



Development of polyol-responsive antibody mimetics for single-step protein purification



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ABSTRACT

The purification of functional proteins is a critical pre-requisite for many experimental assays. Immunoaffinity chromatography, one of the fastest and most efficient purification procedures available, is often limited by elution conditions that disrupt structure and destroy enzymatic activity. To address this limitation, we developed polyol-responsive antibody mimetics, termed nanoCLAMPs, based on a 16 kDa carbohydrate binding module domain from *Clostridium perfringens* hyaluronidase. nanoCLAMPs bind targets with nanomolar affinity and high selectivity yet release their targets when exposed to a neutral polyol-containing buffer, a composition others have shown to preserve quaternary structure and enzymatic activity. We screened a phage display library for nanoCLAMPs recognizing several target proteins, produced affinity resins with the resulting nanoCLAMPs, and successfully purified functional target proteins by single-step affinity chromatography and polyol elution. To our knowledge, nanoCLAMPs constitute the first antibody mimetics demonstrated to be polyol-responsive.

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1. Introduction

An important starting point for mechanistic, structural and functional biochemistry is the purification of enzymatically active proteins and protein complexes [1–3]. For example, structural determinations by Cryo-electron microscopy (CryoEM), depend upon purification procedures that preserve the tertiary and quaternary structure of large, multi-subunit protein complexes (reviewed in Refs. [4–6]). As another example, structure-function and other mechanistic biochemical studies often depend upon the characterization and comparison of a large number of protein variants purified under conditions that preserve enzymatic activity.

Methods of purification typically require tradeoffs between enrichment, yield, activity and convenience. For example, affinity tags enable effective, efficient and rapid purification but require genetic modification of the target protein to attach the tag (e.g., AviTag, FLAG tag, GFP, His tag, MBP, TAP-tag, and Strep-tag) [7,8]. In

addition to the effort required to add the tag, this approach has the potential to disrupt the tagged protein's function, conformation, and expression level. As an alternative, immunoaffinity chromatography avoids genetic modification but requires the development of a suitable antibody. Immunoaffinity chromatography resins can be used to purify native proteins in high yield in a single step but generally require elution with pH extremes, denaturants, competing antigen or other conditions that may interfere with subsequent assays. In addition, the capture antibody can contaminate the eluate in instances when the antibody cannot be covalently crosslinked to the support. Immunoaffinity chromatography often results in a loss of activity, a disruption of protein complexes, or a need for additional purification steps to remove undesirable contaminants.

An exceptional class of monoclonal antibodies, called polyol-responsive antibodies, has a higher probability of preserving the activity and subunit interactions of target proteins or protein complexes [9–11]. Polyol-responsive antibodies have the distinctive property of enabling elution at neutral pH without denaturing agents. Although the mechanism is not well understood, polyol-responsive antibodies release antigen when exposed to neutral buffers containing propylene glycol or glycerol and a nonchaotropic salt such as ammonium sulfate. Polyol-responsive

Abbreviations used: CBM, carbohydrate binding module; SEC, size exclusion chromatography; WCL, whole cell lysate; PEB, polyol elution buffer; PG, propylene glycol; AS, ammonium sulfate.

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antibodies have been used to purify active, multi-subunit complexes such as *E. coli* RNA polymerase, eukaryotic RNA polymerase II, and the *Saccharomyces cerevisiae* Set1 complex [10,12,13]. One of these antibodies was successfully converted to a single-chain variable fragment (scFv) and was shown to retain polyol responsivity, although the affinity for the antigen was reduced in this format [14].

An emerging alternative to traditional immunoaffinity chromatography is the use of single-domain antibodies or antibody mimetic scaffold proteins. Antibody mimetics bind to targets with high specificity and affinity and can be genetically modified to add new functions or properties. The genetic modification enables precise, site-specific chemical conjugation. For example, the antibody mimetics may be engineered to facilitate the addition of a single fluorophore at a unique site or the irreversible attachment to a solid support in a defined orientation. Antibody mimetics are also typically resilient to denaturation and can be produced in high yield, microbial expression systems. Over the past 30 years, antibody mimetics based on several different scaffolds have been extensively characterized as alternatives to traditional antibodies. Examples include Affibody molecules, Affilins, Affimers, Adhirons, Affitins, Alphabodies, Anticalins, Avimers, DARPinS, Fynomers, Ubvs, Nanobodies, and Monobodies [15–28]. Compared with traditional antibodies, antibody mimetics often offer advantages in speed, cost and performance, but, to our knowledge, none have been shown to be polyol-responsive [16].

To combine the advantages of polyol-responsive antibodies and antibody mimetics, we sought to develop a single-domain antibody mimetic scaffold for which polyol-responsiveness would be a general rather than an exceptional property. We screened a series of small protein domains for potential use as scaffolds for antibody mimetics and identified a scaffold enabling the isolation of antibody mimetics with the following properties: (1) Selectivity and affinity comparable to published antibody mimetics, (2) Polyol-responsiveness, and (3) High-yield production in bacteria. The scaffold we selected for further development is a beta-sandwich fold domain derived from the Type 32 carbohydrate binding module of the NagH hyaluronidase of *Clostridium perfringens* [29,30]. Because the 16 kD size is comparable to nanobodies, we call these single domain antibody mimetics nanoCLAMPs (**n**ano **C**lostridial **A**ntibody **M**imetic **P**roteins). This report describes the development of the scaffold, the general procedure used to isolate nanoCLAMPs against a variety of target proteins, and the use of nanoCLAMPs for the single-step capture and polyol elution of a diverse set of proteins.

2. Materials and methods

2.1. Library construction

A cDNA coding for residues 807 to 946 of a carbohydrate binding module (Protein Data Bank 2W1Q) was codon optimized for expression in *E. coli* and synthesized by IDT (Coralville, IA). The cDNA was cloned into the phagemid pComb3X (Fig. 2A) such that the CBM contained an N-terminal His tag and a C-terminal FLAG tag, and was fused N-terminally to a truncated form of pIII (gene 3 product). To construct a phagemid library of variants of the scaffold CBM, we employed degenerate primers constructed using pools of 18 phosphoramidite trimers (equal mix of all amino acid codons except Cys and Met; Glen Research, Sterling, VA; TriLink Biotechnologies, San Diego, CA) at each variable position to amplify the phagemid and introduce variable positions in the loops. We amplified 1 ng of this phagemid using degenerate primer 397T-F and the non-degenerate primer 398-R (Table 1), which randomized Loop V, in a 50 μ l reaction with ClonAmp HiFi PCR Mix, according to

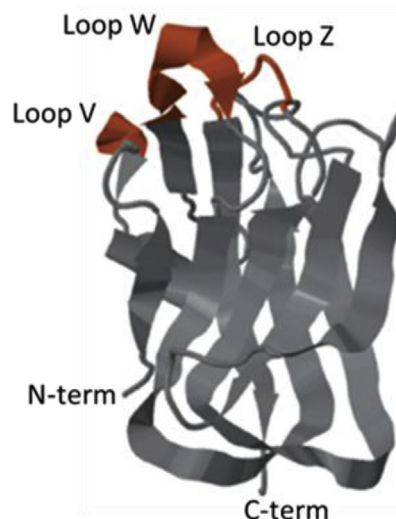


Fig. 1. Ribbon diagram of the second Type 32 carbohydrate binding module of NagH hyaluronidase of *Clostridium perfringens* (PDB 2W1Q). Loops variabilized during library construction are shown in orange.

