

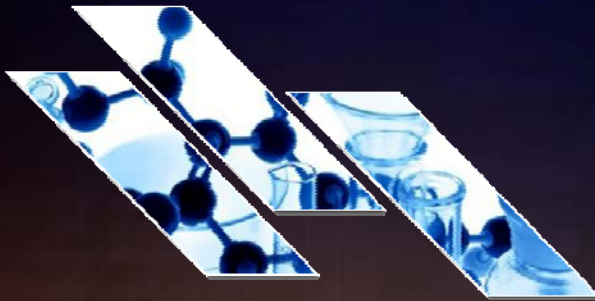


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DESIGN AND TESTING OF A THERMOSTABLE PLATFORM FOR MULTIMERIZATION OF SINGLE DOMAIN ANTIBODIES



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14. ABSTRACT-LIMIT 200 WORDS Immunoaffinity reagents are used to detect biothreat agents, but one limitation is the thermal sensitivity of standard antibodies. The abilities to maintain recognition and remain thermostable provide enhanced capability for fielding immunoaffinity-based sensors. One technology capable of delivering thermostability is single domain antibodies. These molecules have substantial thermostability, but affinity is often reduced as compared to monoclonal antibodies. Using recombination, we created single domain antibodies (sdAb's) in a thermostable platform and used the sequences for two sdAb's that recognized unique epitopes on the ricin A chain. We tethered them to a platform on the basis of the structural maintenance of chromosome proteins. The engineered molecules were characterized by surface plasmon resonance, and the thermoplasticity of the molecules was tested up to 55 °C. The engineered constructs did bind to ricin. They demonstrated an avidity increase over the sdAb's. The heated sample maintained half of the biological activity. These results indicate that by multimerizing the sdAb's on a thermostable platform, it is possible to increase avidity and maintain some thermoplasticity. This platform allows for a "plug and play" approach to multimerization of sdAb's, and it has the potential for application to signaling and mitigating activities.					
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EXECUTIVE SUMMARY

Immunoaffinity reagents have been demonstrated to be powerful tools for the detection of biothreat agents. Despite this success, one limitation of these tools is the inherent thermal sensitivity of the molecules. Therefore, the ability to maintain specific and sensitive recognition characteristics in combination with increased thermostability provides an enhanced capability for fielding immunoaffinity-based sensors. A potential technology capable of delivering this improved thermostability is the use of single domain antibodies (sdAb's), which are generated from the blood of sharks and camelids (camels and llamas). Largely due to their small size (12–14 kDa), these molecules have substantial thermostability as well as the ability to refold after exposure to extreme temperatures (thermoplasticity). However, the current lack of hybridoma technology for these species forces the use of in vitro panning methods for their selection. This often leads to a general reduction in affinity toward the target antigen when compared to standard mouse monoclonal antibodies. One solution to this challenge is to multimerize the antibodies, which increases the number of binding sites and leads to enhanced recognition.

We used recombinant technology to multimerize sdAb's in a flexible, scalable, and thermostable platform. We then used the sequences for two sdAb's that recognized unique epitopes on the ricin A chain and tethered them to a platform based on the structural maintenance of chromosome (SMC) proteins. The SMC portion of the molecule was derived from the thermophilic organism *Pyrococcus furiosus*. The engineered molecules were produced in *Escherichia coli*, and their characteristics were determined by surface plasmon resonance. In addition, the thermostability and thermoplasticity of the molecules were tested up to 55 °C. The engineered constructs exhibited very tight binding to ricin and demonstrated an avidity increase over the sdAb's, alone. Upon exposure to high temperatures, the constructs maintained half of the biological activity. These results indicate that by multimerizing the sdAb's on a thermostable platform, it is possible to increase avidity and maintain some thermoplasticity. This platform will allow for a “plug and play” approach to multimerization of sdAb's, and it has the potential for application to signaling and mitigating activities.

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PREFACE

The work described in this report was started in October 2008 and completed in September 2010.

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DESIGN AND TESTING OF A THERMOSTABLE PLATFORM FOR MULTIMERIZATION OF SINGLE DOMAIN ANTIBODIES

1. INTRODUCTION

The development of antibody-based detection systems has proven to be of great use in the detection and initial characterization of biothreat agents. The specificity and sensitivity of antibodies have made them a valuable resource in the continued evolution of sensor development. Despite the successes achieved by using antibodies generated from mammalian sources (e.g., mice, goats, and rabbits), several drawbacks exist. The primary drawbacks relate to the need for maintaining the antibodies in a refrigerated environment and the inherent instability associated with these molecules, leading to reduced shelf-life. These reasons limit the use of antibodies in the field of immunology.

