4B.
6. Similarly, competitive ELISA with 4 nM – 10 μ M nTIMP-2 can be performed. Typical results are shown in Fig 4C.

3.3.4 Inhibition Potency Measurement by FRET Assays

1. Prepare 2 nM cdMMP-14 in assay buffer and 100 μ M peptide M-2359 in 20% DMSO.
2. Starting at 10 μ M, two-fold serially dilute Fabs in 96-well black microplate.
3. Aliquot 25 μ l 2nM cdMMP-14 solution into each well containing 25 μ l Fab solutions. Incubate the mixtures for 30 min to form antibody/antigen complex.
4. Add 1 μ l peptide M-2359 solution to each well to start the reaction.
5. Monitor hydrolysis of the fluorogenic peptide with excitation at 328 nm and emission at 393 nm. Fluorescence is recorded continuously for 30 min, and the initial reaction rates are measured. Inhibition constants can be calculated by fitting the data to equation below, where V_i is the initial velocity in the presence of inhibitor, V_o is the initial velocity in the absence of inhibitor, and [I] is the inhibitor concentration. Typical results are shown in Fig 4A.

$$\frac{V_i}{V_o} \% = \frac{1}{1 + \frac{[I]}{IC_{50}}} \times 100$$

6. Similarly, monitor hydrolysis reaction with 10 nM cdMMP-14, 0–40 μ M M-2359, and fixed concentrations of Fab which show 35–70% inhibition. Determine kinetic parameters K_m and V_{max} by fitting to Lineweaver-Burk equation. Typical results are shown in Fig 4D.

Acknowledgments

This work was supported by National Science Foundation the Faculty Early Career Development (CAREER) Program 1453645 and National Institutes of Health Grant R01 GM115672 to X.G.

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Figure 1. Scheme that convex antibody paratope formed by an extended CDR-H3 mediates enzyme inhibition (reprinted from Ref [24]).

Figure 2.

Construction of synthetic antibody libraries with long CDR-H3s. (Step 1) Hybridization of degenerate oligonucleotides (Table 1) encoding long CDR-H3s with 23, 25 and 27 aa. (Step 2) CDR-H3 fragment assembly by T4 DNA polymerase and T4 DNA ligase. (Step 3) Assembled long CDR-H3 genes were subjected for full-length in-frame selection by fusing with β-lactamase (pVH-bla). (Step 4) Cloning the in-frame CDR-H3s into an existing Fab library to construct synthetic antibody library carrying long CDR-H3s with 23, 25 and 27 aa.

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Figure 4. Typical results of MMP inhibitory Fab characterizations (reprinted from Ref [24]). (A) Binding affinity and inhibition potency measured by ELISA and FRET assays. (B) Selectivity tests. (C) Competitive ELISA with n-TIMP-2. (D) Determination of mode of inhibition.

Table 1

List of oligonucleotides for long CDR-H3 assembly

Name	Oligonucleotide sequences
VH1	Ggccgtttcactataagcgcagacacatccaaaacacagcctacctgcagatgaacagc
VH2_23	P- <u>ccgtgtattattgcgcgcgt</u> (XYZ) ₁₈ (TAT)(GBN) <u>atggactactgggtcaggg</u>
VH2_25	P- <u>ccgtgtattattgcgcgcgt</u> (NNS) ₂₀ (TAT)(GBN) <u>atggactactgggtcaggg</u>
VH2_27	P- <u>ccgtgtattattgcgcgcgt</u> (NNS) ₂₂ (TAT)(GBN) <u>atggactactgggtcaggg</u>
VH3	P- <u>acgcgcgcaataatacacggcagtgctcctcagctcttaagctgttcactgcaggtaggc</u>
VH4	Tggatgaccgaagcttgcggaggagacggtgaccagggtccctgacccagtagtccat

Notes:

1. Overlapping regions are underlined with annealing temperatures of ~58 °C.
2. VH2 and VH3 are 5'-phosphorylated.
3. For CDR-H3 with 23 aa, XYZ codons were used, which contains unequal nucleotide ratios at each position of the codon triplet (X = 38% G, 19% A, 26% T and 17% C; Y = 31% G, 34% A, 17% T and 18% C; and Z = 24% G and 76% C). For CDR-H3 with 25 or 27 aa, NNS codon was used.