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14. ABSTRACT Conventional antibodies, widely used in detection schemes, such as point detectors for biological threats, are large 150 kDa proteins that aggregate irreversibly under harsh conditions such as excessive heating. There is a need for the development of high affinity, highly specific robust recognition elements to overcome the limitations of conventional antibodies. One option is single domain antibodies (sdAb), the molecularly engineered binding elements derived from camelids and shark. SdAb are small 12 – 16 kDa robust recognition elements able to					
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Report Title

Development and testing of recombinant single domain antibodies

ABSTRACT

Conventional antibodies, widely used in detection schemes, such point detectors for biological threats, are large 150 kDa proteins that aggregate irreversibly under harsh conditions such as excessive heating. There is a need for the development of high affinity, highly specific robust recognition elements to overcome the limitations of conventional antibodies. One option is single domain antibodies (sdAb), the molecularly engineered binding elements derived from camelids and shark. SdAb are small 12 – 16 kDa robust recognition elements able to function after exposure to chemical denaturants and elevated temperatures. These antibody fragments contain only 3 hypervariable loops but can still bind with affinity equivalent to conventional antibody fragments having twice the number of complementarity-determining regions (CDRs) available to interact with the target. We developed and characterized sdAb towards toxins (ricin, abrin and SEB) and viral (filovirus) targets. We employed a combination of strategies, including sandwich immunoassays, surface plasmon resonance, circular dichroism, mutagenesis, and crystallography to help us to understand the mechanism of this bio-molecular recognition and provide critical insights to the basis of the sdAb-target interaction and sdAb properties.

List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Structures of a key interaction protein from the *Trypanosoma brucei* editosome in complex with single domain antibodies. Wu M, Park YJ, Pardon E, Turley S, Hayhurst A, Deng J, Steyaert J, Hol WG. *J. Struct Biol.* 2010 Oct 20. [Epub ahead of print]

Anderson G.P., Bernstein, R.D., Swain, M.D., Zabetakis, D., Goldman E.R. 2010 Binding Kinetics of anti-Ricin Single Domain Antibodies and Improved Detection Using a B-chain Specific Binder. *Analytical Chemistry*, 82: 7202–7207.

Slocik J.M., Kim S.N., Auvil T., Goldman E.R., Liu J., Naik R.R. 2010. Single Domain Antibody Templated Nanoparticle Resistors for Sensing Biosensors and Bioelectronics 25: 1908-1913.

Goldman E.R. Anderson, G.P., Bernstein, R.D., Swain, M.D. 2010 Amplification of Immunoassays using Phage-Displayed Single Domain Antibodies *Journal of Immunological Methods* 352: 182-185.

Goldman, E.R., Liu, J.L., Bernstein, R.D., Swain, M.D., Mitchell, S.Q., Anderson, G.P. 2009 Ricin Detection using Phage Displayed Single Domain Antibodies. *Sensors* 9(1), 542-555.

Anderson, G.P., Liu, J.L., Hale, M.L., Bernstein, R.D., Moor, M., Swain, M.D., Goldman, E.R. 2008 Development of Anti-Ricin Single Domain Antibodies Towards Detection and Therapeutic Reagents *Analytical Chemistry* 80: 9604-9611.

Liu, J.L., Anderson, G.P., Goldman, E.R. 2007 Isolation of anti-toxin single domain antibodies from a semi-synthetic spiny dogfish shark display library. *BMC Biotechnology* 7:78.

Anderson, G.P., Matney, R., Liu, J.L., Hayhurst, A., Goldman, E.R. 2007 Multiplexed fluid array screening of phage displayed anti-ricin single domain antibodies for rapid assessment of specificity. *BioTechniques*. 43:806-811.

Liu, J.L., Anderson, G.P., Delehanty, J.B., Baumann, R., Hayhurst, A., Goldman, E.R. 2007 Selection of cholera toxin specific IgNAR single domain antibodies from a naïve shark library. *Molecular Immunology*. 44:1786-1794.

Sherwood, L.J., Osborn, L., Carrion, R., Patterson, J., Hayhurst, A. 2007 Rapid assembly of sensitive antigen-capture assays for Marburg virus, using in vitro selection of llama single-domain antibodies, at biosafety level 4. *Journal of Infections Diseases* 196: s213-s219.

Goldman, E.R., Anderson, G.P., Liu, J.L., Delehanty, J.D., Sherwood, L.J., Osborn, L.E., Cummins, L.B., Hayhurst, A. 2006 Facile Generation of Heat Stable Antiviral and Antitoxin Single Domain Antibodies from a Semi-synthetic Llama Library. *Anal. Chem.* 78:8245-55

Number of Papers published in peer-reviewed journals: 11.00

(b) Papers published in non-peer-reviewed journals or in conference proceedings (N/A for none)

Liu, J.L., Anderson, G.P., Hayhurst, A., Goldman, E.R. "Single Domain Antibodies: Rugged Recognition Elements for Tomorrow's Biosensors" in *Optical Biosensors Today and Tomorrow*, F.S. Ligler and C.R. Taitt (eds), Elsevier, Amsterdam, the Netherlands. p 469-492(2008).