Recent developments in immunology have demonstrated the existence of single domain antibodies (sdAb's), which are made up of a single heavy-chain molecule from several organisms, including sharks, camels and llamas (1). It is now possible to isolate small variable domains (12–14 kDa) that are highly specific to a particular antigen (2, 3). The small size of the isolated variable domains results in sdAb's that are extremely thermostable. The use of sdAb's is limited by the lack of tumor cell lines to produce hybridomas, and the arduous isolation by phage display technology often leads to reduced binding affinity. One method that has been developed to overcome this limitation is the multimerization of a given single variable domain to allow for enhanced affinity (4). Although this has proven valuable, it has not been conducted in a manner that maintains the thermostability of a molecule because the domains used for dimerization are not thermostable, which results in a molecule that still has the inherent limitations of standard mammalian antibodies (4). We attempted to overcome these limitations by designing a platform for multimerizing sdAb's by using the structural maintenance of chromosome (SMC) proteins. This conserved class of proteins forms a flexible intramolecular coiled-coil and intermolecular dimerization with a second SMC protein as shown in Figure 1 (5). This family of proteins is highly conserved, and there are well-characterized SMC proteins from the archael kingdom, including the thermophiles (6).

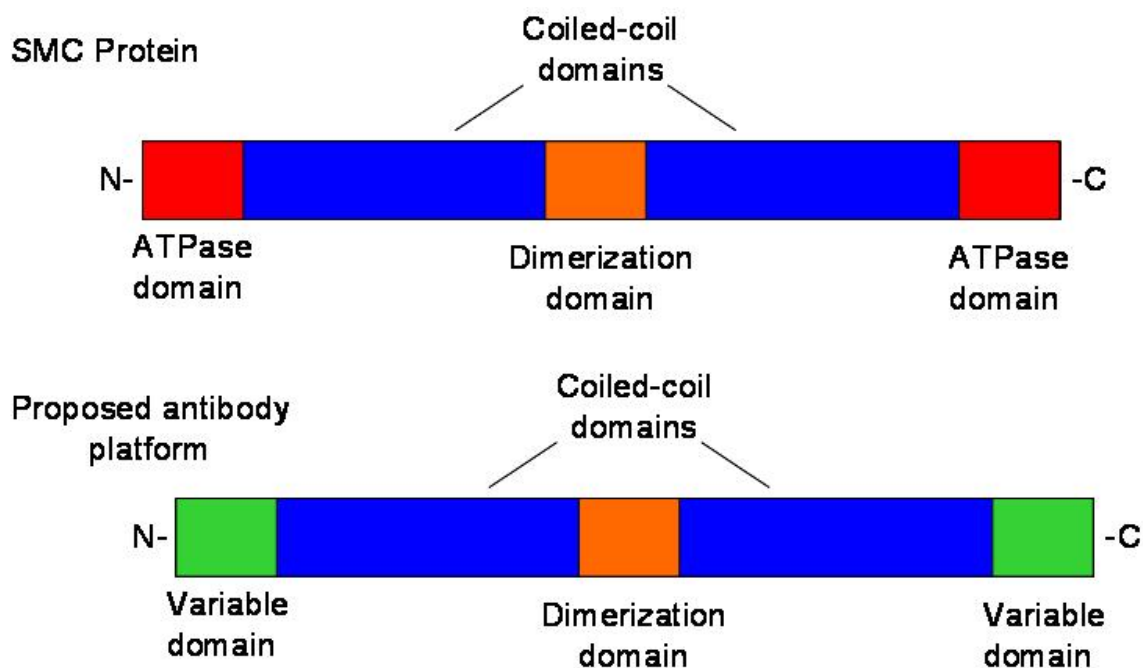


Figure 1. Comparison of SMC molecules and the antibody nanoassembly. The upper portion shows the schematic of a standard SMC protein. The lower portion indicates how the antibody nanoassembly was created by substituting the ATPase domain of the SMC protein with variable domains of a llama single domain antibody. This scaffolding allowed for two different variable domains to be incorporated into the molecule.

2. METHODOLOGY

2.1 Design of C8smcF11*

Published sequences of *Pyrococcus furiosus* were used with structural and functional data to design the construct (6–8). The program Coils (version 2.2 by A. N. Lupas, and programmed by J. M. Lupas, Tübingen, Germany) was used from the Swiss EMBnet node server to help determine where the coils and the dimerization domain were located. The sequence, which calculated the probability of the sequence adopting a coiled-coil conformation, was uploaded into the Coils program. Dr. Ellen Goldman (U.S. Naval Research Laboratory, Washington, DC) provided the sequences for α ricin C8 and α ricin F11 and two sdAb's (derived from a llama) that recognize distinct epitopes of the ricin A chain.

* C8smcF11 was made inhouse by Alena Calm of the Biotechnology Branch, Research and Technology Directorate, U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD.