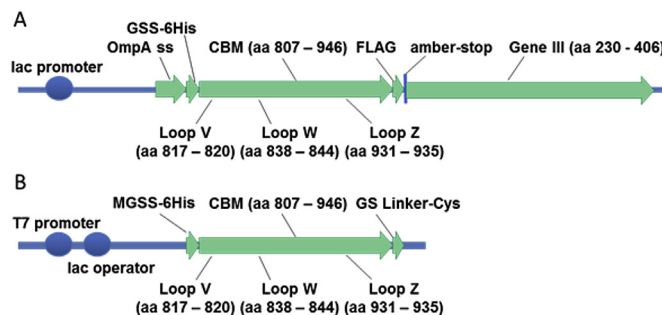


Fig. 2. Schematic of phagemid and expression constructs. A: Region of phagemid pComb3X modified to contain CBM scaffold library with randomized Loops V, W, and Z. CBM corresponds to amino acids 807 to 946 (PDB 2W1Q). The displayed GSS-6His-CBM-pIII fusion protein is approximately 36.3 kDa after cleavage of the OmpA signal sequence. B: pET expression system for nanoCLAMPs. GS-Linker-Cys amino acid sequence is GGGGSGGGGSGGGC. The expressed nanoCLAMPs are 163 aa after Met cleavage with a MW of 17.6 kDa (MW with native loops).

manufacturer's instructions (Takara Bio, Mountain View, CA). The reaction cycle was 98 °C for 10 s, 65 °C for 10 s, and 72 °C for 30 s, repeated 30 times. The resulting Amplicon 1 was gel purified on a 1.1% agarose gel using Qiagen (Qiagen, Germantown, MD) Qiaquick Gel Extraction Kit columns, and eluted in 12 μ l elution buffer. These primers contained overlapping regions so that the resulting amplicon could be fusion cloned and ligated *in vivo* using Takara's InFusion HD Enzyme kit, with the resulting phagemid a mini-library with 4 variable codons in Loop V, which consists of residues 817 through 820. Briefly, 495 ng of the gel purified Amplicon 1 was fusion cloned in a 50 μ l reaction with 10 μ l of 5X InFusion HD Enzyme and incubated at 50 °C for 15 min, and then put on ice. The DNA was then concentrated and purified using a Qiaquick PCR Purification Kit column, and eluted in 10 μ l EB. The DNA was then desalted on a Millipore (EMD Millipore, Billerica, MA) VSWP 0.025 μ m membrane floating on 100 ml ddH₂O for 30 min, changing the water and repeating for 30 more min.

The DNA library was electroporated into electrocompetent TG1 cells (Lucigen, Middleton, WI) by adding 1 μ l of DNA at 40 ng/ μ l to each of 6 aliquots of 25 μ l of cells on ice in 0.1 cm electroporation cuvettes. The DNA was electroporated using a MicroPulser (Bio-

Table 1
Primers used to construct the phage display library CNL-2 in pComb3X.

Primer	Sequence (5'–3')	Used to Construct:
397-TF	CGTTCTGAATCTGGNNNNNNNNNNNGGGAATGAAGCCAATTTATTAGATG	Amplicon 1
398 R	CCAGGATTGAGAACGAATTAAGAAG	Amplicon 1
404 F	TCTCTGTCAGGAGAATTCATTGGATTG	Amplicon 2
405A R	TTCCAGATTAGTCAGACGAATGTACTTAG	Amplicon 2
402T R	TTCTCTGCAAGAGANNNNNNNNNNNNNNNNNNNNNNNATACCAACACCGGTGTTATCGTC	Amplicon 3
403T F	CTGACTAATCTGGAANNNNNNNNNNNNNNNNNNNNNNCTGACTTTTAGTGAGTTGCAATTG	Amplicon 3
391 F	CATCATCATCATCACAACCTTCTTTAATTCGTTCTGAATC	Amplicon 4
450 R	GGCTTTGTTAGCAGCTCAGCAGTCAGACACAATTGCAAACTCACTAAAAG	Amplicon 4
390 R	GTGATGATGATGATGATGGCTGCTGCCC	Amplicon 5
387 F	GCTGCTAACAAAGCCCGAAAGGAAGCTG	Amplicon 5

Note: NNN denotes phosphoramidite trimer.

Rad, Hercules, CA) on setting Ec1, producing a Tau of approximately 5.4 msec, after which the cells and DNA were diluted with 1 ml per electroporation of Lucigen Recovery Media, pooled, and incubated at 37 °C, at 275 rpm, for 1 h in a shaking incubator. To titer the sub-library, 10 µl of recovered culture was diluted ten-fold and 10 µl aliquots spotted onto 2xYT/glucose (2%)/Carbenicillin (100 µg/ml) (2xYT/glu/Carb), and incubated overnight at 30 °C. The remaining mini-library was expanded to 50 ml 2xYT/glu/Carb and incubated overnight at 30 °C, 250 rpm. The cells were pelleted and re-suspended in 2xYT/18% glycerol at an OD₆₀₀ of 75 and stored at –80 °C.

The phagemid mini-library was prepared by inoculating 50 ml 2xYT/glu/carb with 5 µl glycerol stock and growing overnight, and then preparing phagemid using a Qiagen Plasmid Midiprep Kit, resulting in 100 µl of 156 ng/µl phagemid DNA. To prepare this phagemid library to serve as template for construction of the library with Loops W and Z also randomized, 5 µg of phagemid was digested with 30 Units of PstI in 50 µl reaction with Buffer 3 (NEB, Ipswich, MA) and bovine serum albumin (BSA), incubated for 1 h at 37 °C, and gel purified. The “insert” was prepared by amplifying the region of CBM between Loops W and Z, which contained no variabilized residues, by amplifying 2 ng of native CBM using primers 404 F and 405 AR, which mutated M929L, using ClonAmp HiFi PCR Mix in a 100 µl reaction according to manufacturer's instructions, and cycled at 98 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s, cycling 30 times (Amplicon 2). Both the insert and the purified phagemid were gel purified on a 1.1% agarose gel using Qiagen Qiaquick Gel Extraction Kit columns.

The phagemid was then amplified using the phosphoramidite primer primers containing randomized codons for loops W and Z, and also overlapping regions for annealing to the insert, which contained the interior, non-random region of CBM M929L. Briefly, 420 ng of PstI digested and purified phagemid was amplified with phosphoramidite primer primers 402-TR and 403-TF, using ClonAmp HiFi PCR Premix, according to manufacturer's instructions, in 42 reactions of 25 µl, cycling 15 times 98 °C for 10 s, 65 °C for 10 s, and 72 °C for 30 s (Amplicon 3). Primer 402-TR variabilized codons in Loop W, which code for residues 838–844. Primer 403-TF variabilized codons in Loop Z, which code for residues 931–935. Amplicon 3 was gel purified on 1% agarose using 8 Qiaquick Gel Extraction Kit columns, eluting each one with 50 µl EB and combining. Both the amplified phagemid (Amplicon 3) and the insert (Amplicon 2) were purified using Qiaquick PCR Purification Kit columns and eluted with 100 µl and 20 µl EB, respectively, yielding the phagemid at 152 ng/µl and the insert at 174 ng/µl.

The phagemid library CNL-2 was created by Gibson Assembly cloning the linear phagemid library, containing 4 variable codons in Loop V (residues 817–820), 7 variable codons in Loop W (residues 838–844), and 5 variable codons in Loop Z (residues 931–935), for a total of 16 variable residues in 3 loops, to the insert region

between loops W and Z. Briefly, 4.17 µg of phagemid and 1.52 µg of insert were combined in an 830 µl reaction containing 415 µl of Gibson Assembly Master Mix (2X) (NEB), and incubated at 50 °C for 15 min and then put on ice. The ligated DNA was then purified and concentrated in one MinElute PCR Purification Kit column, and eluted in 25 µl EB. The DNA was then desalted on a VSWP 0.025 µm membrane (EMD Millipore) on ddH₂O for 1 h with a water change at 30 min. The desalted DNA was then adjusted to 75 ng/µl with ddH₂O and used to electroporate electrocompetent TG1 cells (Lucigen). Approximately 51 µl of DNA was added to 1.25 ml ice cold TG1 cells and pipetted up and down 4 times to mix on ice, after which 25 µl aliquots were transferred to 50 electroporation cuvettes (with 1 mm gaps) on ice. The cells were electroporated as described, and immediately quenched with 975 µl Lucigen recovery media, pooled, and incubated at 37 °C, 250 rpm for 1 h. To titer the library, 10 µl of recovered culture was serially diluted in 2xYT and 10 µl of each dilution spotted on 2xYT/glu/carb and incubated at 30 °C overnight. The remaining library was expanded to 3 L 2xYT/glu/carb and amplified overnight at 30 °C, 250 rpm. The next day, the library was pelleted at 10 k × g, 10 min, 4 °C and the media discarded. The pellet was re-suspended to an OD₆₀₀ of 75 in 2xYT/2% glucose/18% glycerol, aliquoted and stored at –80 °C.