(c) Presentations

1. Hayhurst et al. February 15-18 2006 poster presentation ASM Biodefense Research Meeting in Washington DC, Developing Recombinant Single Domain Antibodies as Heat Stable, High Affinity, Reusable Recognition Elements
2. Goldman, E.R. March 22, 2006 invited seminar at the Army Research Laboratory, Adelphi MD. Development of recombinant single domain antibodies to provide heat stable, high affinity, regenerable recognition elements.
3. Hayhurst et al. June 17-22, 2006 poster presentation 13th Negative Strand Conference in Salamanca Spain, Heat Stable Antibodies for Virus Hunting in Hot Climates:-Towards rapid and inexpensive tests for Marburg and Ebola viruses
4. Sherwood et al., September, 2006 poster presentation at the 2nd International Filovirus meeting in
5. Winnipeg, Canada. Rugged filoviral diagnostics using heat stable single domain antibodies.
6. Anderson et al. October 8-12, 2006 poster presentation HSARPA Chemical and Biological Countermeasure Technologies for Homeland Security in Boulder, CO, Generation of heat stable antitoxin single domain antibodies from a llama library evaluation using the Luminex xMAP system
7. Goldman, E.R. November 14, 2006 oral presentation at the Joint Services Scientific Conference on Chemical and Biological Defense Research. Single domain antibodies selected from naive llama and shark-based libraries as small, rugged recognition elements
8. Sherwood et al., November, 2006 poster presentation at the 7th Asia Pacific Congress of Medical Virology in New Delhi, India. Heat stable antibodies for viral diagnostics in hot climates
9. Sherwood, L.J., Osborn, L, Carrion, R., Patterson, J.P and Hayhurst, A. April 22, 2007 Rapid assembly of filovirus antigen detection assays at biosafety level four. 1st UK-US Conference on Chemical and Biological Sensors and Detectors, London, England.
10. Hayhurst, A. May 1, 2007 Disruptive technologies for antibody development against pathogens and toxins. 6th Biodefense Summit of the San Antonio and Austin Life Sciences Association, New Bruanfels, TX.
11. Sherwood, L.J., Osborn, L., Carrion, R., Patterson, J.P. and Hayhurst, A. May 14, 2007 Phage display selections at biosafety level four generate rapid and sensitive antigen assays for filoviruses in under three weeks. 9th annual CHI Phage Display of Antibodies and Peptides Meeting, Boston, MA.
12. Goldman, E.R., Liu, J.L., Hayhurst, A., Anderson, G.P. May 16, 2007 Development of Recombinant Single Domain Antibodies for Toxin Detection. 8th annual CHI Recombinant Antibodies meeting, Boston, MA.
13. Sherwood, L.J., Osborn, L., Carrion, R., Patterson, J.P. and Hayhurst, A. June 14, 2007 Rapid aassembly of sensitive capture assays for Marburg virus using in vitro selection of llama single domain antibodies at biosafety level four. 10th International Biodetection Technologies Conference, Atlanta, GA.
14. Liu, J.L., Anderson, G.P., Goldman, E.R. December 13, 2007 Shark single domain antibodies as rugged recognition elements against a variety of toxins NanoBiotechnology for Defense Applications Workshop, College Park, MD
15. Liu, J.L., Anderson, G.P., Hayhurst, A., Goldman, E.R. "Single Domain Antibodies: Rugged Recognition Elements for Tomorrow's Biosensors" in Optical Biosensors Today and Tomorrow, F.S. Ligler and C.R. Taitt (eds), Elsevier, Amsterdam, the Netherlands. p 469-492(2008).
16. Liu, J.L. February 26, 2008 presentation of the ASM biodefense meeting, Baltimore Maryland, Heavy chain antibodies and single domain binding elements for ricin detection.
17. Goldman, E.R. September 18, 2008 invited seminar at Washington State University School of Molecular Biosciences, Pullman Washington, Llama derived single domain binding elements specific for toxins.
18. Goldman, E.R. October 9, 2008 invited seminar at KPL, Gaithersburg, Maryland Llama derived single domain binding elements specific for toxins.
19. Anderson, G.P, Liu, J.L., Swain, M.D., Bernstein, R.D., Moor, M., Goldman, E.R. November 19, 2008 Selection and Characterization of Anti-Ricin Single Domain Antibodies Chemical and Biological Defense Physical Sciences and Technology Conference, New Orleans, LA
20. E.R. Goldman et al, February 24, 2009 Oral presentation at the ASM Biodefense and Emerging Diseases Research Meeting (Baltimore, MD) Improved toxin detection using Single Domain Antibodies
21. L.J. Sherwood and A. Hayhurst February 23, 2009 Poster presentation at the ASM Biodefense and Emerging Diseases Research Meeting (Baltimore, MD) Rapidly Formulated Antigen Capture Assays for Ebola Viruses Based on in vitro Antibody Selections at BSL4
22. Ellen Goldman, April 27, 2009, invited presentation at Single domain antibody workshop, Frederick Maryland, Single domain antibodies for toxin detection
23. Andrew Hayhurst, "Selections on live BoNT and Filoviruses yield promising diagnostic ligands", Invited Speaker, Single domain antibody workshop, USAMRIID, Maryland, April 27th, 2009
24. Ellen Goldman, invited presentation 2009 Biothreat Agent Workshop at Chapel Hill, May 21, 2009, North Carolina, Single domain antibodies for biothreat detection.
25. isolating anti-Ebola single domain antibodies and transitioning to Luminex. Sherwood, L. and Hayhurst, A. Poster at Xmap 2009, Austin, Texas, May 4th-6th, 2009.
26. Ellen Goldman, invited presentation at 14th international conference Biodetection technologies 2009 Baltimore, June 25, 2009, Maryland, Single domain antibodies for biothreat detection.
27. Andrew Hayhurst "Towards heat stable diagnostics for Filoviruses", lecture at National Public Health Laboratory, Brazzaville,

Republic of Congo, 28th July 2009.

