EDGEWOOD CHEMICAL BIOLOGICAL CENTER
U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND
Aberdeen Proving Ground, MD 21010-5424

ECBC-TR-1339

DARPA ANTIBODY TECHNOLOGY PROGRAM STANDARDIZED TEST BED FOR ANTIBODY CHARACTERIZATION: CHARACTERIZATION OF AN MS2 HUMAN IGG ANTIBODY PRODUCED BY ANAPTYSBIO, INC.

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February 2016

U.S. ARMY RDECOM

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
XX-02-2016	Final	Sep 2010 – Oct 2010

4. TITLE AND SUBTITLE
DARPA Antibody Technology Program Standardized Test Bed for Antibody Characterization: Characterization of an MS2 Human IgG Antibody Produced by AnaptysBio, Inc.

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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Excet, Inc., 8001 Braddock Road, Suite 303, Springfield, VA 22151-2110

8. PERFORMING ORGANIZATION REPORT NUMBER
ECBC-TR-1339

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Defense Advanced Research Projects Agency (DARPA)

10. SPONSOR/MONITOR'S ACRONYM(S)
DARPA

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT:
The Defense Advanced Research Projects Agency (DARPA) Antibody Technology Program (ATP) focused on developing technologies to enhance the thermal stability and binding affinity of an antibody. In this study, the U.S. Army Edgewood Chemical Biological Center (ECBC) functioned as an independent testing laboratory to provide specific technical support on immune reagents and assist in the definition of government-supplied, antibody–antigen pairs. The project goal was to select, develop, and standardize methods for characterizing the de novo thermal and binding properties of select reagents to be used by DARPA-funded investigators and to use those methods to validate changes in antibody thermal stability and binding affinities. The antibody chosen was the ECBC MS2 recombinant antibody, which detects an MS2 coat protein that forms the capsid for MS2 bacteriophage. The focus of the work described herein was to evaluate the MS2 antibodies supplied by the DARPA-funded investigator, AnaptysBio, Inc. (San Diego, CA), for affinity and stability enhancements. The study results provide standardized parametric data on antibody properties and performance. This information will also help develop a decisional analysis tool to expand confidence levels for the selection of antibody-based reagents that will optimize field operational and performance metrics for future detection and diagnostic platforms.

15. SUBJECT TERMS

Antibody	Biosurveillance	Enzyme-linked immunosorbent assay (ELISA)
Quality	Testing	MS2 coat protein (MS2CP)

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Renu B. Rastogi
U	U	U	UU	32	**19b. TELEPHONE NUMBER** *(include area code)* (410) 436-7545

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PREFACE

The work described in this report was supported by the Defense Advanced Research Projects Agency funding. The work was started in September 2010 and completed in October 2010.

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STANDARDIZED TEST BED FOR ANTIBODY CHARACTERIZATION: CHARACTERIZATION OF AN MS2 HUMAN IGG ANTIBODY PRODUCED BY ANAPTYSBIO, INC.

1. INTRODUCTION

The current platforms for detection and diagnosis of biothreat agent exposure depend on the use of antibodies to recognize and bind to specific antigens. To date, selecting antibodies for inclusion in a final assay format has primarily relied on the performance of an antibody in an enzyme-linked immunosorbent assay (ELISA), with little regard for quantifying the full spectrum of variables that affected antibody–antigen interaction. The Joint Project Management Office Biosurveillance (JPMO BSV) Critical Reagents Program (CRP) members instituted a quality program for the standardization of test methods to more fully characterize and compare the physical and functional properties of antibody reagents in its repository. The development and standardization of antibody testing provides the JPMO BSV with an invaluable platform for providing consistent, high-quality assays and reagents, not only for use in current biodetection platforms, but also for developing and validating future systems. This antibody characterization platform will be used to characterize the MS2 recombinant antibody, produced at AnaptysBio, Inc. (San Diego, CA) for the Defense Advanced Research Projects Agency (DARPA) Antibody Technology Program (ATP).

The DARPA ATP members focused on developing technologies to enhance the thermal stability and binding affinity of a given antibody. In this study, the U.S. Army Edgewood Chemical Biological Center (ECBC) functioned as an independent testing laboratory to provide specific technical support on immune reagents and assist in the definition of government-supplied, antibody–antigen pairs. The goal of this project was 2-fold: (a) to select, develop, and standardize the methods for characterizing the de novo thermal and binding properties of select reagents to be used by DARPA-funded investigators and (b) to then use those methods to validate the changes in antibody thermal stability and binding affinity achieved by the DARPA investigators. The antibody chosen for this project was the recombinant MS2 single-chain variable fragment (scFv) antibody produced at ECBC (*1*), which detects an MS2 coat protein (MS2CP) that forms the capsid for the MS2 bacteriophage. The focus of the work described herein was to evaluate the MS2 antibodies supplied by the DARPA-funded investigator, AnaptysBio, Inc. for affinity and stability enhancements. The results of this study provide standardized parametric data on antibody properties and performance. This information will also contribute to the development of a decisional analysis tool to expand confidence levels for the selection of antibody-based reagents that will optimize field operational and performance metrics for future detection and diagnostic platforms.