2.2. Example of library panning: maltose binding protein (MBP) as target

For the first round of panning, 3 L of 2xYT/glu/carb was inoculated with 4 ml of the CNL-2 glycerol stock (OD₆₀₀ = 75), to an OD₆₀₀ of approximately 0.1 and grown at 37 °C, 250 rpm until the OD₆₀₀ reached 0.5. From the initial culture, 750 ml was super-infected with 466 µl of the M13 filamentous helper phage VCSM13 (1E13 phage/ml) (Agilent, Santa Clara, CA), at a ratio of approximately 20 phage to 1 cell, and incubated at 37 °C for 30 min at 100 rpm, and then for 30 min at 250 rpm. The cells were pelleted at 10 k × g, 10 min, and the media discarded. The cells were then resuspended in 1.5 L 2xYT/Carb (100 µg/ml)/Kan (70 µg/ml), and incubated overnight at 30 °C, 250 rpm. The cells were pelleted at 10 k × g for 10 min and the phage containing supernatant transferred to clean tubes containing 5X PEG/NaCl (20% polyethylene glycol 6000/2.5 M NaCl), mixed well and incubated on ice for 25 min. The phage was pelleted at 13 k × g, 25 min and the supernatant discarded. The phage was resuspended in 60 ml PBS and centrifuged at 13 k × g, 10 min to remove insoluble material. The supernatant was precipitated with 5X PEG/NaCl again and incubated on ice for 5 min before spinning down the phage again at 13 k × g, 20 min. The supernatant was discarded and the pellet resuspended in 30 ml PBS, with an A₂₆₈ of 6.6.

For solution panning of biotinylated MBP, two sets of 100 µl of Dynabeads MyOne Streptavidin T1 (ThermoFisher Scientific, Waltham, MA) streptavidin coated magnetic beads slurry were washed

2 × 1 ml with PBS-T (applying a magnet in between washes to remove the supernatant), and then blocked in 1 ml of 2% dry milk in PBS with 0.05% Tween20 (2% M-PBS-T) for 1 h, rotating, at room temp. Unless stated otherwise, all panning and screening incubations are carried out at room temperature. After blocking the beads, the magnet was applied and the blocking agent removed. To pre-clear the phage solution before incubating with the biotinylated antigen, 1 ml of phage solution (prepared in the previous step) was incubated on one set of the blocked beads for 1 h, rotating. The magnet was applied and the precleared phage transferred to a clean tube. The biotinylated MBP (Avidity, Aurora, CO) was then added to the precleared phage solution at a concentration of 100 nM (4.4 µg/ml) and incubated for 1.5 h rotating to allow the phage to bind to the antigen.

The phage/antigen solution was then transferred to the second set of blocked beads and incubated for 20 min to capture antigen bound phage. The magnet was applied and the supernatant discarded. The beads were then washed and resuspended 8 times with 1 ml PBS-T, switching to fresh tubes after the third, fifth, and seventh wash, and precipitating the beads with the magnet in between each wash for approximately 2 min. The beads were eluted with 800 µl 0.1 M glycine, pH 2 for 10 min, the magnet applied, and the supernatant aspirated into a tube with 72 µl 2 M Tris base to neutralize before adding the entire neutralized eluate to 9 ml of mid-log phase XL1-blue cells ($OD_{600} = 0.44$). The cells were infected for 45 min at 37 °C, 150 rpm. The unamplified output titer was measured by preparing ten-fold serial dilutions of 10 µl of culture and spotting 10 µl of each on 2xYT/flu/carb agar plates and incubating overnight at 30 °C. The culture was expanded to 100 ml 2xYT/flu/carb and incubated overnight at 30 °C, 250 rpm, and then for a few hours at 37 °C in the morning.

The overnight cultures were harvested by measuring the OD_{600} , centrifuging the cells at 10 k×g, 10 min, and then resuspending the cells to an OD_{600} of 75 in 2xYT/18% glycerol. To prepare phage for the next round of panning, 5 ml of 2xYT/flu/carb was inoculated with 5 µl of the 75 OD_{600} glycerol stock and incubated at 37 °C, 250 rpm until the OD_{600} reached 0.5. The cells were superinfected at 20:1 phage:cell, mixed well, and incubated at 37 °C, 30 min, 150 rpm, then 30 min at 250 rpm. The cells were pelleted at 5500 × g, 10 min, the glucose containing media discarded and the cells resuspended in 10 ml 2xYT/Carb/Kan and incubated overnight at 30 °C, 250 rpm.

The overnight phage prep was centrifuged at 10 k×g, 10 min, and the supernatant transferred to 2.5 ml 5X PEG/NaCl, mixed, and incubated on ice for 25 min to precipitate the phage. The phage was then pelleted at 13 k × g for 20 min, and the supernatant discarded. The phage was then resuspended in 1 ml PBS and the insoluble material removed by centrifugation at 20 k×g for 5 min. The supernatant was applied to 0.25 ml of 5X PEG/NaCl and precipitated a second time for 5 min on ice. The phage was pelleted at 13 k × g, 5 min, 4 °C, the supernatant removed, and the pellet resuspended in 750 µl PBS. The phage was then prepared at $A_{268} = 0.8$ in 2% M-PBS-T, and the panning continued as described, except in the third round the concentration of biotinylated antigen incubated with the precleared phage was lowered to 10 mM, the phage concentration was lowered to an A_{268} of 0.2, and the number of washes was increased to 12 to increase selectivity of higher affinity phage.

2.3. ELISA of individual clones following panning

At the end of the last panning round (usually after round 3 or 4), individual colonies were plated on 2xYT/flu/carb plates following the 45 min 37 °C, 150 rpm recovery of the infected XL1-blue cells with the eluted phage. The next day 96 colonies were inoculated into 400 µl 2xYT/flu/carb in a 96-well, deep-well culture plate, and

grown overnight at 37 °C, 300 rpm to generate a master plate, to which glycerol was added to 18% for storage at –80 °C. To prepare an induction plate for the ELISA, 5 µl of each masterplate culture was inoculated into 400 µl fresh 2xYT/0.1% flu/carb and incubated for 2 h 45 min at 37 °C, 300 rpm. IPTG was then added to 0.5 mM and the plates incubated at 30 °C, 300 rpm overnight. Because the phagemid contains an amber stop codon, some CBM protein is produced without the pIII domain, even though XL1-blue is a suppressor strain, resulting in the periplasmic localization of some CBM, of which some percentage is ultimately secreted to the media. The media can then be used directly in an ELISA. After the overnight induction, the plates were centrifuged at 1200×g for 10 min to pellet the cells.

Streptavidin or neutravidin coated microtiter plates (ThermoFisher) were rinsed 3 times with 200 µl PBS, and then coated with biotinylated MBP at 1 µg/ml at 100 µl/well and incubated 1 h. For blank controls, a plate was incubated with 100 µl/well PBS. The wells were then washed 3 times with 200 µl PBS-T, and blocked with 200 µl 2% M-PBS-T for 1–3 h. The block was removed and 50 µl of 4% M-PBS-T added to each well. At this point 50 µl of each induction plate supernatants were transferred to both a blank and an MBP coated well and pipetted 10 times to mix, and incubated 1 h. The plates were washed 4 times with 250 µl PBS-T in a plate washer using the dispense only function, and the plates dumped and slapped on paper towels in between washes. After the washes, 75 µl of 1/2000 dilution anti-FLAG-HRP in 4% M-PBS-T was added to each well and incubated 1 h. The secondary was dumped and the plates washed as before. The plates were developed by adding 75 µl TMB Ultra substrate (ThermoFisher), and analyzed for positives compared to controls. Positives were then grown up from the masterplate by inoculating 1 ml 2xYT/flu/carb with 3 µl glycerol stock and incubated for at least 6 h at 37 °C, 250 rpm. The cells were then pelleted and the media discarded. Plasmid DNA was prepared from the pellets using the Qiaprep Spin Miniprep Kit, and the sequences determined by Sanger sequencing at Genewiz (South Plainfield, NJ).