28. Plastic ligands for fieldable and rugged Filoviral diagnostics. Sherwood, J., Garza, J.A. and Hayhurst, A., Poster at DMID/NIAID/NIH workshop "Bats and Emerging Viral Diseases", Rockville, Maryland, September 10th -11th , 2009.
29. George Anderson, Jinny Liu, Rachael Bernstein, Marla Swan, Ellen Goldman "Single domain antibodies as robust recognition elements", invited speaker, September 28, 2009, nanoelectronic devices for defense & security conference, Fort Lauderdale, Florida.
30. Ellen Goldman, November 10, 2009 Oral presentation at the 1st Bio-Sensing Technology Conference (Bristol UK) Llama derived single domain recognition reagents specific for toxins
31. Andrew Hayhurst, "Llama single domain antibodies specific for polyvalent nucleoprotein may offer rugged yet sensitive virosensing elements for Ebola and Marburg viruses", speaker at 1st Biosensing Conference, Bristol, England, November 10th-12th, 2009.
32. Plastic ligands for fieldable and rugged Filoviral diagnostics. Sherwood, L., Garza, J. and Hayhurst, A. Poster at 3rd Chemical and Biology Defense Science and Technology Conference, Dallas, Texas, November 16th-20th, 2009.
33. High resolution crystal structure of an autocrystallising llama VH domain at 1.7 Angstroms. Garza, J., Taylor, A., Galaledeen, A., Hart, P.J. and Hayhurst, A. Poster at 3rd Chemical and Biology Defense Science and Technology Conference, Dallas, Texas, November 16th-20th, 2009.
34. Rapid assembly of crystalline mega-structures from salt solutions by an unusual mutant nanobody. Conway, J., Sherwood, L., Garza, J., Grigorian, A. and Hayhurst, A. Poster at 3rd Chemical and Biology Defense Science and Technology Conference, Dallas, Texas, November 16th-20th, 2009.
35. George Anderson et al, November 18, 2009 Poster presentation at the 2009 Chemical and Biological Defense Science and Technology Conference (Dallas, Texas) Characterization of toxin specific llama derived single domain antibodies
36. February 22, 2010 Poster presentation at the ASM Biodefense and Emerging Diseases Research Meeting (Baltimore, MD) Alkaline Phosphatase Mutants Fused to Single Domain Antibodies for Botulinum Toxin Detection (E.R. Goldman, presenter)
37. March 1, 2010 Oral presentation to DARPA MTO workshop on Chip-Scale Photonic Resonance for Biological Detection (Chantilly, VA) Antibodies and Binding Ligands For Biosensor Applications. (G.P. Anderson, presenter)
38. Llama single domain antibodies as rugged ligands for the detection and intervention of high consequence pathogens and toxins", speaker at the University of Texas Health Science Center San Antonio, San Antonio, Texas, May 13th (Andrew Hayhurst, presenter)
39. Selection and Evaluation of Abrin Binding Single Domain Antibodies Using the Luminex 100. George P. Anderson, Richard H. Glaven, Jinny L. Liu, Rachael D. Bernstein, Marla D. Swain, Ellen R. Goldman. Poster at Planet X-map, May 12-14, 2010 Baltimore, Maryland.
40. Plastic ligands for fieldable and rugged Filoviral diagnostics. Sherwood, L., Garza, J. and Hayhurst, A. Poster at the University of Texas at San Antonio College of Sciences 2010 Research Conference, May 28th.
41. High resolution crystal structure of an autocrystallising llama VH domain at 1.7 Angstroms. Garza, J., Taylor, A., Galaledeen, A., Hart, P.J. and Hayhurst, A. Poster at the University of Texas at San Antonio College of Sciences 2010 Research Conference, May 28th.
42. Goldman et al poster presentation at the 4th annual Botulinum Symposium (Dartmouth MA) Llama derived single domain antibodies for the detection of BoNT A, ricin, and abrin August 20, 2010.
43. Andrew Hayhurst Invited speaker at first "Single Domain Antibodies Come of Age" Conference, Het Pand, Ghent, Belgium, October 14-15.
44. Goldman et al. Poster presentation "Single Domain Antibodies for the Detection of Biothreat Agents Ricin and Abrin" at the first "Single Domain Antibodies Come of Age" Conference, Het Pand, Ghent, Belgium, October 14-15, 2010.
45. George P. Anderson, Invited Presentation at the Luminex MAGPIX Road Show, Philadelphia PA, Dec 13, 2010 Immunoassay development and reagent evaluation using xMAP Technology

Number of Presentations: 45.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

(d) Manuscripts

Several manuscripts in preparation.

