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. Immunofinity 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, immunofinity chromatography avoids genetic modification but requires the development of a suitable antibody. Immunofinity 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. Immunofinity 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 non-chaotropic salt such as ammonium sulfate. Polyol-responsive...
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 mimic 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, Affittins, Alphabodies, Anticalins, Avimers, DARPinS, Fynomers, LVbs, VHHs, 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 mimic 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 (nano CLostridial Antibody Mimetic Proteins). 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 pIIl (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 μl reaction with ClonAmp HiFi PCR Mix, according to 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 μl 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 μl reaction with 10 μl 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 Qiagen PCR Purification Kit column, and eluted in 10 μl EB. The DNA was then desalted on a Millipore (EMD Millipore, Billerica, MA) VSWP 0.025 μm 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 μl of DNA at 40 ng/μl to each of 6 aliquots of 25 μl of cells on ice in 0.1 cm electroporation cuvettes. The DNA was electroporated using a MicroPulsar (Bio-
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 electroportion of Lucigen Recovery Media, pooled, and incubated at 37 °C, at 275 rpm, for 1 h in a shaking incubator. To titrate the sublibrary, 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 containing 5 µl of native CBM using primers 402-TR variabilized codons, by amplifying 2 ng of native CBM using primers 405A R TTCCAGATTAGTCAGACGAATGTACTTAG Amplicon 2

The phagemid mini-library was expanded to 3 L of 2xYT/glu/carb with 5 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, and 4°C and the media discarded. The pelleted 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 glycere 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 superinfected 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 × 10 min and the phage containing supernatant transferred to clean tubes containing SxPEG/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 A280 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 PBS-T. The beads were then washed and resuspended 8 times with 1 ml 2X YT/gluc/0.44% with the eluted phage. The next day 96 colonies were inoculated on 1 ml 2xYT/gluc/carb in a 96-well, deep-well culture plate, and incubated for at least 6 h at 37 °C, 250 rpm. The overnight cultures were harvested by measuring the OD600, centrifuged at 10,000 g, 10 min, the glucose containing media discarded and the media pelleted and the media discarded. Plasmid DNA was prepared from the pellets using the Qiaprep Spin Miniprep Kit, and the sequence 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 plasmid cDNA, including its N-terminal 6-His tag, was amplified from the pComb3X phagemid clones prepared in the previous section using primers 390 R and 387 F in a 50 μl PCR reaction containing 12.5 μl ClonAmp HiFi PCR Premix, and cycling 30 times 98 °C, 60 s, 65 °C, 15 s. 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 pelleted resuspended in 750 μl PBS. The phage was then prepared at A260 = 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 A260 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/gluc/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/gluc/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% gluc/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 plII 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 (Thermo-Fisher) 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. Then, 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 dropped and the plates washed as before. The plates were developed by adding 75 μl TMB Ultra substrate (Thermo-Fisher), and analyzed for positives compared to controls. Positives were then grown up from the masterplate by inoculating 1 ml 2xYT/gluc/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).
overnight cultures were pelleted at 10 k x 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 NaH2PO4, 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 k x 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 k x 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 A280 flattened. 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 nanoclamp 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 because the native CBM possesses a Ca2+ 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.

2.6. Purification of native nanoclamp 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 NaH2PO4, 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 NaH2PO4, pH 8 (no salt), and then 10 CVs of 50 mM NaH2PO4, 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) and incubated rotating for 15 min, allowed to settle for 15 min, 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 CaCl2, pH 6.5. We have historically included CaCl2 in the storage buffer because the native CBM possesses a Ca2+ 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.