2.4. Expression and purification of nanoCLAMPs (1 L scale)

Positives identified from the ELISA using the secreted binders were subcloned into a pET vector coding for a 13 amino acid C-terminal GS-linker followed by a cysteine (Fig. 2B). This construct codes for a mature protein of 163 amino acids with a molecular weight of 17.6 kDa (weight with native loops). The CBM cDNA, including its N-terminal 6-His tag, was amplified from the pComb3X phagemid clones prepared in the previous section using primers 391 F and 450 R, in a 25 µl reaction containing 12.5 µl ClonAmp HiFi PCR Premix, and cycling 30 times 98 °C for 10 s, 65 °C for 10 s, and 72 °C for 30 s (Amplicon 4). A pET15b (EMD Millipore) vector containing native CBM M929L was used as template (although pET15b could be used) for amplifying the vector using primers 390 R and 387 F in a 50 µl rxn containing 25 µl ClonAmp HiFi PCR Premix, cycling the same way (Amplicon 5). These two amplicons were gel purified on 1.1% agarose gel using a Qiagen MinElute Gel Extraction Kit. Between 20 and 100 ng of insert and vector were fusion cloned in a 5 µl InFusion reaction (Takara) at 50 °C, 15 min. Chemically competent BL21(DE3) *E. coli* were heat-shocked with 1.5 µl of the InFusion reaction and recovered in 500 µl SOC for 1 h, 37 °C, 250 rpm. The cells were plated on 2xYT/flu/carb and incubated overnight at 37 °C. Individual colonies were grown up in 10 ml 2xYT/flu/carb cultures for at least 7 h, after which plasmid was purified for sequencing to confirm insertion of the CBM cDNA. Meanwhile, the cultures were seeded into 1 L 2xYT/flu/carb and grown to an OD_{600} of 0.8 at 37 °C, 250 rpm, induced to 0.5 mM IPTG and incubated 20–24 h at 30 °C, 250 rpm. The

overnight cultures were pelleted at $10 \text{ k} \times \text{g}$, 10 min, at 4°C , and the media discarded. The pellets were lysed in 9 ml 6 M guanidine-HCl (GuHCl), 0.1 M NaH_2PO_4 , 10 mM Tris, pH 8.5 (Buffer A, pH 8.5) per g wet weight pellet, and incubated for 1 h to overnight. The insoluble material was pelleted at $30 \text{ k} \times \text{g}$, 30 min, at 15°C , and the supernatant transferred to a clean tube containing 5–10 ml Ni-NTA SF (Qiagen) equilibrated in the same buffer, and incubated 2 h - overnight rotating at room temp. The beads were pelleted at $1 \text{ k} \times \text{g}$, 1 min, and the flow-through discarded. The beads were transferred to a column and washed with 3 column volumes (CVs) Buffer A, pH 8.5 + 1 mM TCEP at 1 ml/min, or until the A_{280} flatlined. The beads were then washed with 3 CVs of same buffer with no TCEP. At this point, a portion of the resin was separated and used to purify native, refolded protein (see next section: *Purification of native nanoCLAMPs for biophysical analysis*). The denatured, purified protein was eluted with 3–4 CVs of Buffer A, pH 8.5 + 250 mM imidazole, and pooled. The protein was quantified by measuring the absorbance at 280 nm and purity assessed by ethanol precipitating 25 μl of the eluate to remove the GuHCl and resuspending the pellet in SDS-sample buffer with reducing agent, then analyzing 10 or more micrograms of protein on a 12% Bis-Tris NuPage gel stained with GelCode Blue (ThermoFisher).

2.5. nanoCLAMP naming convention

The nanoCLAMPs were named according to the Uniprot gene name of the target protein, the product identifier (A1, A2, etc), and the conjugation entity. For example, the nanoCLAMP to MBP has two forms; male-A1(Cys) and male-A1(Resin), where male is the Uniprot name for the *E. coli* gene that produces the protein Maltose-binding periplasmic protein, A1 is our designation of this particular binder, (Cys) indicates a lone, C-terminal cysteine, and (Resin) indicates the protein is covalently conjugated to Sulfolink resin via a thioether bond (described below).

2.6. Purification of native nanoCLAMPs for biophysical analysis

After washing the denatured, bound nanoCLAMPs, and separating 1 ml of the resin (from section *Expression and Purification of nanoCLAMPs*, above), the protein was refolded on the resin by washing with 10 CVs of Qiagen Lysis Buffer, QLB (50 mM NaH_2PO_4 , 300 mM NaCl, pH 8.0) at 1.0 ml/min, followed by 10 CVs of same buffer at flow rate of 0.5 ml/min. The resin was then washed with 10 CVs of 50 mM NaH_2PO_4 , pH 8 (no salt), and then 10 CVs of 50 mM NaH_2PO_4 , 1 M NaCl, pH 8 (high salt). The high salt was then removed with 10 CVs of QLB. The refolded protein was then eluted with Qiagen Elution Buffer (QLB + 250 mM imidazole) at 0.5 ml/min until the $A_{280} < 0.1$. Proteins were dialyzed into 10 mM MOPS, pH 6.5 with at least 4 buffer changes over 8 h and freeze-dried in 10-nmol aliquots (180 μg) with a Labconco (Kansas City, MO) Triad freeze dryer.

2.7. Analysis of nanoCLAMP monodispersity by size exclusion chromatography

Native nanoCLAMPs were resuspended in Nectagen Resuspension Buffer 1, NRB1 (50 mM Tris, 500 mM L-Arg, 5 mM EDTA, 0.05% Tween20, pH 7.2) to a final concentration of 0.18 mg/ml. Prior to analysis the samples were reduced with 5 mM TCEP (made fresh in H_2O) for 30 min at 4°C , centrifuged at $20 \text{ k} \times \text{g}$, 5 min 4°C , and the supernatants transferred to a clean tube. The samples were loaded into a 100 μl sample loop and injected onto a Superdex 75 10/300 GL column (GE Healthcare Life Sciences, Pittsburgh, PA) equilibrated in Tris Buffered Saline (TBS) + 1 mM TCEP, pH 7.2, at a flowrate of 0.65 ml/min. The column was calibrated with Bio-Rad

Gel Filtration Standard per manufacturer's instructions.

2.8. Preparation of nanoCLAMP(Resin) (100 μl scale)

The purified, denatured nanoCLAMPs in Buffer A, pH 8.5 from section *Expression and purification of nanoCLAMPs* were directly conjugated to Sulfolink cross-linked, 6% beaded agarose (ThermoFisher). Briefly, 100 μl of packed resin was equilibrated by washing the beads 3 times with at least 5 CVs of Buffer A, pH 8.5, and transferred to a 1.3 ml column. The protein was added at a concentration of approximately 6–8 mg/ml in a volume of 200 μl , and incubated rotating at room temp for 15 min. The resin was allowed to settle for 30 min, and the column drained to the top of the resin bed. The columns were washed 3 times with 600 μl Buffer A, pH 8.5, then incubated with 800 μl 50 mM L-Cys (prepared fresh) and incubated rotating for 15 min, allowed to settle for 15 min, drained to the top of the resin bed, and washed 2 times with 800 μl 1 M NaCl to remove the L-Cys. The protein was refolded on the column by washing 4X with 800 μl 20 mM MOPS, 150 mM NaCl, 1 mM CaCl_2 , pH 6.5. We have historically included CaCl_2 in the storage buffer because the native CBM possesses a Ca^{2+} binding site (PDB 2W1Q), but further stability studies are required to determine if this is necessary. The beads were then transferred to a clean tube and azide added to the MOPS buffer at 0.05% to inhibit microbial growth.

2.9. Affinity purification of antigen from *E. coli* whole cell lysate using nanoCLAMP(Resin)

BL21(DE3) *E. coli* cells were grown to an OD_{600} of 4–8 at 37°C , 250 rpm, pelleted at $10 \text{ k} \times \text{g}$, 10 min, 4°C , the media discarded, the pellets weighed and frozen. A whole cell lysate was prepared by lysing pellets with BPER, a nonionic, detergent-based cell lysis reagent that disrupts cells and solubilizes native or recombinant proteins without denaturation, supplemented with Pierce™ Universal Nuclease for Cell Lysis, at 4 ml BPER per gram of pellet, per manufacturer's instructions (ThermoFisher). The insoluble material was pelleted at $30 \text{ k} \times \text{g}$, 10 min, 4°C , and the supernatant transferred to a clean tube. The cleared lysate was diluted into PBS so that the final concentration of BPER was 20%, the $\text{OD}_{600} = 8$ (calculated from the dilution of the original culture), and the concentration of the spiked antigen = 0.1 mg/ml. We chose to spike-in purified antigens to prepare the antigen-containing lysates as opposed to using lysates containing over-expressed antigen in order to control the amount of target protein in the crude lysates. To 1.4 ml spiked lysate, 20 μl of a 50% slurry of nanoCLAMP resin was added and incubated at 4°C , rotating, for 1 h. The resin was transferred to a small chromatography column and the flow-through drained to the top of the resin bed. The resin was washed $4 \times 400 \mu\text{l}$ PBS at room temp (passing 400 μl through these small columns took approximately 30 s). The bound antigen was eluted $5 \times 25 \mu\text{l}$ polyol elution buffer, or PEB (10 mM Tris, 1 mM EDTA, 0.75 M ammonium sulfate, 40% propylene glycol, pH 7.9), and the elutions combined. For cleaner analysis by SDS-PAGE, the PEB can be removed by desalting column or TCA/acetone precipitation, as sometimes the PEB causes streaking up the sides of the bands on SDS-PAGE. The PEB should be made fresh each month. The resin can be regenerated by washing with Buffer A, pH 8.5, $4 \times 500 \mu\text{l}$, followed by $2 \times 500 \mu\text{l}$ with 20 mM MOPS, 150 mM NaCl, pH 6.5 (MBS), and finally $4 \times 500 \mu\text{l}$ with MOPS + 1 mM CaCl_2 . Stripping the columns with GuHCl appeared to remove all non-covalently bound protein, as no residual protein was evident in subsequent washes or elutions (data not shown). We typically regenerate working stocks of resins over five times with no apparent loss in binding capacity or specificity. The resin can be stored at 4°C with

azide at 0.05%.