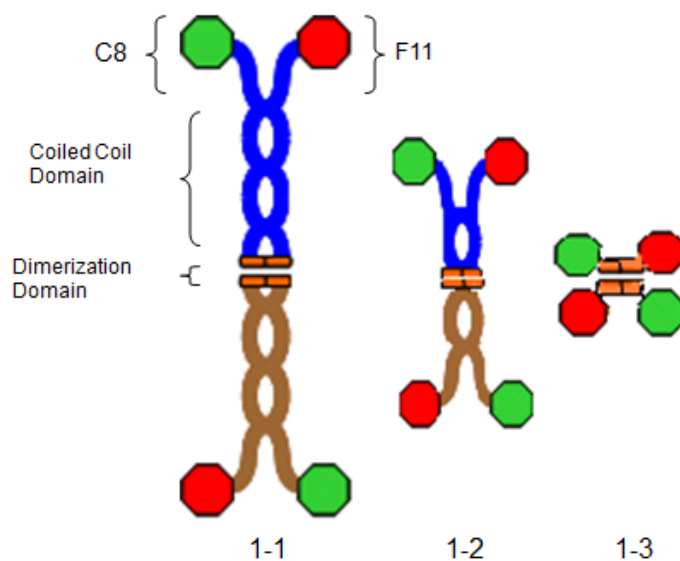


Figure 2. Illustration of C8smcF11. The red and green octagons represent the two different single domain ricin antibodies. The variable domains are tethered to the SMC coiled-coil backbone, which then dimerize. This allows for the creation of a ricin-binding heteropolymeric quadravalent recognition moiety. Three constructs were designed, one with a full length coiled-coil domain, one that was truncated, and one that contained the dimerization domain only.

These sequences were incorporated into the SMC construct at the site of the ATPase domain (2, 3, 9). Three constructs were designed and designated as C8smcF11: 1-1, 1-2, and 1-3. Each construct contained the two sdAb's sequences C8 and F11 with a varied length of coiled coil, and each construct had a distinctive characteristic (Figure 2):

- 1-1 was the full length version
- 1-2 had a truncated length of coiled coil
- 1-3 consisted of the dimerization domain only

Initially, DNA2.0 cloned the sequence for C8smcF11:1-1 into two different vectors, pJexpress401 and pET28a(+), for evaluation. Both plasmids were received lyophilized. Upon receipt, they were rehydrated and transformed into BL21(DE3)SLyD- strains of *E. coli*. A pilot expression study was done to compare the expression levels between the two vectors, and the results indicated that pET28a(+) was the better vector (Figure 3). DNA2.0 was used to clone the sequences for C8smcF11:1-2 and 1-3 in pET28a.

2.2 Protein Expression and Purification

The three C8smcF11 constructs were expressed in BL21(DE3)SLyD-electrocompetent *E. coli*. Transformations were performed using electroporation. Briefly, 100 μ L of electrocompetent cells and 1 μ L of plasmid were thawed on ice. The plasmid was then added to the cells, and the mixture was triturated. The mixture was transferred to a chilled Bio-Rad electroporation cuvette (Bio-Rad Laboratories, Hercules, CA), which was immediately placed in

the electroporator. Two and one-half kilovolts of current were applied to the cells before they were immediately transferred into 1 mL of super optimal broth with catabolite repression ([commonly known as SOC media] Thermo-Fisher Scientific, Waltham, MA). The culture was incubated at 37 °C for 1 h with agitation and then plated on lauria broth (Thermo-Fisher Scientific) with appropriate antibiotic (30 µg/mL kanamycin). The plates were incubated overnight at 37 °C. We used Auto Induction Instant terrific broth (Thermo-Fisher Scientific) media from Novagen (Billerica, MA), which contained components that were metabolized differentially to promote high density cell growth and induce protein expression from *lac* promoters without the usual steps of monitoring cell density and adding isopropyl β-D-1-thiogalactopyranoside (Thermo-Fisher Scientific). Ten milliliters of media, containing 30 µg/mL of kanamycin, were inoculated with a single transformant and grown to log phase before being scaled up to a 500 mL culture. These half-liter cultures were then grown at 37 °C at 300 rpm for 20 h. The cell paste was collected via centrifugation and stored at -80 °C. Crude analysis of the three constructs revealed that C8smcF11:1-2 had poor expression levels. We focused on C8smcF11:1-1 and 1-3 and used the bacterial protein extraction reagents ([Pierce] Thermo-Fisher Scientific) to determine if the proteins were soluble. The 1-3 protein was almost completely insoluble, and it had to be purified under denaturing conditions (using 8 M urea).