Number of Manuscripts: 0.00

Patents Submitted

Patents Awarded

Awards

2006 U.S. Naval Research Laboratory Alan Berman Annual Research Publication Award in recognition of the technical merit of the publication "Facile Generation of Heat Stable Antiviral and Antitoxin Single Domain Antibodies from a Semi-synthetic Llama Library" Anal. Chem. 78:8245-8255

Graduate Students

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Names of Under Graduate students supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

- The number of undergraduates funded by this agreement who graduated during this period: 0.00
- The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 0.00
- The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 0.00
- Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00
- Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00
- The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00
- The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 0.00

Names of Personnel receiving masters degrees

<u>NAME</u>
Total Number:

Names of personnel receiving PHDs

<u>NAME</u>
Total Number:

Names of other research staff

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

1 a. Southwest Foundation for Biomedical Research

1 b. 7620 NW Loop 410

San Antonio TX 78250

Sub Contractor Numbers (c): N00173-05-1-G011

Patent Clause Number (d-1):

Patent Date (d-2):

Work Description (e): Construction of a library of sdAb derived from reserach naive llamas. Selection and characterization

Sub Contract Award Date (f-1): 3/4/2005 12:00:00AM

Sub Contract Est Completion Date(f-2): 3/3/2009 12:00:00AM

Inventions (DD882)

Project Title: Development and testing of single domain antibodies

Principal investigators:

Ellen R. Goldman, Naval Research Laboratory (NRL), Washington DC
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Statement of the problem studied: Conventional antibodies, widely used in detection schemes, such point detectors for biological threats, are large 150 kDa proteins that tend to be fragile, not able to stand up to harsh conditions such as excessive heating. Robust recognition elements are needed to overcome these limitations. One option are single domain antibodies (sdAb) the molecularly engineered binding elements derived from camelids and shark. SdAb are small 12 – 16 kDa robust recognition elements able to function after exposure to chemical denaturants and elevated temperatures. We aim to develop sdAb towards a variety of targets and elucidate a greater understanding of their unique molecular properties.

Many groups, including our own have documented the remarkable ability of sdAb recognition elements to re-gain both their secondary structure and ability to bind antigen, after heat denaturation. SdAb are being developed for many applications including diagnostics, environmental detection and therapeutics; however there is only limited understanding of their binding interactions and the physical chemistry that leads to their unusual properties. These antibody fragments contain only 3 hypervariable loops but can still bind with affinity equivalent to conventional antibody fragments having twice the number of complementarity-determining regions (CDRs) available to interact with the target. We developed sdAb towards toxin and viral targets and used a combination of strategies to help us to understand the mechanism of this bio-molecular recognition and provide critical insights to the basis of the sdAb-target interaction and sdAb properties.

This program is a collaborative effort between Dr. Ellen Goldman at the Naval Research Laboratory (NRL) in Washington, DC and Dr. Andrew Hayhurst at the Southwest Foundation for Biomedical Research (SFBR) in San Antonio, Texas. Our objective was to develop libraries of sdAb derived from camelids and sharks, select sdAb against model and genuine threat targets, and characterize the biochemical properties of sdAb versus conventional antibodies (if available). Through characterization of the developed sdAb we aimed to acquire a fundamental understanding of sdAb properties and binding interactions, towards elucidating sdAb-antigen interactions as well as critical components of sdAb architecture required for plasticity.

Background: Antibodies are unparalleled in their capacity to bind a diverse array of antigens with high specificity and high affinity. These antibodies are large ~150 kDa molecules made up of 2 heavy chains and 2 light chains. Each antibody possesses 2 antigen combining sites formed by the pairing of a variable domain from the heavy chain (VH) with a variable domain

from the light chain (VL). Their complexity in requiring two domains for antigen binding is a weakness for applications involving harsh denaturing conditions such as high temperatures as they aggregate irreversibly on unfolding. Additionally, monoclonal antibody development and production is costly, usually requiring growth of hybridomas in tissue culture, which yields only small amounts of antibody, or use of many mice for production in ascites fluid. While polyclonal antibodies are easily produced in large quantities, affinities and specificities often vary from lot-to-lot.

Recombinant binding elements, such as single chain antibodies (scFv) constructed by joining the VH and VL with a flexible linker, have been constructed to reduce the size of the IgG while retaining its antigen-binding properties (Bird et al., 1988; Huston et al., 1988). These fragments can often be expressed in *E. coli* at reasonable levels and lend themselves well to genetic manipulation. However, as with IgG, their requirement for two domains for antigen binding limits their potential for use under harsh conditions.

In the mid 1990s, it was discovered that in addition to standard antibody isotypes containing both H and L chains, certain animals, such as camelids (i.e., camels, llamas) and sharks possess a class of immunoglobulins consisting of heavy-chain homodimers where antigen binding is mediated through a single V domain (Hamers-Casterman *et al.*, 1993; Greenberg *et al.*, 1995). These V domains, when cloned separately as single domain antibodies (sdAb), comprise the smallest known antigen binding fragments (12-15 kDa).

Despite their small size, sdAb display a high level of specificity and affinity for their antigens, with many displaying nM and sub-nM affinities (Muyldermans et al. 2001, 2006). SdAb have been developed and isolated towards many targets including haptens, proteins, bacteria, and virus (Wesolowski et al, 2009). Due to the fact that association with a partner light domain is unnecessary, the surface of the protein has lost its hydrophobic interface, thus sdAb are extremely well expressed and soluble in *E. coli* when compared to all other antibody fragments (Ghahroudi et al., 1997). These mutations also serve to enhance the solubility of the sdAb and prevent aggregation after denaturation, thereby allowing quantitative refolding (Perez et al., 2001; Ewert et al., 2002).

Brief summary of accomplishments: Both the groups at SFBR and NRL have constructed libraries of sdAb displayed on phage, selected sdAb and characterized the selected binders. The SFBR group constructed a large semi-synthetic library derived from research naïve llamas and mined the library against the surrogates hen egg lysozyme and vaccinia, as well as biosafety level 4 (BSL4) threat agents Marburg virus and four of the Ebola species. The NRL group constructed libraries derived from research naïve sharks as well as from immunized llamas; they concentrated on the selection and characterization of toxin binding sdAb with a focus on ricin.