2.10. Depletion of SlyD from Ni-NTA eluate using slyD-A1(Resin)

A 50 μ l aliquot of a 1.2 mg/ml recombinant protein preparation containing the contaminant SlyD (verified by tryptic digest of SDS gel slice followed by mass spec analysis, data not shown) was incubated with 10 μ l of either slyD-A1(Resin) or a negative control nanoCLAMP(Resin) in small chromatography columns, rotating at room temperature for 1 h. The resins were allowed to settle and the flow through collected. SDS-PAGE samples were prepared by mixing 52 μ l of the eluted protein with 20 μ l 4X LDS buffer and 8 μ l NuPage Sample Reducing Agent (ThermoFisher), and heating to 95 °C for 5 min. The non-depleted protein and the resin flow-throughs were analyzed on 12% SDS-PAGE under reducing conditions with amounts adjusted so that the flow-through contained approximately 20% more target protein than the non-depleted sample, to rule out dilution effect artifacts.

2.11. Beta-galactosidase activity assay following elution from lacZ-A2(Resin)

Beta-galactosidase (Rockland Immunochemicals, Inc., Limerick, PA) was bound to lacZ-A2(Resin) by incubating 20 μ l packed, PBS rinsed resin with 400 μ l beta-galactosidase at 0.1 mg/ml in PBS for 1 h, room temp. The flow-through was drained, the resin washed 4 \times 400 μ l PBS and then resuspended in PBS and split into equal parts in small chromatography columns, and drained to their beds. For the polyol elution, the resin was eluted with 3 \times 25 μ l PEB (5 min per elution). The other set of resin was eluted with low pH by sequentially adding 3 \times 25 μ l 0.2 M glycine, pH 2.5 (quickly), and immediately neutralizing the pooled eluates with 7.5 μ l 1 M Tris base, pH 10.4. A non-processed positive control (Pos Control) was prepared by diluting 20 μ l of 1 mg/ml β -galactosidase to 125 μ l with PBS. A neutralization buffer control (NB Control) was prepared by diluting 20 μ l of 1 mg/ml β -galactosidase to 125 μ l with pre-neutralized low pH elution buffer (prepared by mixing 1 ml of 0.2 M glycine, pH 2.5 with 100 μ l of 1 M Tris, pH 10.4). The concentrations of the resin eluates and the two controls were normalized to 0.07 mg/ml by diluting with their respective buffers and analyzed on SDS-PAGE to ensure all samples contained equal amounts of β -galactosidase. Serial dilutions of each β -galactosidase sample were prepared in PBS, and 50 μ l aliquots of each were added to a 96-well microtiter plate. Aliquots of 150 μ l of ortho-Nitrophenyl- β -galactoside (ONPG, Thermo) substrate were added, mixed by pipetting, developed for 15 min, and the absorbance measured at 450 nm.

2.12. Microscale thermophoresis

Microscale thermophoresis (MST) was carried out by 2bind GmbH (Regensburg, Germany) on a Monolith NT.115 Pico

(NanoTemper Technologies, Munich, Germany) as described in Table 2, using standard methods [31]. This technique requires that one of the proteins of a binding pair (shown in Table 2) be fluorescent so that binding of the non-fluorescent partner causes a measurable change in the thermophoresis of the fluorescent protein. For three of the binding pairs, we had fluorescent constructs available: GFP (for GFP-A1), NusA-GFP (for nusA-A1(Cys)), and SMT3-GFP (for SMT3-A1). These fluorescent proteins were expressed as 6-His fusions and purified under native conditions using Ni-NTA-SF (Qiagen). The nanoCLAMPs GFP-A1 and SMT3-A1 are variants of GFP-A1(Cys) and SMT3-A1(Cys) that were subcloned to remove the C-terminal linker and Cys to prevent homodimerization through disulfides, and were expressed and purified as described for nanoCLAMPs (above). The nanoCLAMP nusA-A1(Cys) was used without removing the C-terminal Cys. To prepare a fluorescent binding partner for the remaining two pairs, we conjugated the nanoCLAMPs AVD-A1(Cys) and malE-A1(Cys) to Alexa Fluor 568 C5 Maleimide or Alexa Fluor 647 C2 Maleimide (Thermo) and desalted 2X with Zeba desalting columns (Thermo) to remove unincorporated dye. The binding partners for these two fluorescent nanoCLAMPs were recombinant Avidin (Thermo) and MBP (Atgen, Gyeonggi-do, South Korea). Serial dilutions of the non-fluorescent binding partner protein were prepared in PBS, and aliquots of 5 μ l of each dilution were mixed with 5 μ l of constant concentration fluorescent binding partner, which filled the MST capillaries. The fluorescence was measured with respect to time over the laser induced thermal gradient at the various ratios of non-fluorescent protein to fluorescent protein, and K_d determined by fitting the resulting binding curve to the non-linear solution of the law of mass action. Each experiment was carried out twice.

2.13. Analysis of polyol elution conditions

GFP-A1(Cys) was reduced with 1 mM TCEP for at least 30 min and immobilized on wells of a black Pierce Maleimide Activated 96-Well Plate (Thermo) per manufacturer's instructions, at a concentration of 5 μ g/ml. Wells were washed and blocked with 2% M-PBS-T for 2 h, then rinsed with PBS-T and allowed to dry at room temp. GFP-A1(Cys) coated wells were incubated with GFP at 20 μ g/ml in 2%M-PBS-T. The wells were washed 5X with PBS and then treated with TE elution buffers with either 0, 20, or 40% propylene glycol containing 0, 0.25, 0.5, or 0.75 M ammonium sulfate and incubated for 1 h at room temp. The elutions were removed and the wells washed 5X with 200 μ l PBS-T. After the last wash, the wells were adjusted to 100 μ l with PBS-T and the fluorescence measured using a Tecan Infinite F200 plate reader (λ_{exc} = 485, λ_{em} = 535). Wells were measured in duplicate and averaged. The fluorescence was reported after subtracting background fluorescence from negative control wells containing no nanoCLAMP, that had been incubated with GFP and washed with PBS. The negative controls were not distinguishable above background, indicating the fluorescent proteins required nanoCLAMPs to bind to the plate.

Table 2
Microscale thermophoresis parameters.

Fluorescent protein ^a	Binding partner	Binding partner conc range (nM)	Constant fluorescent protein conc (nM)	LED %	Laser %
AVD-A1(Cys)-AF	Avidin	5.00–0.153	5.00	30	40
GFP	GFP-A1	5.00–0.153	10.0	15	20
malE-A1(Cys)-AF	MBP	5.00–0.153	5.00	3	80
NusA-GFP	nusA-A1(Cys) ^b	10.0–0.305	100	20	80
SMT3-GFP	SMT3-A1	5.00–0.153	10.0	15	20

^a Fluorescent proteins were prepared by conjugating maleimide-Alexafluor (AF) to a C-terminal Cys, or by expressing and purifying the protein as a GFP-fusion.

^b nusA-A1(Cys) contains a free C-terminal Cys, and could be present to some degree as a homodimer.