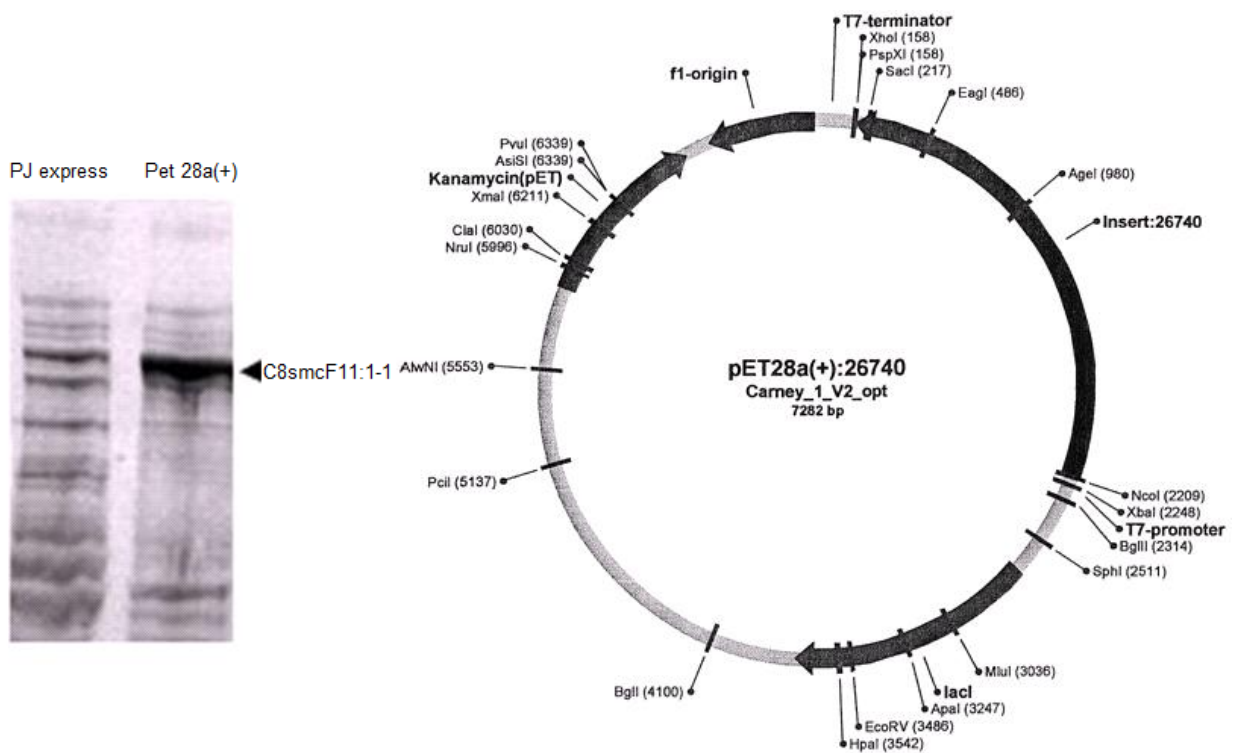


Figure 3. (Left) Pilot expression comparing pJexpress401 and pET28a(+) in BL21(DE3)SLyD-*E. coli*; (the 80 kDa highlighted band corresponds to the predicted molecular weight of C8smcF11:1-1), and (right) vector map supplied by DNA2.0 of C8smcF11:1-1 in pET28a(+).

2.3 Soluble Purification of C8smcF11:1-1

Each construct was designed with a hexahistidine tag to ease purification; therefore, the first step in purification was to perform immobilized metal ion affinity chromatography (IMAC). The initial attempt involved batch affinity purification. The cell paste was thawed on ice and resuspended in lysis buffer (20 mM sodium phosphate, 500 mM sodium chloride, and 10 mM imidazole pH 8.0). The cells were ruptured by French press and centrifuged to pellet the remaining cell debris. The clarified lysate was incubated with nickel-nitrilotriacetic acid (Thermo-Fisher Scientific) agarose (Qiagen, Germantown, MD) with gentle agitation at 4 °C. The agarose was separated from the lysate via gentle centrifugation. The beads were washed, and the protein was eluted off with 300 mM imidazole.

The initial product was noticeably degraded. The purification scheme was modified to use the AKTA Purifier FPLC (GE Healthcare Biosciences, Pittsburgh, PA). The first columns employed were 1 mL HisTrap FF (GE Healthcare) prepacked nickel columns. The cell paste was processed, as described previously, with the addition of ethylenediaminetetraacetic acid free protease inhibitors (Thermo-Fisher Scientific). The clarified lysate was pumped onto the column and washed prior to elution with 300 mM imidazole. The product from these initial FPLC runs was still degraded. Procedural modifications, including faster processing of cell paste and the use of extra protease inhibitors, helped reduce proteolysis. We also switched to a 1 mL HisTrap FF crude column, which helped alleviate problems with high back pressure. The elution was changed from a gradient elution to a step elution. Over 40 column volumes, the concentration of imidazole was increased to 500 mM in four increments. After these modifications, the protein eluted off the nickel columns in an intact condition. A considerable amount of nonspecific protein came off in the elution, which was remediated by adding ion exchange as a final step. The peak fractions from the HisTrap were pooled (Figure 4) and desalted into 25 mM Tris 7.6 pH, 50 mM NaCl using a 5 mL HiTrap desalting column (GE Healthcare). The peak fractions from the desalting column were pooled and loaded onto a 1 mL FF Q column (GE Healthcare). The protein was eluted using a salt concentration gradient from 50 to 500 mM NaCl over 10 column volumes. The fractions were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis ([SDS-PAGE] Bio-Rad). The fractions were pooled and buffer exchanged into phosphate-buffered saline-T 7.4 pH (Bio-Rad) for surface plasmon resonance (SPR) analysis (Bio-Rad). The aliquots were made and snap-frozen in liquid nitrogen.