2. MATERIALS AND METHODS

2.1 MS2 scFv and MS2CP

MS2 scFv antibody was produced from a plasmid supplied by Ellen Goldman at the U.S. Naval Research Laboratory (NRL; Bethesda, MD). The plasmid was designated Gv1, and the sequence was cloned into a pET-22b(+) plasmid (EMD Millipore; Billerica, MA). The protein was produced and eluted in 20 mM sodium phosphate (pH 8.0), 0.5 M sodium chloride, and 0.5 M imidazole. Peak fractions were then collected and separated on a 16/60 Superdex 200 gel filtration column (GE Healthcare Life Sciences; Pittsburgh, PA), and fractions that corresponded with a monomeric protein were collected and flash-frozen in liquid nitrogen. These fractions were then provided (along with sequence data) to AnaptysBio as baseline material.

The MS2CP was produced from a pET-28a(+) plasmid (Novagen, Inc.; Madison, WI) with the MS2CP sequence inserted with an amino acid substitution of an arginine at position 83 construct engineered by DNA2.0, Inc. (Menlo Park, CA, www.DNA20.com). MS2CP was produced and eluted with 300 mM imidazole in pH 7.4 phosphate buffered saline (PBS; Sigma-Aldrich Company LLC; St. Louis, MO). Peak fractions were collected, and buffer was exchanged into PBS (pH 7.4) using 470 mL of packed volume Sephadex G-25 (Amersham Biosciences Corporation; Piscataway, NJ) fine gel chromatography and provided to AnaptysBio as an antigen for the MS2 antibody.

2.2 NanoDrop Spectrophotomer

To determine the concentration of the MS2 human IgGs supplied by AnaptysBio, the NanoDrop ND-1000 (Thermo Fisher Scientific, Inc.; Waltham, MA) spectrophotometer was used. This instrument was used to provide the absorbance at 280 nm (A_{280}) of the sample. Because the A_{280} is influenced by the number of tryptophan and tyrosine residues in a given protein, the extinction coefficient was used in conjunction with A_{280} to determine an accurate concentration. The concentration of each MS2 IgG was determined by taking the average A_{280} data divided by 1.4, which is the extinction coefficient used for an IgG. Each reading required a 2 µL sample, which was placed on the sample pedestal. The arm of the spectrophotometer was lowered, making a liquid column between the top of the arm and the pedestal surface; this created the path length through which the laser passed. The instrument was blanked using PBS and readings were taken in triplicate. As a positive control, bovine gamma globulin (BGG; Bio-Rad Laboratories; Hercules, CA) was also tested to validate the instrument operation.

2.3 Experion Electrophoresis System

Molecular weight and purity data were collected using the Experion (Bio-Rad) automated electrophoresis system. The Experion system employs microfluidic technology to automate electrophoresis for protein analysis. The microfluidic chip, in conjunction with the Experion reagents, electrophoresis station, and software, are designed to accomplish separation, staining, destaining, detection, and basic data analysis. The Experion Pro260 analysis kit uses engineered lower and upper internal alignment markers to provide clean baselines, accurate molecular weight sizing, and quantitative protein analysis (*2*). The Pro260 analytical software

determines sample purity by calculating the percent mass of the separated proteins in a sample. For Experion analysis, each of the AnaptysBio MS2 IgGs was standardized to a final concentration of 1 mg/mL by diluting it in PBS. The control (BGG) and AnaptysBio samples were then processed using the validated procedure specified in the Bio-Rad Experion Pro260 analysis kit, rev. C (*3*). Briefly, a Pro260 microfluidic chip was prepared by adding 12 µL of Pro260 gel and gel stain to the designated wells. The chip was then placed on the priming station and primed for 1 min at the medium (B) pressure setting. Priming filled the fluidic channels with gel, which was used by the instrument to form a barrier between samples during the run. The sample was reduced with dithiothrietol (Sigma-Aldrich) and denatured in the kit-provided sample buffer at 95 °C before being applied to the primed chip. The chip was then placed in the instrument, and the lid was closed, which lowered the sample needles into the wells. The instrument was operated using the Experion software, and each chip took 30 min to complete. All samples were run in triplicate alongside one sample of the control (BGG) and Pro260 ladder (Bio-Rad). All analyses were performed using the Experion software (Bio-Rad).

2.4 Dynamic Light Scattering (DLS)

DLS was used to evaluate how the proteins behaved in solution. DLS data can be used to indicate whether a protein is in solution by the measurement of the polydispersity, hydrodynamic radius, and molecular weight of the sample. Prediction algorithms are used by the DLS software to produce a range of values for the protein under evaluation. For DLS analysis, five 20 µL aliquots of the AnaptysBio MS2 IgGs, along with the control bovine serum albumin (BSA; Sigma-Aldrich) were placed into a quartz 384-well plate (Wyatt Technology Corporation; Santa Barbara, CA) and centrifuged for 2 min at (239×*g*) to remove trapped air bubbles from the samples. Mineral oil (Sigma-Aldrich) was applied to the top of each sample to prevent sample evaporation, and the plate was then placed into a DynaPro temperature-controlled plate reader (Wyatt Technology). Each well was scanned 10 times for 5 s each at 25 °C, and results were averaged to provide measurements of polydispersity, hydrodynamic radius, percent mass, and molecular weight for each sample using the Wyatt Technology Dynamics software. The results of three wells were averaged and reported.