3. Results

3.1. Scaffold properties

We reviewed the RCSB protein databank for immunoglobulin-like protein structures that might serve as a scaffold for the development of antibody mimetics. Several candidates were selected and tested for suitability. The preliminary testing criteria included high level expression in *E. coli*, tolerance of amino acid substitutions in one or more loops, tolerance of N- and C-terminal fusions and compatibility with phage display. The second Type 32 carbohydrate binding module of NagH met all of these preliminary criteria. This domain consists of a sandwich of 10 anti-parallel beta strands and resembles the 7–9 stranded immunoglobulin domain found in many receptors and antibodies (PDB 2W1Q and Fig. 1). Like the complementarity-determining region of antibody variable domains, the domain recognizes its natural sugar ligands via loops connecting the beta-strands [30]. However, unlike the analogous antibody domains, the scaffold's parent domain does not contain any cysteines. In addition, the N- and C-termini of the scaffold are opposite from the end engaged with ligand so fusions are less likely to result in steric interference with ligand binding.

The natural domain has a single methionine at position 929. Because methionine is susceptible to oxidation, we tested whether the scaffold would tolerate replacement of the methionine. We produced a version with a conservative M929L mutation, which we then confirmed did not have a significant qualitative effect on the scaffold's thermal stability or expression level in *E. coli*. In addition, the isolated domain has unstructured N- and C-terminal regions, which are unlikely to contribute to binding but might promote aggregation or be vulnerable to proteolytic attack. For subsequent experiments, we therefore used a minimized version with the unstructured residues deleted.

3.2. Library construction

We next used the refined and minimized scaffold as the basis for an M13 filamentous phage display library based on the pComb3X phagemid (Fig. 2A). The library contained a total of sixteen randomized amino acids distributed in three loops at positions 817–820 (Loop V), 838–844 (Loop W), and 931–935 (Loop Z). The randomized portion of the library was synthesized with degenerate oligos containing phosphoramidite trimers encoding a single codon for every amino acid except for cysteine and methionine. The resulting library contains approximately 10^{10} unique clones and is designated library CNL-2.

3.3. Screening and characterization of candidate nanoCLAMPs

We next screened the phage display library for nanoCLAMPs against a variety of targets, including three solubility enhancing tags (SUMO, NusA, and MBP), two fluorescent proteins (GFP and mCherry), an affinity tag (Avidin), and a common histochemistry marker (β -galactosidase). Each screen required 1 to 3 rounds of panning to yield a set of clones with a workable number of positives. Typically, a sample of 96 clones from each final round contained 1 to 30 unique clones that tested positive in a secondary ELISA. After the ELISA confirmatory screen, positives were sub-cloned into an *E. coli* T7 expression vector (Fig. 2B) with an N-terminal 6His-tag and a C-terminal GS-linker and cysteine. The mature proteins contain 163 amino acids and have a molecular mass of approximately 17.6 kDa. Each construct produced between 50 and 200 mg of the nanoCLAMP per liter of culture. The nanoCLAMPs' facile expression and purification enabled screening to include direct assessment of the performance of the purified binders in

affinity chromatography rather than an indirect assessment of the binders' performance when expressed on the phage surface. The nanoCLAMPs were named according to the convention described in Materials and Methods. Table 3 lists the nanoCLAMPs along with their respective targets.

Each candidate nanoCLAMP was purified and then bound to Ni-NTA resin under denaturing conditions. The denaturant was washed away and the proteins refolded on the column prior to elution under non-denaturing conditions. Some of the isolated nanoCLAMPs showed aggregation in size exclusion chromatography (SEC) so we also screened for those nanoCLAMPs whose preparations consisted primarily of monodisperse monomer (Fig. 3). In this gel filtration experiment, the monomer is theoretically expected to elute at around 15 ml. The dimer is theoretically expected at 13.25 ml. The actual elution volumes measured for nanoCLAMPs varied from 14 to 15.3 ml. When reducing agent was omitted, the elution volume shifted to 13–13.5 ml presumably because of the formation of a dimer linked by a disulfide bond between the C-terminal cysteines (data not shown). We have not further characterized the variation in elution volumes, which may reflect conformational or charge differences caused by differences in the variable loops.

nanoCLAMPs eluting in the monomeric range were then assessed for their ability to serve as capture reagents immobilized on affinity resin. The top candidate was analyzed by microscale thermophoresis in order to quantitate the binding affinity of each nanoCLAMP-target interaction. The measured dissociation constants ranged from 11 to 417 nM (Table 3). The nanoCLAMPs selected were given names using the convention described in Materials and Methods.

3.4. Preparation of chromatography resin and purification of target antigens from *E. coli* lysates

To test the candidates' utility for affinity chromatography, we conjugated the nanoCLAMPs to 6% cross-linked agarose resin via their C-terminal cysteines and assessed the working binding capacity and specificity of each nanoCLAMP. The conditions of the conjugation reaction were consistent across preparations. Each resin was tested for its ability to purify the corresponding recombinant target protein spiked into *E. coli* whole cell lysates so that the target was in excess of the resin. The spiked lysate was incubated with the appropriate resin in batch, washed and then eluted with polyol elution buffer (PEB; 10 mM Tris, 1 mM EDTA, 0.75 M ammonium sulfate, 40% propylene glycol, pH 7.9). The eluted protein was analyzed to assess working binding capacity and specificity. We report the working binding capacity as 75% of the average yield of target protein purified after a 1 h incubation with a lysate containing 0.1 mg/ml of target protein. The intent of calculating the working binding capacity in this manner is to provide a practical lower limit for expected recovery from a complex lysate. The working binding capacities observed ranged from 20 to 180 nmol/ml of packed resin (Table 3). We were unable to discern a readily apparent relationship between the dissociation constant of the nanoCLAMP-target interaction and the working binding capacity of the corresponding resin (Table 3). Presumably, other variables such as steric accessibility, coupling efficiency, or avidity effects differ from nanoCLAMP to nanoCLAMP and confound the relationship between affinity and working binding capacity. For example, nanoCLAMP nusA-A1, with a comparatively low affinity ($K_d = 417$ nM), produces a resin with a comparatively high working binding capacity (43 nmol/ml resin, or 2.93 mg/ml resin).

The resins passing this screen had sufficient affinity, selectivity and polyol-responsiveness to yield a single, predominant Coomassie-stained band representing the target protein when

Table 3
nanoCLAMPs and their target proteins.

nanoCLAMP(Cys)	Target	Target Acc#	K _D (nM)	nanoCLAMP(Resin)	Resin working binding capacity (nmol/ml resin)	Resin working binding capacity (mg/ml resin), (MW of target used)
AVD-A1(Cys)	Avidin	P02701	20	AVD-A1(Resin)	40	2.64 (66 kDa)
GFP-A1(Cys)	GFP	P42212	32	GFP-A1(Resin)	75	2.23 (29.7 kDa)
malE-A1(Cys)	MBP	P0AEX9	11	malE-A1(Resin)	20	0.88 (44.0 kDa)
nusA-A1(Cys)	NusA	P0AFF6	417	nusA-A1(Resin)	43	2.93 (67.5 kDa)
SMT3-A1(Cys)	SMT3	Q12306	163	SMT3-A1(Resin)	176	4.16 (23.6 kDa)
mCher-A2(Cys)	mCherry	X5DSL3	n.m.	mCher-A2(Resin)	58	1.68 (28.9 kDa)
lacZ-A2(Cys)	Beta-gal	P00722	n.m.	lacZ-A2(Resin)	6.7	3.12 (465 kDa)

^a (Cys) indicates the nanoCLAMP has a C-terminal GS-linker followed by a free cysteine.

^b Target Acc#'s refer to UniProt accession numbers.

^c Dissociation constants of nanoCLAMPs and their targets were determined by microscale thermophoresis (MST). nanoCLAMPs were conjugated to maleimide conjugated fluorescent dyes through their C-terminal cysteine prior to analysis in the cases where the targets were not fluorescent proteins or conjugated to fluorescent proteins (see Table 2 for details). Interactions that were not measured are denoted n.m.

^d (Resin) indicates the nanoCLAMPs were conjugated to Sulfolink resin via their C-terminal cysteines.

^e Resin working binding capacity was determined by incubating resin in spiked *E. coli* lysate with the target in excess for 1 h, 4 °C, washing, eluting with polyol elution buffer, and quantifying eluate yield and purity. The Resin working binding capacity presented here is 75% of the average recoverable target protein per ml of resin from multiple experiments.