At the start of our program we constructed libraries of shark and llama-derived sdAb derived from research naïve animals and successfully selected sdAb against several toxin and viral targets (Goldman et al, 2006; Liu et al, 2007a; Liu et al, 2007b; Sherwood et al, 2007). Isolated

sdAb were characterized in terms of specificity, utility as immunoreagents in sandwich assays, and their ability to bind antigen after heat denaturation. SdAb selected at both laboratories showed superior heat stability over conventional IgG and single chain (scFv) antibody fragments. When subjected to prolonged heating some sdAb retained 100% of their binding ability even after 45 minutes at 95 °C. Other sdAb retained close to 100% binding activity for the first ~10 minutes and then slowly lost activity over the course of 60-90 minutes. The great majority of conventional antibodies lost ~90% of their binding activity after only 5 minutes of heating, with none lasting more than 20 minutes. However, most sdAb isolated from the naïve libraries had relatively low affinities and performed poorly in antigen binding assays relative to conventional antibodies. One exception to this was a panel of four unique sdAb selected at SFBR under BSL4 conditions against Marburg virus that revealed exquisite sensitivity and specificity in a simple antigen capture ELISA format. The group at NRL turned to immune libraries for the selection of toxin binding sdAb while the SFBR team continued working with filovirus binders derived from their naïve llama library to rationalize the mechanism of sdAb high performance.

NRL: Ideally sdAb towards any threat could be rapidly selected from extremely large libraries derived from unimmunized animals. Binders towards ricin, cholera toxin (CTX) and staphylococcal enterotoxin B (SEB) were selected from the naïve semi-synthetic library constructed at SFBR, and CTX binders were selected from the naïve shark library constructed at NRL (Goldman et al, 2006; Anderson et al, 2007; Liu et al, 2007a; Liu et al, 2007b). These binders were shown to perform as captures and tracers in sandwich assays, be specific and able to function after heating. Toxin binding sdAb isolated from the naïve libraries constructed for this project, however, were poor binders; the NRL team chose to construct libraries derived from animals immunized with toxoids in an effort to isolate higher affinity recognition reagents.

Using libraries derived from immunized llamas, the NRL group isolated sensitive, high affinity sdAb towards ricin (Anderson et al 2008; 2010). The ricin-binding sdAb were determined to fall into 9 sequence families; the majority of binders were found to be specific for ricin with minimal binding to irrelevant proteins. Using ricin sandwich assays we were able to determine pairs of sdAb that appear to bind non-overlapping epitopes, we found that the isolated sdAb bind to at least 4 different epitopes on ricin. Further analysis showed that 2 of the epitopes are on the ricin A chain, one is on the ricin B chain, and the fourth seems to overlap both chains, with the sdAb recognizing the intact toxin but neither of the isolated chains. Many of the ricin binding sdAb have sub-nM affinity for ricin based on surface plasmon resonance (SPR) measurements. The best capture/tracer pair allowed ricin detection down to 0.1 ng/ml in sandwich assays and could discriminate between ricin and the highly similar castor bean plant protein RCA120. We examined the binding specificity of the best pairs in non-standard matrices (such as milk and tuna fish blend) as well as buffer as a measure of their specificity and found that binding ability was maintained in all the matrices in which we tested.

Ricin binders were mobilized into a modified pet22b vector with a triple histidine (his) tail for attachment to CdSe/ZnS core/shell quantum dots (QDs) synthesized at NRL. We found that where sdAb expressing a single repeat of 6 his did not self assemble on the surface of the QDs,

adding multiple repeats of 6 his separated by short linkers allowed good attachment. The sdAb could also be assembled on commercial QDs which we functionalized with Ni-NTA. We demonstrated sensitive fluoroimmunoassays for ricin using both types of sdAb-QD constructs. The ability to genetically modify the sdAb allows them to be tailored for specific applications as conventional antibodies cannot be.

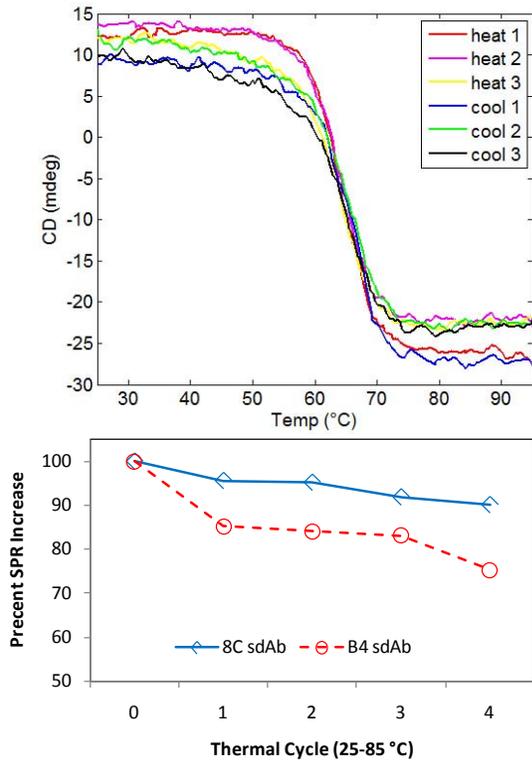


Figure 1. Thermostability of anti-ricin sdAb. Top panel. Heating and cooling cycles of clone H1 monitored by CD spectroscopy. SdAb H1 was heated to 95 °C and then cooled while the ellipticity at 205 nm was monitored, results are typical of the isolated sdAb. Bottom panel. Measurement of binding activity following heating to 85 °C. The SPR signal (resonance units) generated by the addition of 10 nM sdAb to a surface coated with ricin was measured following the indicated number of heating cycles.