2.5 Differential Scanning Calorimetry (DSC)

DSC was used to obtain a quantitative melting temperature (T_m) for each of the AnaptysBio MS2 IgG proteins. The T_m was determined to predict results of subsequent ELISA and surface plasmon resonance (SPR) thermostability testing. A T_m above 70 °C is predictive for antibody activity after the thermal stress test remains above 50%. A T_m below 70 °C predicts, at minimum, a 50% decrease in antibody activity after the thermal stress test. For DSC experiments, samples were diluted to 0.5 mg/mL and dialyzed overnight in PBS (pH 7.4). Before analysis, samples were degassed for 5 min and then injected into the sample cell of a MicroCal VP-DSC (Malvern Instruments Ltd.; Worcestershire, UK). Dialysis buffer was added to the reference cell of the calorimeter, and a buffer scan was used as the baseline for all experiments. The samples were scanned from 15 to 100 °C at a rate of 60 °C/h, in duplicate. The transition midpoint of the curve was determined using the Origin 7.0 software to analyze the data.

2.6 Thermal Stress Test

Before applying heat, all samples were diluted to a concentration of 1 mg/mL to negate any protective effects that may be due to concentration (*2*). AnaptysBio thermally stabilized antibody (APE 686) was diluted to 1 mg/mL in 1X PBS and divided into five tubes. One aliquot was kept on ice for the duration of the experiment and marked "time 0". The remaining four aliquots were heated to 70 °C on a calibrated heat block for 15, 30, 45, and 60 min each. After each time point, the corresponding aliquot was removed and placed in an ice bath. These samples were then tested for activity using ELISA.

2.7 ELISA

ELISAs were performed in triplicate using standard techniques. After thermal stress testing, each sample was diluted to 1 µg/mL in PBS and used to coat one row each of three Maxisorb 96-well plates (NUNC products; Thermo Fisher Scientific, Inc.). Samples were then incubated at 4 °C overnight. In the morning, each plate was washed in 1X wash buffer (KPL, Inc.; Gaithersburg, MD) using a standard wash protocol on an AquaMax 200 plate washer (MDS Analytical Technologies; Sunnyvale, CA), and then blocked with 1X milk diluent block (MDB) (KPL, Inc.) for 30 min at 37 °C. The plates were then washed again using the same procedure, and 100 µL of PBS-T (PBS with 0.05% Tween 20; Sigma-Aldrich) was added to all wells. MS2CP was diluted in PBS-T to 2 µg/mL, and 100 µL of this solution was applied to the first well of each row. A 2-fold serial dilution was performed across each plate, which were then incubated for 1 h at 37 °C.

After washing, the rabbit anti-MS2, which was supplied by the CRP Joint Program Executive Office for Chemical and Biological Defense, was diluted to 5 µg/mL in 1X MDB. After the dilution, 100 µL of this solution was added to each well and then the plate was incubated at 37 °C for 1 h. After incubation, the plates were washed. Goat anti-rabbit IgG (H+L)*-horseradish peroxidase (HRP; KPL, Inc.) was diluted to 0.2 µg/mL in 1X MDB, and 100 µL of this solution was added to each well and incubated at 37 °C for 30 min. After washing, 100 µL of room temperature 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) one-component HRP substrate (KPL, Inc.) was added to each well. After 20 min at 37 °C, the optical density (OD) at the 405 nm light wavelength was determined using a Synergy H4 Hybrid Multi-Mode microplate reader (BioTek Instruments, Inc.; Winooski, VT). Data analysis was performed using the Prism graph pad software (GraphPad Prism, version 5.00 for Windows, GraphPad Software, Inc.; San Diego, CA; www.graphpad.com).

2.8 SPR Method

SPR is a method used to determine the kinetic parameters of an antibody–antigen interaction. This rapid methodology monitors biomolecular interactions through the excitation of surface plasmons that results when polarized light is shone through a prism on a sensor chip with a thin metal film coating. The metal film reflects the light by actig as a mirror, and when the angle of light shining through the prism is changed, the intensity of the reflected light can be

* H+L, heavy and light chains of the antibody.

monitored and the differences in intensity can be recorded. Although the refractive index at the prism side of the sensor chip does not change, the refractive index in the immediate vicinity of the metal surface will change when accumulated mass (bound proteins) adsorbs onto the metal surface. Therefore, if binding occurs, the resonance angle (SPR angle) changes, and this shift of the SPR angle provides information on the kinetics of the protein adsorption on the metal surface. The SPR software then provides an accurate analysis of the association (k_a) and dissociation (k_d) rate constants for the antibody interactions and calculates the overall affinity constant (K_D) between the antibody and antigen.