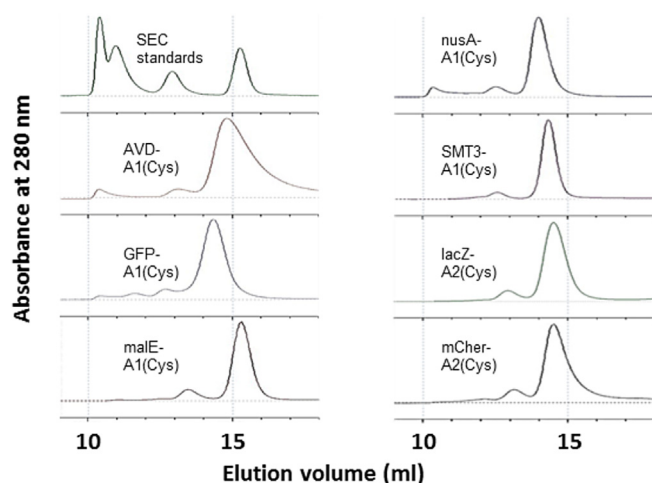


Fig. 3. Size exclusion chromatography of purified nanoCLAMPs. SEC standard proteins: from left to right, 670, 158, 44, and 17 kDa standards. Proteins analyzed are indicated on chromatographs. Monomers are expected to elute near 15 ml, dimers near 13 ml and higher order aggregates between 13 ml and the void volume of 10 ml.

analyzed by SDS-PAGE (Fig. 4). The extra bands observed in some cases are likely to represent degradation products or multimers of the target protein because the bands are only present when the target was spiked-in. Finally, GFP and mCherry exhibited strong fluorescence activity while bound to the resin and in the eluate. These observations indicate that the polyol elution buffer did not irreversibly and completely disrupt the protein conformations required to produce fluorescence.

3.5. Selectivity in whole cell lysates from different species

To explore the applicability of nanoCLAMP affinity resins to other species, we tested the selectivity of a GFP-targeted nanoCLAMP resin in human, mouse, and insect cell lysates. Like the *E. coli* lysate, purification from these sources recovered the spiked-in protein without apparent contaminants. These results indicate that the selectivity of at least one nanoCLAMP is sufficient to avoid co-purifying contaminants from whole cell lysates from a diverse set of organisms (Fig. 5).

3.6. Purification of enzymatically active protein with polyol elution buffer

The fluorescent proteins eluted in polyol elution buffer (Fig. 4) retained fluorescence activity and were not irreversibly inactivated. Indeed, the polyol elution buffer composition used in this study has previously been used by other laboratories to purify a diverse set of enzymatically active, multi-subunit complexes [10,12,32]. To assess the purification procedure's ability to preserve the structure and activity of native complexes, we applied the procedure to the purification of β -galactosidase, a homo-tetrameric enzyme whose activity depends on its quaternary structure [33].

We bound purified β -galactosidase to the appropriate nanoCLAMP resin and eluted the protein with either low pH glycine elution buffer or polyol elution buffer. The eluate was neutralized immediately in the case of the low pH buffer, and both eluates were assayed for β -galactosidase activity. For controls, we tested unprocessed β -galactosidase in PBS, as well as in neutralized glycine buffer. As shown in Fig. 6, β -galactosidase eluted by low pH buffer and then neutralized did not retain detectable activity. In contrast, the β -galactosidase eluted by polyol elution buffer retained activity comparable to untreated positive controls. Because the activity of β -galactosidase is dependent upon tetramerization, these results indicate that the polyol-based purification procedure preserves both activity and quaternary structure in at least one case.

3.7. Removal of a contaminant with a nanoCLAMP affinity resin directed towards an endogenous protein

Because many researchers might wish to isolate nanoCLAMPs against a protein of interest, we determined the time and effort required to use our optimized procedure to isolate a nanoCLAMP against an endogenous protein. As a test case, we isolated nanoCLAMPs against the *E. coli* protein SlyD. In the course of purifying nanoCLAMPs by Ni-NTA chromatography, we often observed contamination by SlyD, a 24 kDa, histidine-rich peptidyl-prolyl cis-trans isomerase that has been reported as a common contaminant of 6-His protein preparations [34]. Removal of SlyD contamination from 6-His preparations is useful in cases where SlyD's physical presence or enzymatic activity interferes with subsequent experiments so we sought to develop a nanoCLAMP SlyD resin as a general tool for removing the contaminant. To do so, we first screened library CNL-2 against recombinant SlyD, isolated several binders, and prepared an affinity resin with one. We then tested the ability of this resin to remove native SlyD from one of our Ni-NTA eluates

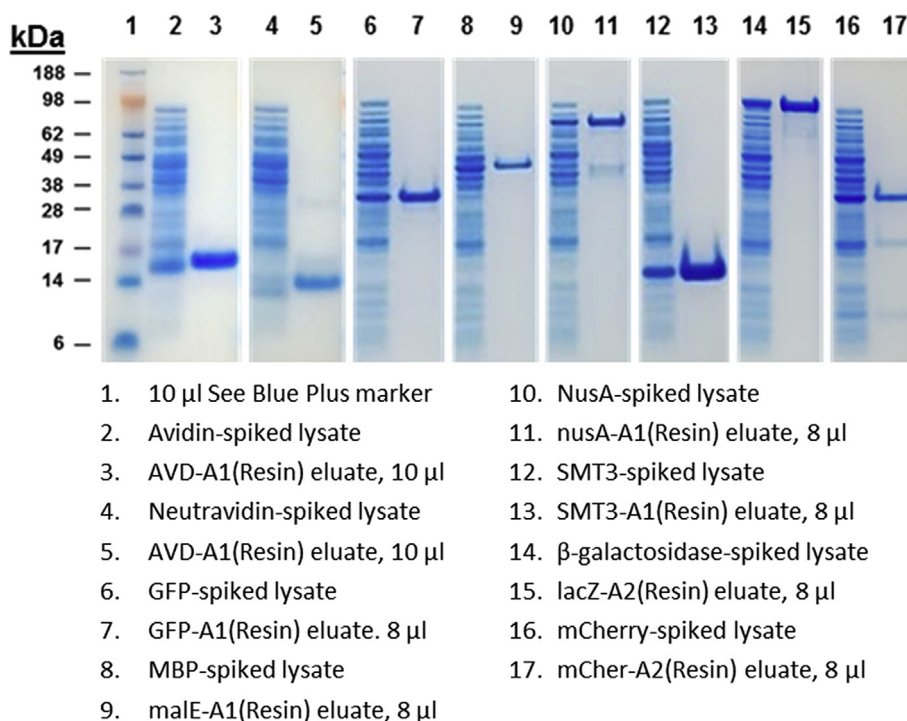


Fig. 4. SDS-PAGE analysis of one-step purification of spiked proteins from *E. coli* BL21(DE3) whole cell lysates using nanoCLAMP chromatography resin. *E. coli* were lysed with BPER, cleared by centrifugation, and diluted with PBS to 1.2 mg total protein/ml. The lysate was spiked with recombinant antigen to 0.1 mg/ml in 1.5 ml and incubated with 10 μ l (packed vol) nanoCLAMP resin. Beads were washed with PBS and eluted with polyol elution buffer at near neutral pH. The gel was loaded with lysate samples containing approximately 10 μ g total protein or with indicated volumes of eluates (from a total of approximately 125 μ l eluate) from nanoCLAMP resins, in reducing SDS-sample buffer. Eluates were acetone precipitated or buffer exchanged prior to SDS-PAGE analysis. Note: the band in lane 11 near 40 kDa was determined by mass spec to be a fragment of NusA.

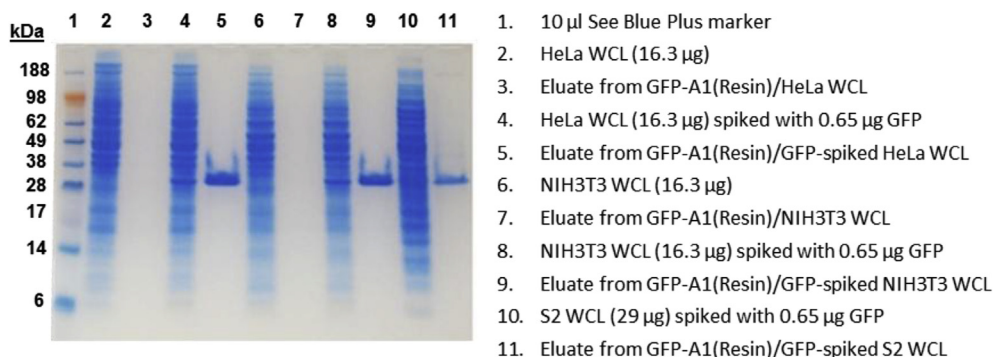


Fig. 5. SDS-PAGE analysis of one-step purification of spiked proteins from human, mouse, and insect whole cell lysates using GFP-A1(Resin) chromatography resin. The indicated whole cell lysates (WCL) were prepared with and without spiked recombinant GFP to 0.1 mg/ml and then incubated with GFP-A1(Resin). The resin was washed and eluted with 60 μ l polyol elution buffer (HeLa and NIH3T3) or 30 μ l (S2). WCLs and 16 μ l of polyol eluates from GFP-A1(Resin) were analyzed on 12% SDS-PAGE in reducing SDS sample buffer.

by incubating the eluate with the nanoCLAMP and collecting the flow-through. As shown in Fig. 7, the flow through does not contain detectable amounts of a Coomassie-stained SlyD contaminating band when analyzed by SDS-PAGE.