Circular dichroism (CD) can be used to monitor the secondary structure of proteins and was used to characterize the anti-ricin sdAb. We monitored the signal at 205 nm while subjecting them to repetitive cycles of heating and cooling; sdAb functionality (antigen-binding) was tested as well. Results from the ricin binders are shown in figure 1. In general, we have observed that most sdAb denature between 60 and 70 °C on heating and then refold quickly as they are cooled to room temperature. Perhaps most importantly, the majority of our isolated sdAb binders retained their ability to bind antigen after heat denaturation, in contrast to conventional antibodies which lose activity after identical heating.

In addition to isolating ricin binders, the group at NRL immunized llamas with abrin toxoid and selected abrin binders. Binders were isolated that were specific for both abrin and its less toxic agglutinin. Only one binder recognized a recombinant version of the abrin A chain, while several cross-reacted with ricin B chain, suggesting that they might have epitopes on the B chain of abrin. As with the ricin binders, CD showed that they refolded after thermal denaturation.

In collaboration with MITRE, the NRL group characterized a SEB binding sdAb. This clone bound its target with sub nM affinity, and showed

a melting temperature of 85 C, higher than any of the ricin or abrin binders. As with the majority of other sdAb, it re-folded and was able to bind antigen after heating cycles.

Progress this quarter

- Constructed system for producing sdAb with triple his tag.
- Self assembled sdAb with triple his tag on QDs and showed fluorimmunoassays for the detection of ricin.

- Functionalized commercial QDs and demonstrated fluoroimmunoassays for the detection of ricin.

Presentations / Publications this quarter

Goldman et al. Poster presentation “Single Domain Antibodies for the Detection of Biothreat Agents Ricin and Abrin” at the first “Single Domain Antibodies Come of Age” Conference, Het Pand, Ghent, Belgium, October 14-15, 2010.

George P. Anderson, Invited Presentation at the Luminex MAGPIX Road Show, Philadelphia PA, Dec 13, 2010 Immunoassay development and reagent evaluation using xMAP Technology

SFBR: We focused on engineering a semi-synthetic library of llama sdAb as, long-term, we believe single pot libraries will enable the rapid delivery of sdAb and bypass immunizations which are costly, time-consuming and are often not possible with many of the targets we deal with (live Filoviruses, live BoNT). Small blood samples were obtained from 3 pet llamas at SFBR and used to amplify sdAb genes, which were relatively scarce among a population of contaminating intron bearing fragments. Following open reading frame selection we managed to develop a million member sdAb gene library, but still were unable to generate binders. Using error prone PCR and shuffling of the hypervariable domains we evolved this 1e+6 library to 1e+9 members and began to isolate binders (figure 1). We selected on hen egg lysozyme and vaccinia virus to show that the sdAb were more thermostable than a scFv and CRP IgG respectively (Goldman et al., 2006). We then sent the library to NRL so they could select binders against their targets of choice.

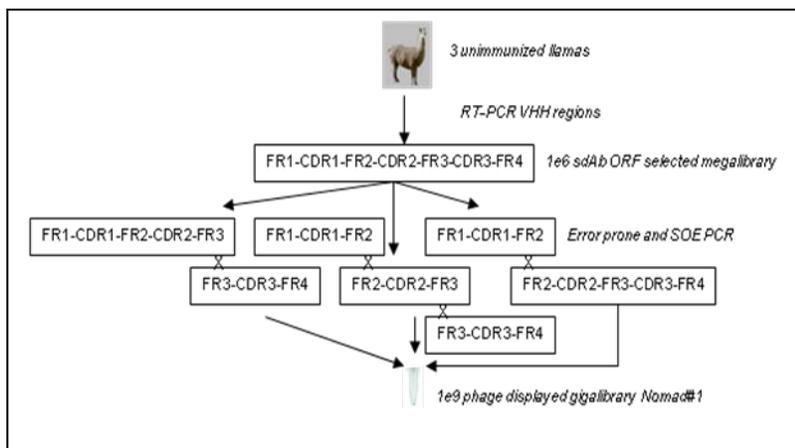


Figure 1. Scheme of salvaging poor sdAb gene pool through a combination of in vivo open reading frame selection and in vitro diversification to generate a productive single-pot library called Nomad.

Sure enough, targeting many normal proteins proved to be unfruitful since the affinities of the resulting sdAb were poor.

One aspect where low affinity of sdAb is not a hindrance to progress is in crystallization of difficult to crystallize proteins, where the binding sdAb creates a hydrophilic scaffold which templates high quality crystal formation of a lattice that allows the target protein to crystallize. Our collaborators at University of Washington Seattle recently used Nomad sdAb to achieve high resolution structures of one such protein of a Trypanosome (Wu et al., 2010).

Since we had just purified *Marburgvirus* in the SFBR BSL-4 we thought to apply the panning technology at this level of containment to see if we could even isolate any binders, whether high

or low affinity. We managed to isolate 4 unique clones which showed remarkable sensitivity and specificity to all strains of *Marburgvirus* to hand and approached RT-PCR sensitivity with the Angola 2005 strain (figure 2) (Sherwood et al., 2007).