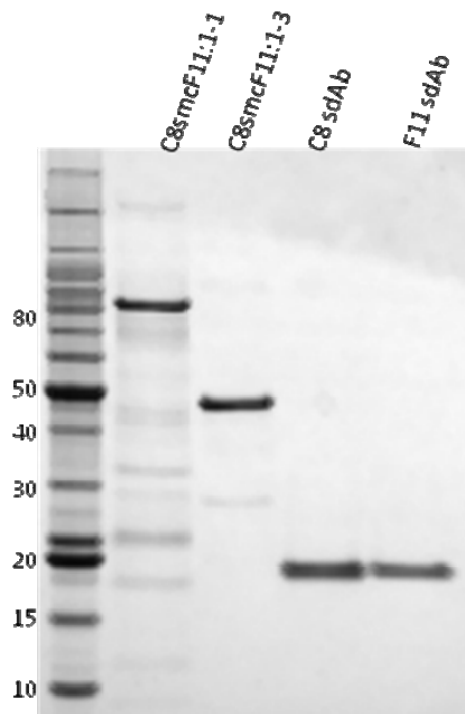


Figure 4. 4–12% SDS-PAGE: lanes one and two are pooled fractions from IMAC; lanes three and four are samples supplied to us by Ellen Goldman.

2.4 Insoluble Purification of C8smcF11:1-3

Frozen cell paste was thawed on ice and resuspended in lysis buffer (20 mM sodium phosphate, 500 mM sodium chloride, and 10 mM imidazole, pH 8.0). The cells were ruptured by French press and centrifuged to pellet the insoluble material. The lysate was discarded and the insoluble material was resuspended in lysis buffer containing 8 M urea. Once the cell material was solubilized, the lysate was clarified via centrifugation. The clarified lysate was pumped over a 1 mL His trap crude column at room temperature. The column was washed, and the protein was refolded on the column by decreasing the concentration of urea by 10% every 10 column volumes. At 4 M urea, the column was attached to a refrigerated AKTA purifier to complete the refolding. Once the urea was washed out of the column, the protein was eluted with 500 mM imidazole. The yield was very poor and, upon further analysis, it was discovered that about 50% of the protein remained in the flow-through. To recover the protein, the flow-through was incubated with 5 mL of packed BD Talon resin (BD Biosciences, San Jose, CA) for 3 h at room temperature. The Talon resin was then washed extensively, and the protein was refolded on the beads by decreasing the concentration of urea in the buffer with each wash. Once the concentration reached 4 M urea, the work was continued using ice. After refolding, the protein was eluted off in eight 2 mL elution steps using 500 mM imidazole elution buffer with 1 min of incubation before centrifugation. The recovered protein was analyzed by SDS-PAGE (Figure 4). The fractions were pooled and buffer exchanged into PBS-T 7.4 pH (Bio-Rad) for SPR analysis. The aliquots were made and snap-frozen in liquid nitrogen.

2.5 SPR Analysis

The ProteOn system (Bio-Rad) was used to assess whether or not our constructs would recognize the ricin and bind to it and the *Ricinus communis* agglutinin (RCA) 120. The ProteOn system was also used to measure the activity of the proteins after they were exposed to thermal stress.

2.5.1 Assay for Protein Activity

Two hundred and seventy-six response units (RUs) of ricin were covalently coupled to a ProteOn GLC sensor chip using amine chemistry. Threefold serial dilutions of C8smcF11:1-1 and 1-3 were used as the analyte. The dilutions were streamed across the surface of the chip at 100 μ L/min for 2 min with 10 min dissociation.

2.5.2 Thermal Stress Test

Two hundred and seventy-six RUs of ricin were tethered to a ProteOn GLC sensor chip using amine chemistry. A concentration scouting experiment was run using C8smcF11:1-1, C8smcF11:1-3, α ricin C8 sdAb's, and α ricin F11 sdAb's as analytes to obtain an R_{Max} (the maximum response of a given analyte on a ligand) between 50 and 100 RUs. The proteins were diluted to the following concentrations: C8smcF11:1-1 (4 nM), C8smcF11:1-3 (12 nM), α ricin C8 (800 nM), and α ricin F11 (419 nM). Once diluted, the proteins were exposed to each of the following temperatures for 15 min: 4, 37, 45, 55, and 65 $^{\circ}$ C. After the exposure was complete, all the samples were placed on ice and immediately run on the ProteOn system at a flow rate of 100 μ L/min for 60 s with 10 min dissociation. Binding was measured in RUs.

2.5.3 Assay for Avidity

Nine hundred and sixty-five RUs of RCA 120 were tethered to a ProteOn GLC sensor chip surface using amine-coupling chemistry. Fifty nanomoles of C8smcF11:1-1, 150 nM of C8smcF11:1-3, 3 μ M of sdAb's C8, and 1 μ M of sdAb's F11 were interacted with the surface of the RCA 120 chip for 60 s at a flow rate of 100 μ L/min with a dissociation time of 10 min. Dissociation constants were determined using Scrubber software (BioLogic Software, Campbell, Australia).

3. RESULTS AND DISCUSSION

3.1 Assay for Protein Activity

C8smcF11:1-1 and 1-3 were evaluated by SPR to determine whether the engineered sdAb's portion of the molecule retained activity once it was tethered to the SMC backbone. The results indicated that C8smcF11:1-1 and 1-3 recognized the ricin and bound to it (Figure 5) and the RCA 120.