2.7. analysis of nanoclamp monodispersity by size exclusion chromatography

Native nanoclamps were resuspended in Nectagen Resuspending Buffer 1, NR81 (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 H2O) for 30 min at 4 °C, centrifuged at 20 k x 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, Pittsburg, 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 nanoclamp 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 CaCl2, pH 6.5. We have historically included CaCl2 in the storage buffer because the native CBM possesses a Ca2+ 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 OD600 of 4–8 at 37 °C, 250 rpm, pelleted at 10 k x 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 k x 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 OD600 = 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 lyses as opposed to using lyses 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 x 400 µl PBS at room temp (passing 400 µl through these small columns took approximately 30 s). The bound antigen was eluted 5 x 25 µl 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/acetate 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 x 500 µl, followed by 2 x 500 µl with 20 mM MOPS, 150 mM NaCl, pH 6.5 (MBS), and finally 4 x 500 µl with MOPS + 1 mM CaCl2. 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 μl 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 μl 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 μl of the eluted protein with 20 μl 4X LDS buffer and 8 μl NuPage Sample Reducing Agent (ThermoFisher), and heating to 95 °C for 5 min. The non-depleted protein and the flow-throughs were analyzed on 12% SDS-PAGE using 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 μl packed, PBS rinsed resin with 400 μl beta-galactosidase at 0.1 mg/ml in PBS for 1 h, room temp. The flow-through was drained, the resin washed 4 × 400 μl PBS and then resuspended in PBS and split into equal parts in small chromatography columns, and drained to their beds. For the poloy elution, the resin was eluted with 3 × 25 μl PEB (5 min per elution). The other set of resin was eluted with low pH by sequentially adding 3 μl 0.2 M Tris, pH 2.5 (quickly), and immediately neutralizing the pooled eluates with 7.5 μl 1 M Tris base, pH 10.4. A non-processed positive control (Pos Control) was prepared by diluting 20 μl of 1 mg/ml β-galactosidase to 125 μl with PBS. A neutralization buffer control (NB Control) was prepared by diluting 20 μl of 1 mg/ml β-galactosidase to 125 μl with pre-neutralized low pH elution buffer (prepared by mixing 1 ml of 0.2 M Tris, pH 2.5 with 100 μl 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 μl aliquots of each were added to a 96-well microtiter plate. Aliquots of 150 μl of ortho-Nitrophenyl-β-D-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 Zebar desalting columns (Thermo) to remove unincorporated dye. The binding partners for these two fluorescent nanoCLAMPS were recombinant Avidin (Thermo) and MBP (Axygen, Gyeonggi-do, South Korea). Serial dilutions of the non-fluorescent binding partner protein were prepared in PBS, and aliquots of 5 μl of each dilution were mixed with 5 μl 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 Kd 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 μg/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 μg/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 μl PBS-T. After the last wash, the wells were adjusted to 100 μl with PBS-T and the fluorescence measured using a Tecan Infinite F200 plate reader (λex = 485, λem = 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.

<table>
<thead>
<tr>
<th>Fluorescent protein</th>
<th>Binding partner</th>
<th>Binding partner conc range (nM)</th>
<th>Constant fluorescent protein conc (nM)</th>
<th>LED %</th>
<th>Laser %</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVD-A1(Cys)-AF</td>
<td>Avidin</td>
<td>5.00–0.153</td>
<td>5.00</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>GFP</td>
<td>GFP-A1</td>
<td>5.00–0.153</td>
<td>10.0</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>malE-A1(Cys)-AF</td>
<td>MBP</td>
<td>5.00–0.153</td>
<td>5.00</td>
<td>3</td>
<td>80</td>
</tr>
<tr>
<td>NusA-GFP</td>
<td>nusA-A1(Cys)b</td>
<td>10.0–0.205</td>
<td>100</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>SMT3-GFP</td>
<td>SMT3-A1</td>
<td>5.00–0.153</td>
<td>10.0</td>
<td>15</td>
<td>20</td>
</tr>
</tbody>
</table>

*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^{16} 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](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2633998/)). 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 subcloned 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 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.

Some of the 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 (Kd = 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.

<table>
<thead>
<tr>
<th>nanoCLAMP(Cys)</th>
<th>Target</th>
<th>Target Acc#</th>
<th>K_D (nM)</th>
<th>nanoCLAMP(Resin) Resin working binding capacity (nmol/ml resin)</th>
<th>Resin working binding capacity (mg/ml resin), (MW of target used)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVD-A1(Cys)</td>
<td>Avidin</td>
<td>P20701</td>
<td>20</td>
<td>AVD-A1(Resin)</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.64 (66 kDa)</td>
</tr>
<tr>
<td>GFP-A1(Cys)</td>
<td>GFP</td>
<td>P42212</td>
<td>32</td>
<td>GFP-A1(Resin)</td>
<td>75</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.23 (29.7 kDa)</td>
</tr>
<tr>
<td>malE-A1(Cys)</td>
<td>MBP</td>
<td>P0AE9X</td>
<td>11</td>
<td>malE-A1(Resin)</td>
<td>20</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td>0.88 (44.0 kDa)</td>
</tr>
<tr>
<td>nusA-A1(Cys)</td>
<td>NusA</td>
<td>P0A5F6</td>
<td>417</td>
<td>nusA-A1(Resin)</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.93 (67.5 kDa)</td>
</tr>
<tr>
<td>SMT3-A1(Cys)</td>
<td>SMT3</td>
<td>Q12306</td>
<td>163</td>
<td>SMT3-A1(Resin)</td>
<td>176</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>4.16 (23.6 kDa)</td>
</tr>
<tr>
<td>mCher-A2(Cys)</td>
<td>mCherry</td>
<td>X5DSL3</td>
<td>n.m.</td>
<td>mCher-A2(Resin)</td>
<td>58</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1.68 (28.9 kDa)</td>
</tr>
<tr>
<td>lacZ-A2(Cys)</td>
<td>Beta-gal</td>
<td>P00722</td>
<td>n.m.</td>
<td>lacZ-A2(Resin)</td>
<td>6.7</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.12 (465 kDa)</td>
</tr>
</tbody>
</table>

a (Cys) indicates the nanoCLAMP has a C-terminal CS-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.

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 peptide-yl-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.

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).
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
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 Nagl. 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 scaleable 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.

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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](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.

**References**