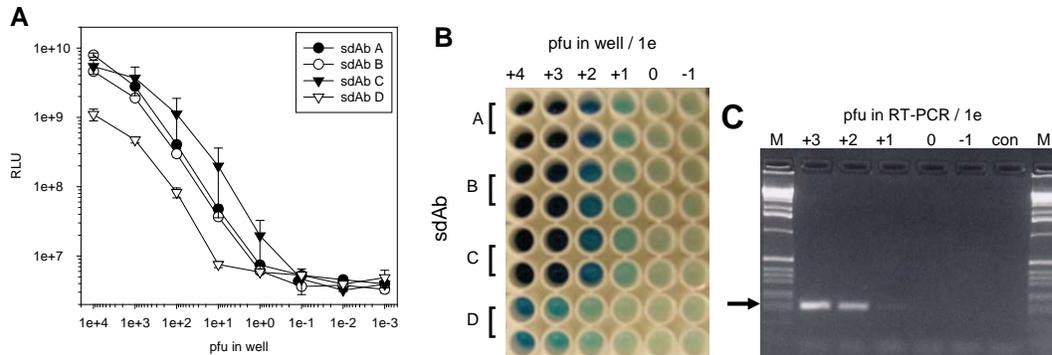


Figure 2. *A: Chemiluminescent titration of Marburg Angola in a sdAb based antigen capture assay with each of four clones (a-d) as both captor and tracer. B: colorimetric titration . C: The same dilutions of virus were Trizol extracted and used in RT-PCR reactions to show matching sensitivity.*

We identified the antigenic target to be nucleoprotein and have since narrowed down the epitopes of all four sdAb to a C-terminal region of the protein. In order to fully rationalize the behavior of the sdAb we are attempting to crystallize the antibody-antigen complex. So far, we have structures of A and B (figure 3) and promising crystals of C.

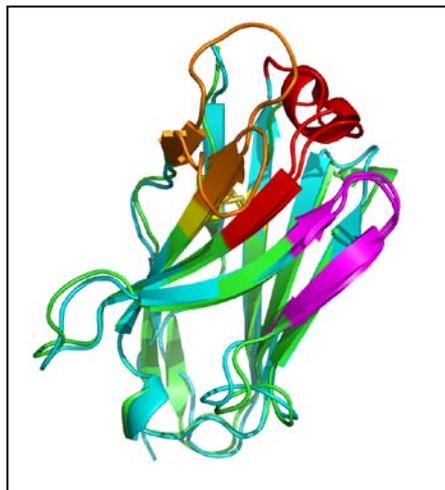


Figure 3. *Overlay of anti-Marburgvirus sdAb A (green) and B (cyan) showing CDR1 (red), CDR2 (magenta) and CDR3 (orange). The structural similarity of the CDR2 region may hint to the one of the shared epitopes the sdAb appear to bind.*

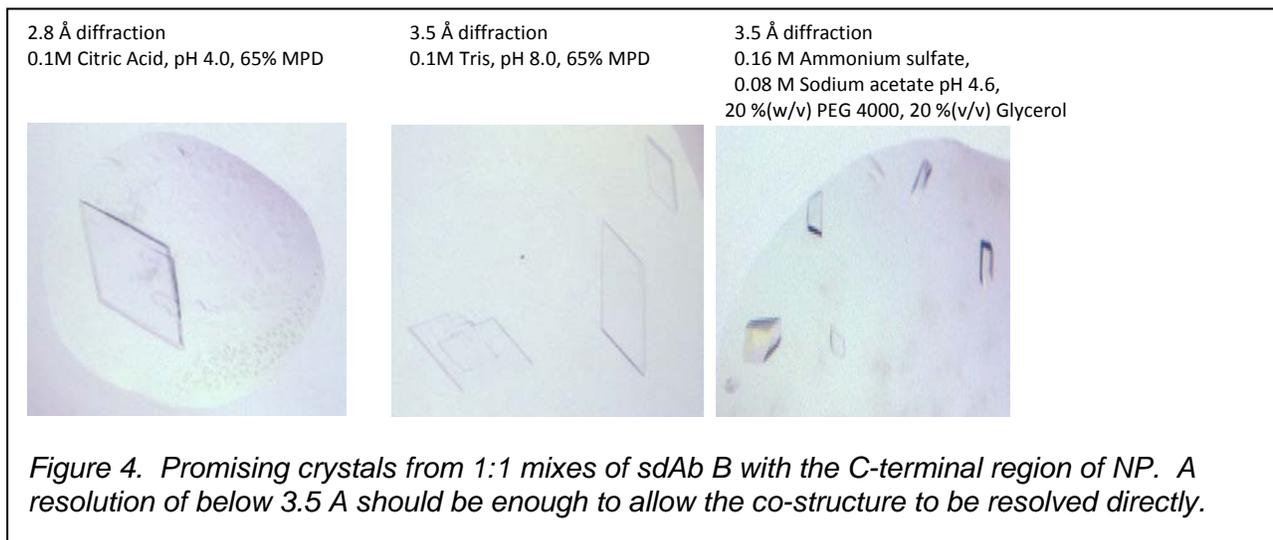
MBG A – 1.1 Å

Crystallized in space group P212121 (unit-cell parameters a=41.6, b=49.4, and c=58.8) with one monomer in the asymmetric unit.