2.8.1 Thermostability Testing Using SPR

Using the Biacore T200 (Biacore AB Corporation; Uppsala, Sweden [subsidiary of GE Healthcare]), 6500 response units (RU) of MS2CP were tethered to one flow cell of a CM5 chip (Biacore) using standard amine-coupling chemistry. After thermal stress testing, samples were centrifuged at 5 °C, 2000×*g* for 5 min. The analyte was then run at 10 µL/min for 120 s. A calibration curve was created by injecting eight concentrations of the time 0, unheated, AnaptysBio MS2 samples (APE 686) at 400, 350, 300, 250, 200, 150, 100, and 50 nM and plotting their respective maximum analyte-binding capacity of the surface in RU (R_{Max}). Unheated and heated samples were then diluted 1:90 and 1:180 so that the time 0 control points fell on the linear calibration curve. All samples were run in triplicate. The chip surface was regenerated using an 18 s injection of 0.85% phosphoric acid at a flow rate of 30 µL/min. Data collection was performed using the Biacore Concentration Analysis software (Biacore T200 Evaluation software), and the active concentration of heated sample was recorded. The running buffer used for this experiment was the Biacore HBS-EP, 1X buffer.

2.8.2 Kinetic Analysis Using SPR

Using a Biacore T200 system and 1X HBS-EP running buffer, 102 RU of MS2CP was tethered to a CM5 chip (GE Healthcare) using standard amine-coupling chemistry. AnaptysBio MS2 IgG (APE 686) was injected across the chip's surface for 120 s at a flow rate of 75 µL/min with a 900 s dissociation at 60, 20, 6.67, and 2.2 nM and 700 pM. The chip's surface was regenerated using an 18 s injection of 0.85% phosphoric acid at 30 µL/min with a 60 s stabilization period. Data were analyzed using a Langmuir 1:1 fit.

3. RESULTS

3.1 NanoDrop Results

Both of the AnaptysBio MS2 human IgGs were read in triplicate on the NanoDrop ND-1000 spectrophotometer. The A_{280} readings are shown in Table 1.

Table 1. NanoDrop A_{280} Readings

Replicate	APE 686 (mg/mL)	APE 987 (mg/mL)
1	2.43	2.52
2	2.42	2.53
3	2.44	2.53

After taking an average of these three values for each antibody (Table 1) and dividing by the extinction coefficient of 1.4, the final concentrations were determined to be 2.4 mg/mL for the thermally stabilized APE 686 and 2.5 mg/mL for the affinity-matured APE 987.

3.2 Experion Electrophoresis Results

The molecular weights of the AnaptysBio MS2 IgGs were determined using the Experion Pro260 analysis kit (Bio-Rad), as shown in Figure 1. The thick band seen at the top of the second lane corresponds to the AnaptysBio MS2 IgG antibody APE 686 heavy chain. The thinner band at the bottom of the second lane corresponds to APE 686 light chain. According to the software analysis, APE 686 is 99.5% pure, with the heavy chain weighing 61.5 kDa and the light chain weighing 29.0 kDa. The thick band seen at the top of the fourth lane corresponds to the AnaptysBio MS2 IgG antibody APE 987 heavy chain. The thinner band at the bottom of the second lane corresponds to APE 987 light chain. According to the software analysis, APE 987 is 99.3% pure, with the heavy chain weighing 61.7 kDa and the light chain weighing 28.7 kDa.

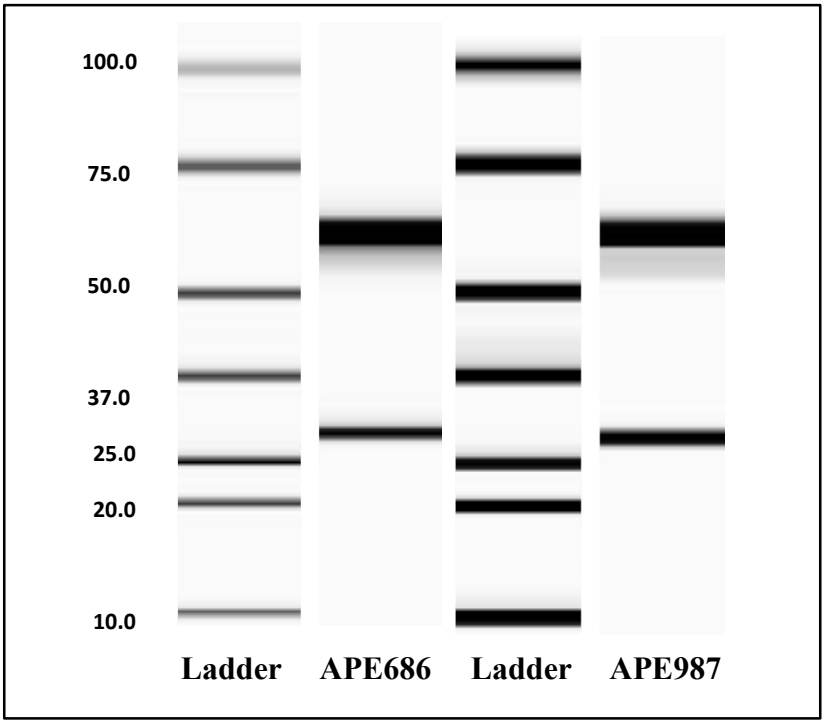

Figure 1. Molecular weight and purity. Digital gel of Anaptys MS2 IgGs produced by the Experion Pro260.