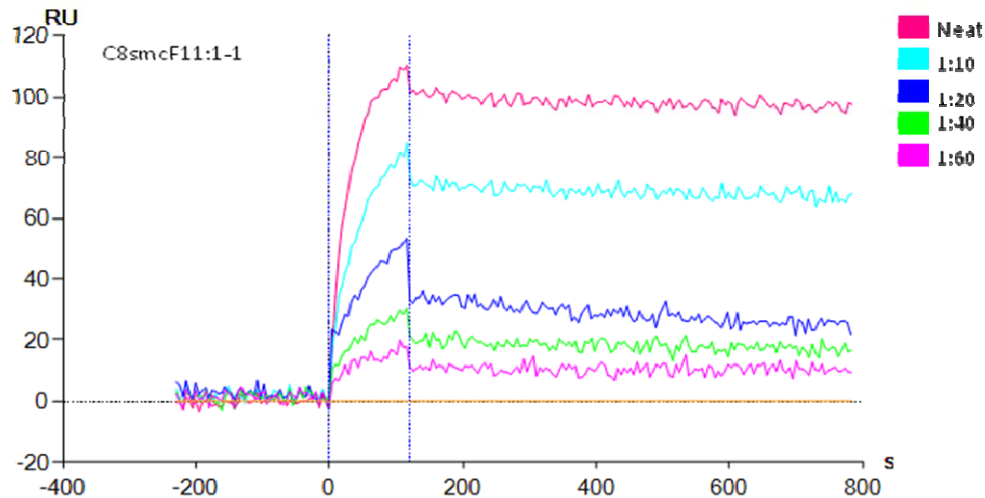


Figure 5. SPR sensogram depicting a scouting experiment. Dilutions of C8smcF11:1-1 are shown binding to a chip that had ricin covalently linked to its surface. (−200–0 is the baseline; 0–150 is the association phase when binding occurs; 150–600 is the dissociation phase.)

3.2 Assay for Avidity

One of our goals in constructing these proteins was to improve upon the binding of the sdAb's. Because of the lack of hybridoma technology, the creation of some sdAb's relies on in vitro panning methods, which often leads to a general reduction in affinity toward the target antigen when compared to standard mouse monoclonal antibodies (1). By fusing C8 and F11 to a coiled-coil backbone attached to a dimerization domain, we were able to create a tetrameric protein that has a greater avidity for RCA 120 when compared to the sdAb's. Analysis of SPR data (Figure 6) shows an order of magnitude decrease in the dissociation constant between the constructs we made and the sdAb's F11. This experiment illustrates that while both of our constructs and the sdAb's F11 bind to RCA 120, the C8smcF11:1-1 and 1-3 exhibit an increase in avidity, which causes them to dissociate from RCA 120 at a slower rate. In this experiment, we also attempted to demonstrate the same affect on sdAb's C8; however, we did not get a high enough R_{Max} to determine the dissociation constant.