The production of the SlyD resin required approximately 8 weeks and approximately 120 person-hours of effort to produce purified, biotinylated target protein, pan library CNL-2, isolate nanoCLAMPs, and produce and test the affinity resin.

3.8. Analysis of polyol-responsiveness

The polyol elution buffer used up to this point consisted of TE buffer containing 40% propylene glycol (PG) and 0.75 M ammonium sulfate (AS), a composition which has been previously documented

as an effective polyol elution buffer. To determine which buffer components are required to dissociate nanoCLAMPs from their target proteins, we chose one representative, GFP-A1(Cys), and covalently immobilized it on a black polystyrene microtiter plate. We added GFP, washed, exposed the complex to varying combinations of PG and AS to elute the GFP, and then measured the remaining fluorescence on the plate. As shown in Fig. 8, wells eluted with no PG had a remaining fluorescence of over 27,000 au. Increasing the concentration of AS in the absence of PG did not significantly improve the elutions. Increasing the concentration of PG improved the elution, with over 50% of the initial fluorescence eluted with 40% PG and no AS. Addition of AS to the PG containing buffers improved the elutions, with nearly all of the fluorescence eluted after elution with 40% PG and 0.75 M AS. This result indicates

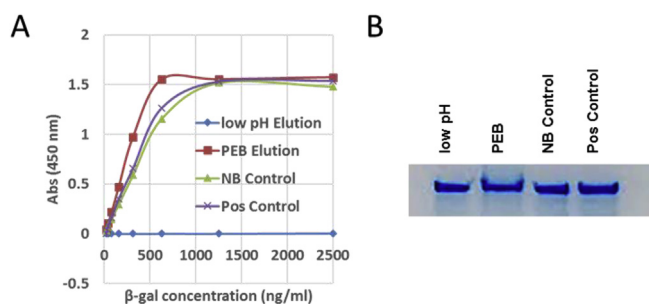


Fig. 6. Activity of β -galactosidase eluted from lacZ-A2(Resin). β -galactosidase was eluted from lacZ-A2(Resin) using traditional low pH elution followed by neutralization or polyol elution buffer (PEB). Panel A: the eluted protein was quantified, normalized, and analyzed for activity using ONPG substrate. Non-processed β -galactosidase was similarly quantified and normalized and analyzed for activity in PBS (Pos Control) or in neutralized buffer (NB Control). Panel B: the protein used in the ONPG assay was analyzed by SDS-PAGE as a control to ensure β -galactosidase was present in each assay, and normalized correctly.

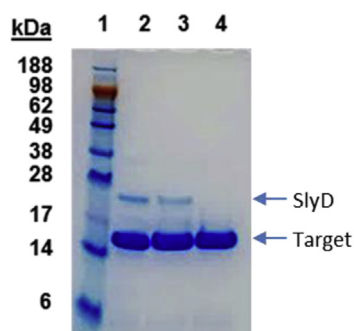


Fig. 7. Depletion of SlyD contaminant from Ni-NTA eluate. A protein purified by Ni-NTA chromatography (lane 2) was incubated with a non-SlyD specific nanoCLAMP(Resin) (lane 3) and with slyD-A1(Resin)(lane 4), allowed to flow through the resin, and analyzed on Coomassie-stained SDS-PAGE. Lane 1 is the MW marker.

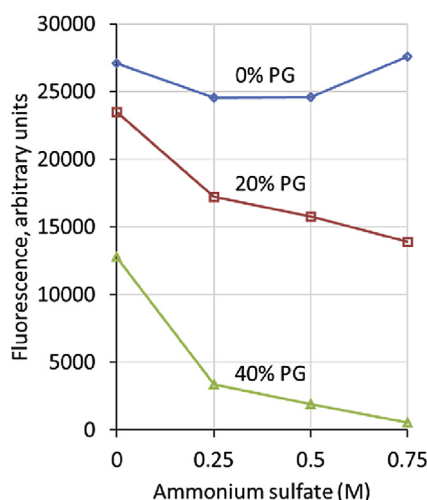


Fig. 8. Response of GFP-A1(Cys) release of GFP to varying concentrations of propylene glycol (PG) and ammonium sulfate (AS). GFP-A1(Cys) was covalently immobilized on polystyrene, reacted with GFP, washed, treated with TE containing varying concentrations of PG and AS, and then analyzed for remaining fluorescence.

this polyol-responsive nanoCLAMP requires both 40% PG and 0.75 M AS for optimal elution of GFP, with incremental decreases in efficiency of elution upon lowering either PG or AS.

4. Discussion and conclusions

This report describes a single-step protein purification procedure based on nanoCLAMP single domain antibody mimetics derived from a carbohydrate binding module from the *Clostridium perfringens* hyaluronidase NagH. The affinity, selectivity and polyol-responsiveness of these nanoCLAMP antibody mimetics are sufficient to purify a variety of proteins from bacterial, mammalian and insect whole cell lysates in a single step. The purification procedure has the additional benefit of avoiding exposure of the target protein to non-physiological pH, denaturants, proteases or competing antigens. We believe that the nanoCLAMP scaffold is inherently polyol-responsive because 95% of the nanoCLAMPs we have tested exhibit this property despite their isolation by a procedure that does not enrich for polyol-responsiveness. To the best of our knowledge, the nanoCLAMP scaffold is the first reported to possess the general property of polyol-responsiveness. To date, we have sought and obtained functional nanoCLAMPs against 13 of 14 target proteins. In addition to producing useful reagents for protein purification, the nanoCLAMP technology is expected to be accessible to most laboratories. The current screening procedure for new nanoCLAMPs uses standard molecular biology techniques and requires approximately 3 man-weeks of effort and 8 weeks of elapsed time. Once isolated, nanoCLAMPs can be produced economically in gram-quantities using shake flasks, with typical yields of 50–300 mg of protein per liter of *E. coli* culture, which usually yields between 8 and 11 g of wet weight cells.

The work described here is significant in describing novel affinity chromatography reagents useful for the rapid and effective purification of a diverse set of commonly used proteins and protein tags. Taken together, our results are also significant in establishing the general utility of a novel technology platform for developing polyol-responsive affinity reagents.

Because of their ability to preserve activity and quaternary structures, polyol-responsive antibodies and antibody mimetics represent an attractive approach to the purification of functional multi-subunit protein complexes. Polyol-responsive affinity reagents support the development of scalable purification procedures whose speed, simplicity and effectiveness enable the functional or structural analysis of large numbers of protein variants. With emerging techniques such as CryoEM, functional proteomics, and high throughput structure-function assays dependent upon the ready availability of purified protein preparations, the nanoCLAMP technology described here represents a timely, significant and complementary addition to established approaches for protein purification.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.pep.2017.04.008>.

Funding

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Competing interests

Richard Suderman, Daren Rice, Shane Gibson, and Eric Strick are employees of Nectagen, Inc. Richard Suderman, Daren Rice, and David Chao are inventors on patents covering the nanoCLAMP technology. David Chao and Richard Suderman hold significant equity stakes in Nectagen, Inc. The Stowers Institute for Medical Research or BioMed Valley Discoveries did not fund or oversee this work, which is independent of and unrelated to David Chao's

employment at those institutions.

Availability of research materials

For academic researchers agreeing to maintain derivatives as open source, all DNA and amino acid sequences are shared without charge at (<https://www.nectagen.com>). Plasmids are distributed at a nominal price under an open source license. nanoCLAMPs and affinity chromatography resins are available for sale.

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