3.3 DLS Results

Both of the AnaptysBio MS2 IgGs were analyzed in triplicate using the DynaPro plate reader. The radius of APE 686 was determined to be 5.7 nm, with a polydispersity of 17.2% (Table 1.). The radius of APE 987 was determined to be 6.1 nm with a polydispersity of 20.5%. Figures 2 and 3 contain representative correlation and regularization graphs for each of the MS2 IgGs. The correlation graphs (Figures 2A and 3A) depict a sigmoidal curve indicative of a valid size distribution. The regularization graphs (Figures 2B and 3B) illustrate the monodispersity found in both samples. Table 2 shows the raw data produced for each replicate. Because 99.8 or 100% of the mass displays favorable polydispersity and hydrodynamic radius, both of these sample preparations are considered to be monodisperse.

Table 2. Features of MS2 scFv In Solution

Sample	Replicate	Radius (nm)	% Polydispersity
APE 686	1	5.8	18.7
	2	5.8	17.3
	3	5.7	16.4
	4	5.8	18.7
	5	5.6	15.0
	Average	5.7	17.2
APE 987	1	6.1	17.5
	2	6.0	18.9
	3	6.5	27.8
	4	6.0	17.9
	Average	6.1	20.5

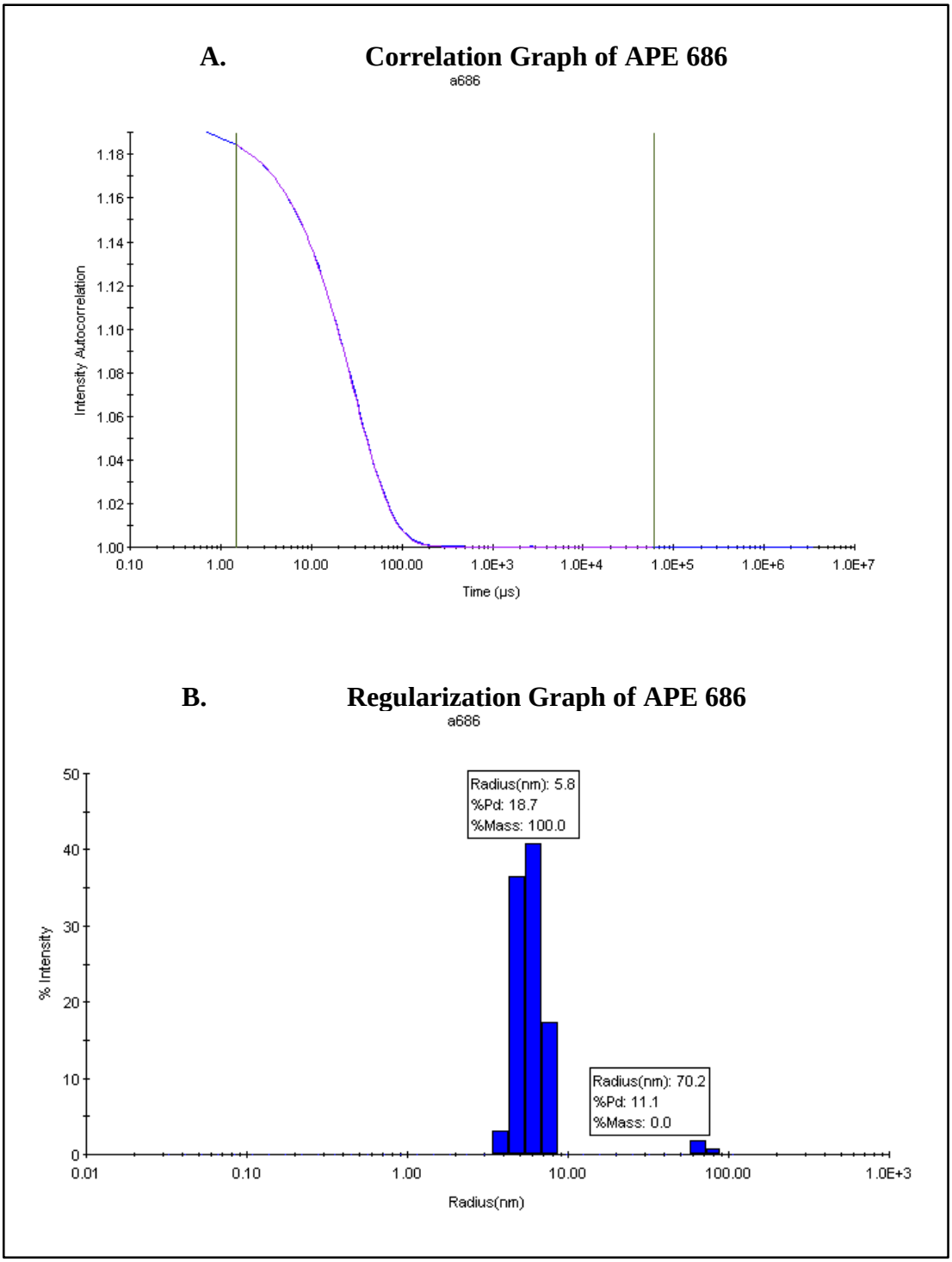

Figure 2. Radius and polydispersity representation. (A) Correlation and (B) regularization graphs of the AnaptysBio thermostable IgG APE 686.