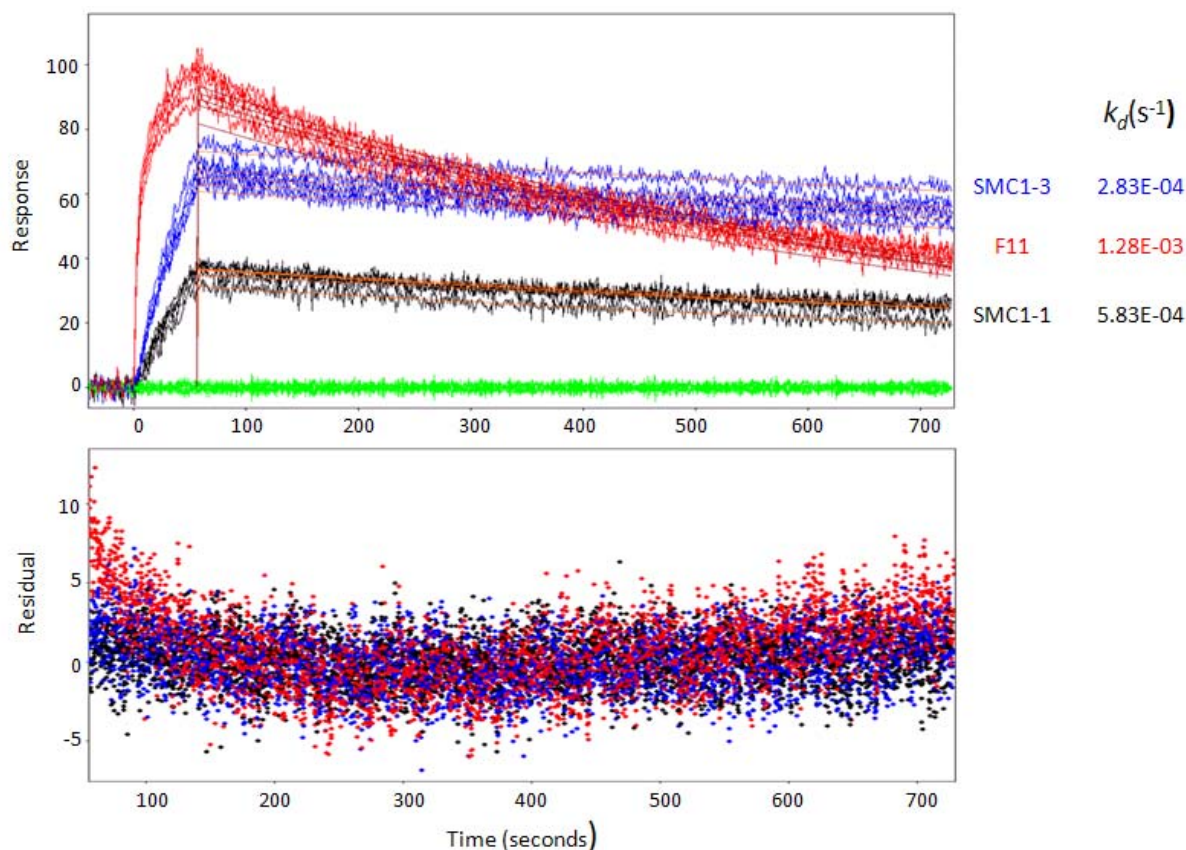


Figure 6. Sensogram with Langmuir 1:1 fit of C8smcF11:1-1, 1-3, and sdAb's F11 binding to RCA 120 with residuals. Dissociation constants are shown at the top right.

3.3 Assay for Thermoplasticity

A unique feature of sdAb's is its ability to unfold and refold efficiently upon heating and cooling. This thermoplasticity is likely the result of the small size of the molecules and increased hydrophilicity (*I*). By constructing a protein that incorporated the structure of a single domain antibody on a platform derived from a thermophile, we tried to maintain thermoplasticity while increasing the avidity due to multimerization. The results from the thermal stress test were extrapolated from SPR data. Percent activity was determined by obtaining report points at R_{Max} for each protein and temperature combination (Figure 7) and assigning 100% activity to the 4 °C sample. When percent activity was plotted against temperature (Figure 8), trend lines showed that as temperature increased, the percent activity of C8smcF11:1-1 and 1-3 decreased. SdAb's C8 and F11 retained 100% of their activity throughout the thermal stress test and actually appeared to exhibit an increase in specific activity. This was most likely due to instrument noise and was not a significant increase. Both C8smcF11:1-1 and 1-3 retained about 50% of their activity up to 55 °C.

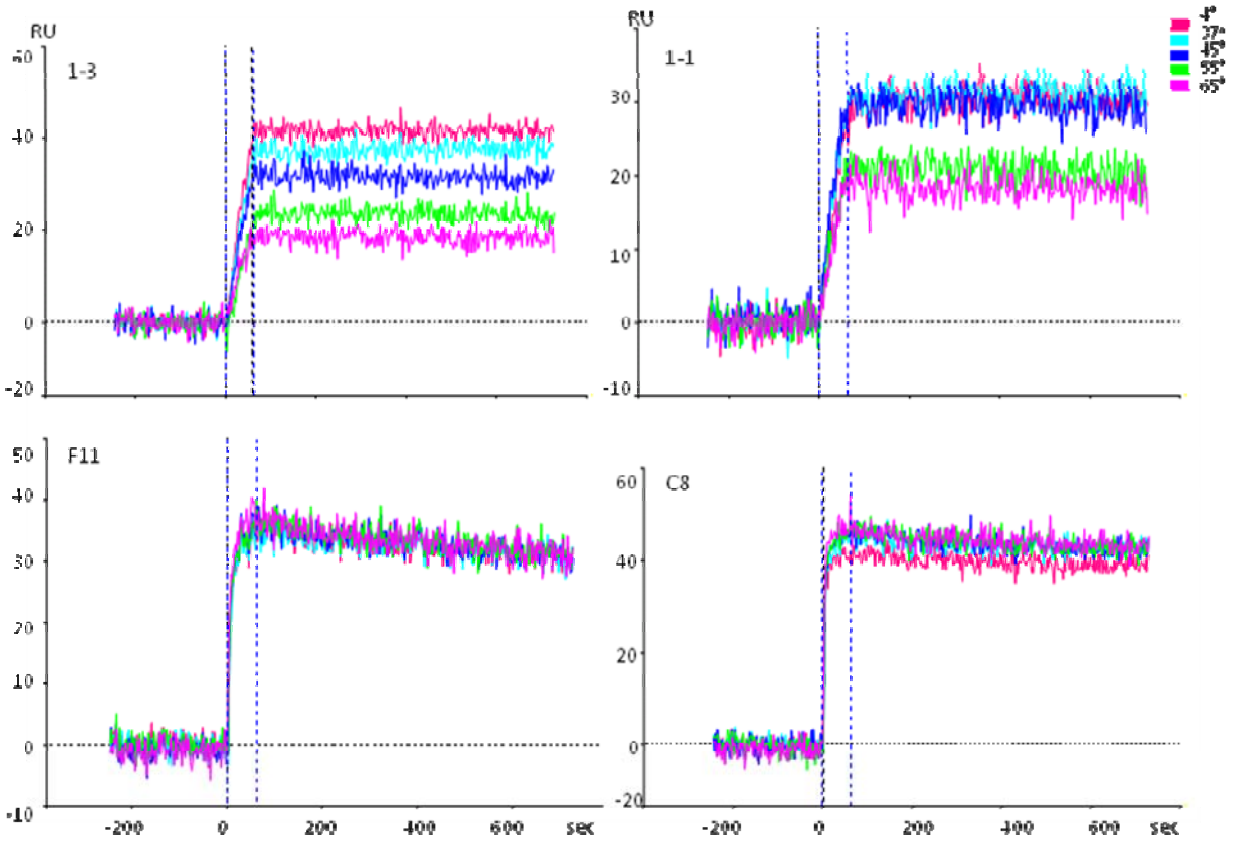


Figure 7. SPR sensograms of C8smcF11:1-1, 1-3, and C8 and F11 of sdAb's after the thermal stress test.