MBG B – 2.0 Å

Crystallized in space group P6522 (unit-cell parameters a=b=80.0, and c=89.8) with one monomer in the asymmetric unit.

We have not yet succeeded in crystallizing the NP C-terminus alone, but promising co-crystals are appearing, again perhaps owing to the hydrophilic stabilizing effects of the sdAb (Figure 4) and we aim to solve the co-structure and confirm the contact points from results of peptide scans and alanine substitution.



A complementary approach to understanding the mechanism of high sensitivity of these sdAb is to see how generic the phenomenon is towards other viruses. The closest relative of *Marburgvirus* is *Ebolavirus* which has 5 distinct species yet shares high structural homology with Marburg and the same filamentous morphology and long NP encapsulated genome. We amplified and purified the 4 species we had on hand and used the Nomad sdAb library to target these preparations. A battery of clones was isolated for Zaire, Ivory Coast and Reston viruses and one clone for Sudan. All were able to act as both tracer and captor in the same antigen capture assay indicating they too bound at least a dimeric if not polymeric target. All clones again showed remarkable sensitivity and all clones did indeed bind NP. The sdAb were a mixture of cross-reactivities among the *Ebolavirus* group and we have since used peptide arrays to precisely define the regions recognized and shared/not shared among the different *Ebolavirus* species. Importantly, many of our sdAb are able to recognize NP synthesised based upon the 5th, recently emerged species, Bundibugyo (Figure 5).

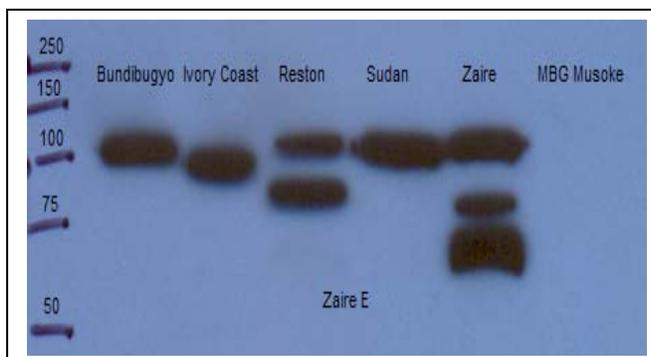


Figure 5. sdAb alkaline phosphatase fusions are convenient probes to assay cross-reactivity of E. coli expressed NP from the 5 Ebolavirus species. Here, anti-Zaire clone E demonstrates good pan reactivity to all NP, and is non-cross reactive with Marburgvirus NP.

Although we would really like to demonstrate pan-reactivity on Bundibugyo virus itself we have not been sent that species by CDC-Special Pathogens Branch despite asking several times

over the past 2 years up to and including writing Gerberding when director of CDC. Consequently, we are resigned to finishing up the Ebola work without access to this particular virus.

An orphan project arising from a DTRA medical program funded chance discovery was also studied during this last funding period as it fit more with basic science of sdAb as heat stable soluble recognition elements. A sdAb that had 4 unusual amino acid mutations kept dropping out of solution overnight to form large crystalline aggregates visible to the naked eye, following gel filtration in PBS. We had never seen such a phenomenon despite purifying some 130 different sdAb proteins in exactly the same manner. The phenomenon occurred in a variety of buffers to yield a mix of protein and salt that usually diffracted poorly after X-ray analysis. However, one lucky crystal diffracted to 1.7 Angstroms and gave insight into potential packing enhancement (figure 6) and we are currently trying to prove this via site directed mutagenesis and conventional crystallisation.

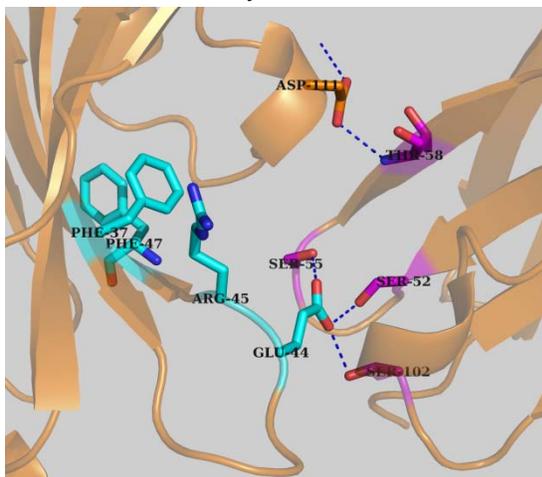


Figure 6. Two adjacent sdAb molecules from the autocrystallising mutant protein show glutamate 44 of one monomer interacting with serines of an adjacent monomer. A wild type sdAb that fails to autocrystallize has substitutions close by this residue and these may alter the disposition of the acidic side chain.

Progress this quarter

- Solved structure of MBG sdAb A, including trip to synchrotron beam-line for post-doc.
- Promising crystals of MBG sdAb B plus NP600 obtained with sufficient resolution for data collection.
- MBG sdAb C purified and in crystal trials, promising crystals.

Presentations / Publications this quarter

Invited speaker at first “Single Domain Antibodies Come of Age” Conference, Het Pand, Ghent, Belgium, October 14-15.

Structures of a key interaction protein from the *Trypanosoma brucei* editosome in complex with single domain antibodies. Wu M, Park YJ, Pardon E, Turley S, Hayhurst A, Deng J, Steyaert J, Hol WG. *J. Struct Biol.* 2010 Oct 20. [Epub ahead of print]

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