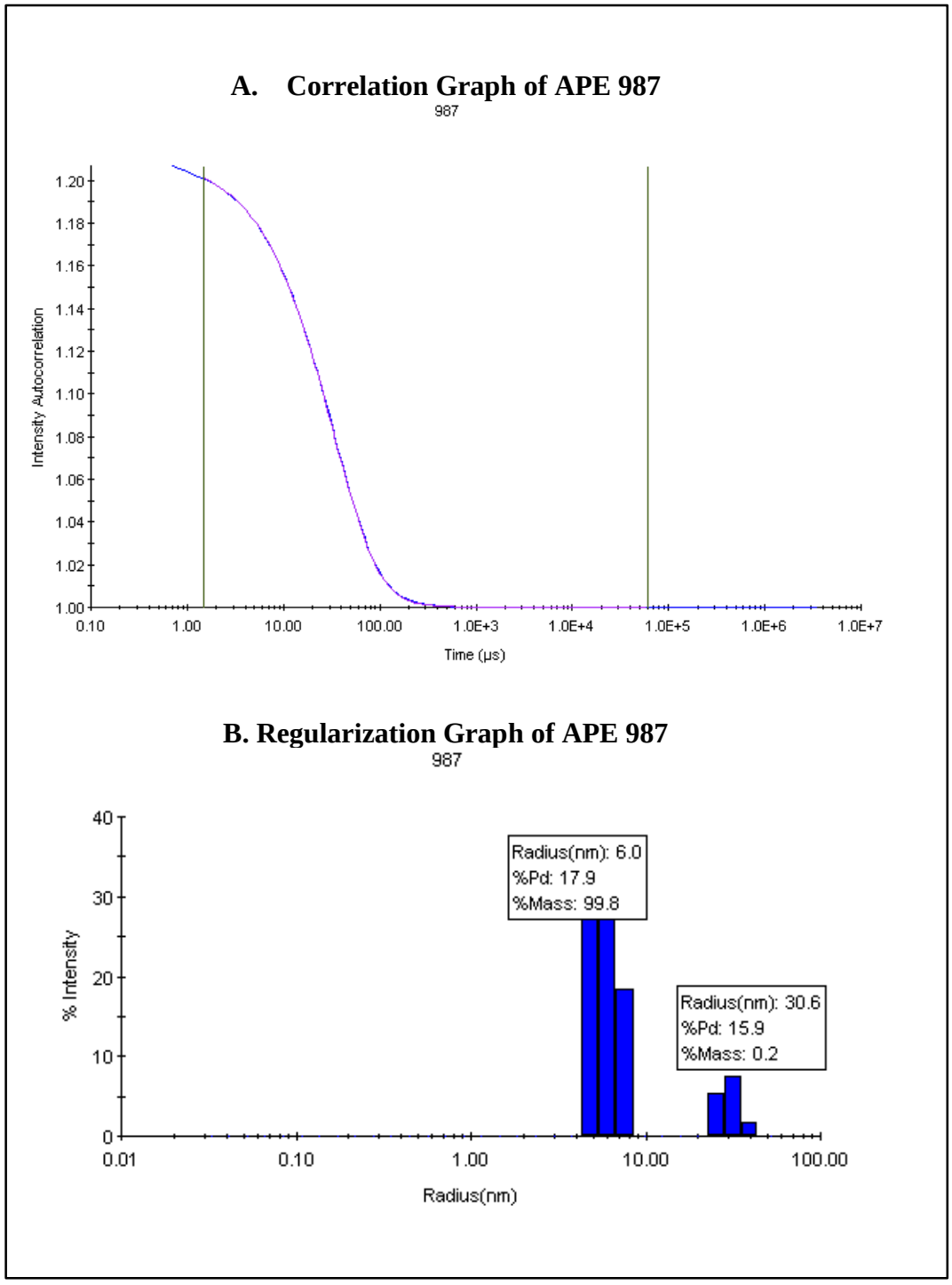

Figure 3. Radius and polydispersity representation. (A) Correlation and (B) regularization graphs of the AnaptysBio, affinity-matured IgG APE 987 used for determining the radius and polydispersity of the samples.

3.4 DSC Results

The MS2 scFv was read in duplicate on the MicroCal VP-DSC. The peak T_ms were as follows:

- APE 686 Replicate 1: 82.76 °C;
- APE 686 Replicate 2: 82.56 °C;
- APE 987 Replicate 1: 78.86 °C; and
- APE 987 Replicate 2: 79.01 °C.

The final T_m of APE 686 was determined to be 82.6 °C (Figure 4) and that of APE 987 was determined to be 78.9 °C (Figure 5).