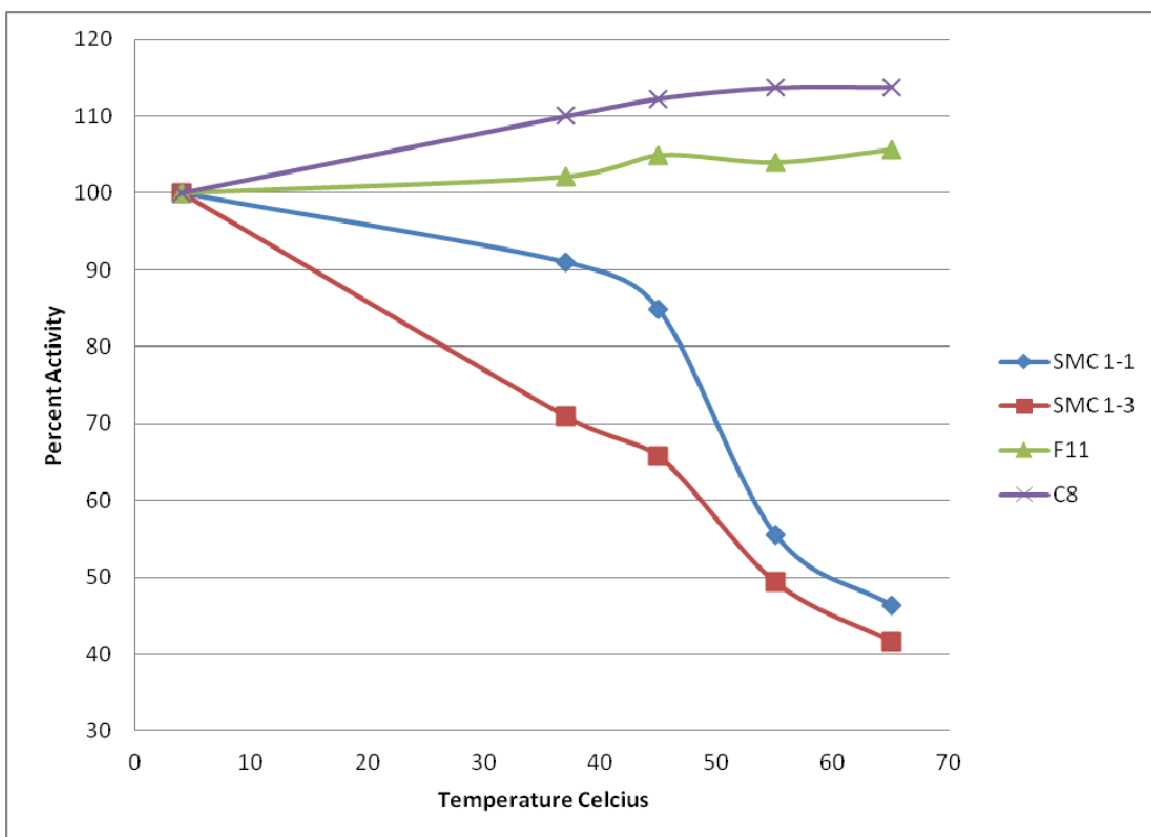


Figure 8. Percent activity of proteins after exposure to various temperatures.

4. CONCLUSIONS

We designed two proteins with two unique binding sites derived from sdAb's specifically for ricin. The binding sites were tethered to a coiled-coil backbone with a dimerization domain derived from the SMC protein of the thermophile, *Pyrococcus furiosus*. When compared to the anti-ricin sdAb's, analysis of both recombinant proteins showed an increase in avidity for RCA 120, which was demonstrated by the 10-fold decrease in the dissociation rate. The avidity improvement was due to the heteropolymeric quadravalent nature of these unique ricin-binding molecules. The thermoplasticity of the sdAb's was compromised when tethered to the coiled-coil backbone. Both C8smcF11:1-1 and 1-3 showed a loss of specific activity when they were exposed to increasing temperatures. The act of fusing the sdAb's to the SMC backbone increased the size of the protein dramatically, which likely affected its ability to refold after heating. The place where the sdAb's and the SMC backbone join may be critical for proper refolding. The fact that both constructed proteins retained 50% activity after being heated to 55 °C is promising. The results from this study indicate that by multimerizing the sdAb's on a thermostable platform, it is possible to increase avidity and maintain some thermoplasticity. This platform will allow for a "plug and play" approach to the multimerization of sdAb's (1), and it has the potential for signaling and mitigating activities. Our future efforts will be focused on improving the construct design to maximize protein refolding after exposure to high temperatures, and testing other sdAb's's using this platform.

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