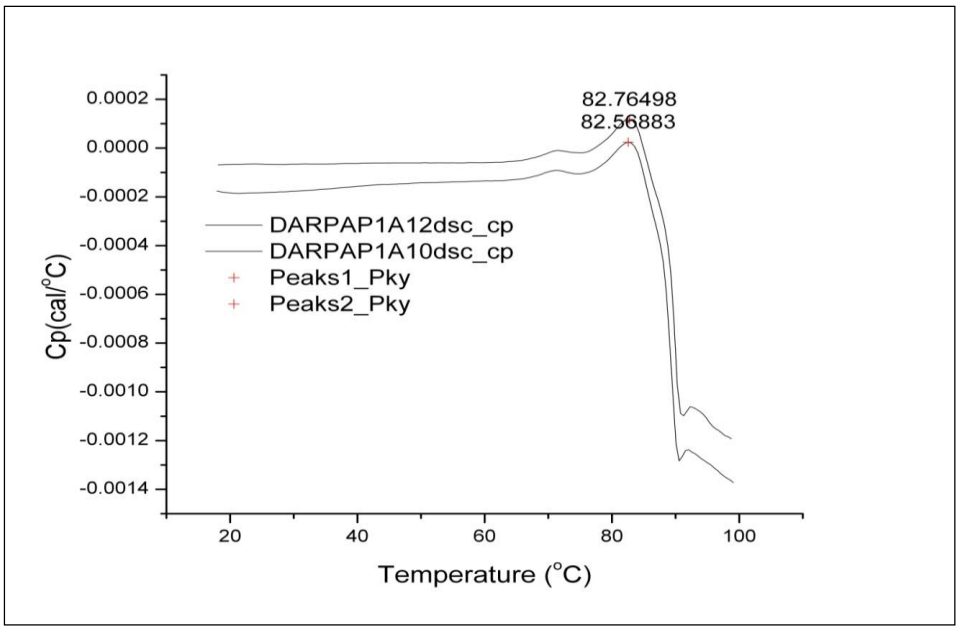

Figure 4. Transition midpoint curve for APE 686. Curve was generated on the MicroCal VP-DSC and analyzed using the Peak Find function in the Origin 7.0 software. The T_m was calculated to be 82.6 °C for APE 686.

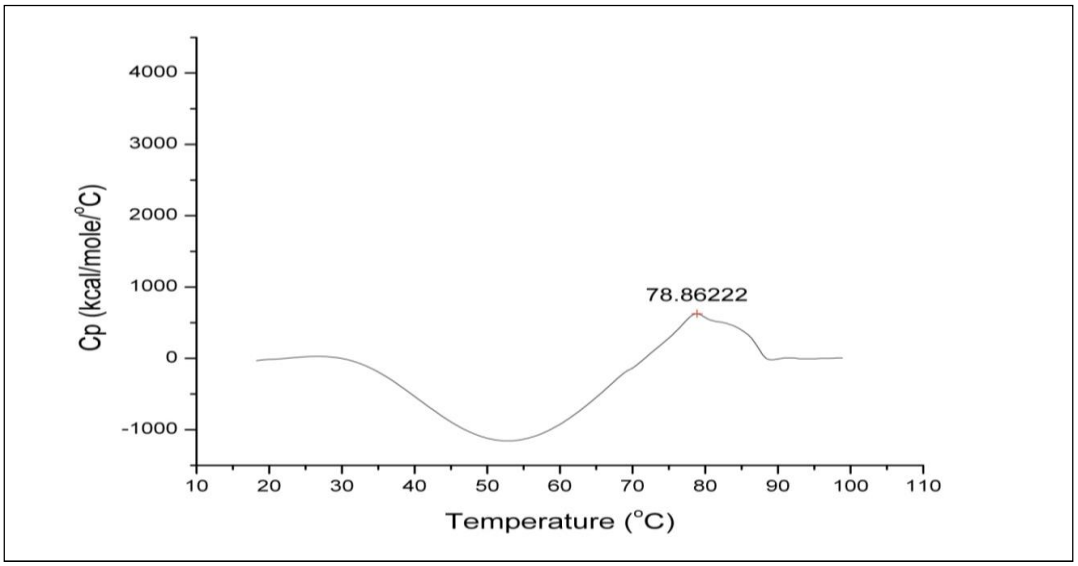

Figure 5. Transition midpoint curve for APE 987. Curve was generated on the MicroCal VP-DSC, and analyzed using the Peak Find function in the Origin 7.0 software. The T_m was calculated to be 78.9 °C for APE 987.

3.5 ELISA Results

ELISA assays were used to test the functional interaction of antibodies and antigens after thermal stress at 70 °C. The ELISA data (Figure 6) show that when the Anaptys IgG APE 686 was heated to 70 °C, it maintained all activity across all time periods of thermal stress, unlike the government-supplied MS2 scFv. The curves in Figure 6A show antibody activity for different time points at 70 °C as a function of the concentration of antigen supplied. The areas under the curve for each time point at 70 °C were calculated, averaged and graphed to depict how the MS2 IgG reacted over time to thermal stress. The graph in Figure 6B shows that all of the MS2 IgG remains functionally capable of binding to antigen after a 60 min exposure to 70 °C, unlike the original scFv, which lost activity within 15 min of heating.

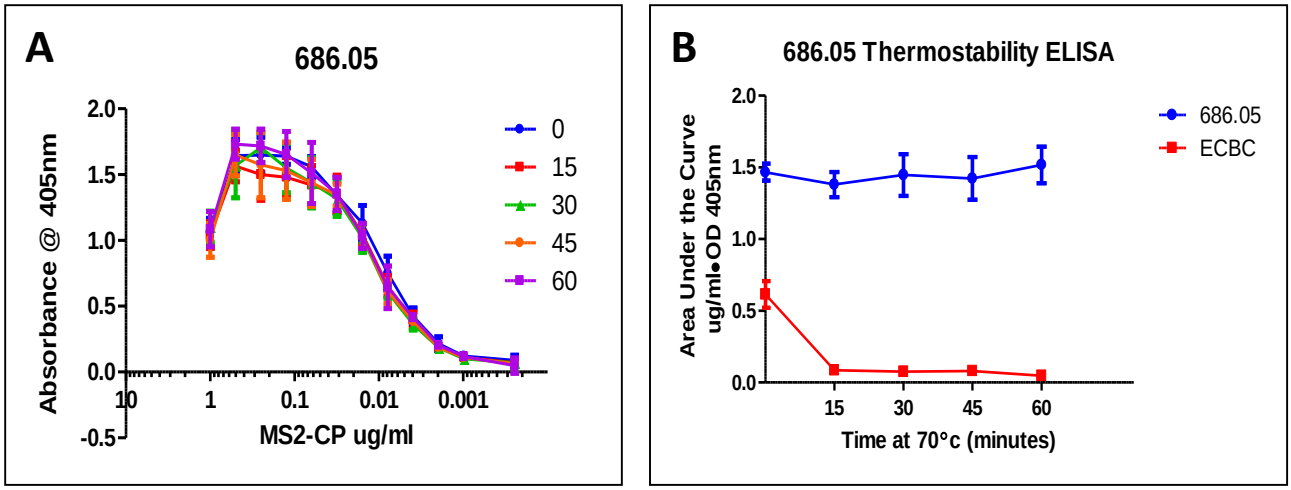

Figure 6. Thermostability of Anaptys APE 686 using ELISA. (A) Activity of APE 686 as a function of antigen concentration after thermal stress. The different curves are represented on the legend by the number of minutes the sample was held at 70 °C. (B) Area under the curve analysis depicting the effect of thermal stress.

3.6 SPR Results

3.6.1 Thermostability Testing Results Using SPR

After heating the antibody–antigen complex to 70 °C for several time periods, the functional binding between the AnaptysBio MS2 IgG (APE 686) and the antigen was assessed using SPR. Five tubes of 1 mg/mL MS2 IgG were prepared and heated to 70 °C for the following time periods: 15, 30, 45, and 60 min, followed by quenching the tube contents on ice. The activity of each sample was compared to a calibration curve of unheated sample using a Biacore T200 system. The percent activity of the heated samples was plotted over time (Figure 7). The results indicate that the activity of the IgG remains active over the entire 60 min; whereas, the activity of the scFv drops off completely within the first 15 min at 70 °C.

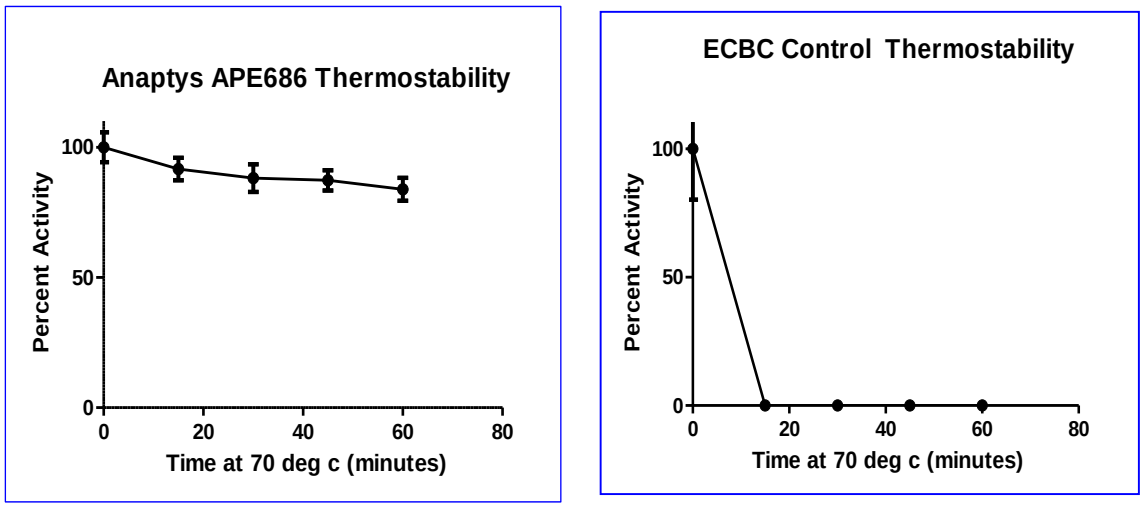

Figure 7. Thermostability of the AnaptysBio MS2 IgG (APE 686) compared to the ECBC MS2 scFv using SPR. The AnaptysBio antibody maintained over 85% of its activity after heating to 70 °C for 60 min, compared with the original ECBC MS2 scFv antibody, which lost all ability to recognize the MS2CP target within 15 min of heating.

3.6.2 Kinetic Analysis Results Using SPR

Kinetic analysis of the affinity-enhanced AnaptysBio MS2 IgG (APE 987) binding to the MS2CP antigen was performed as a direct-binding SPR experiment on the Biacore T200 system, and the results are presented in Figure 8A. Data were normalized to a blank-immobilized reference flow cell and fit to a Langmuir 1:1 model using Biacore T200 software. The K_D was determined to be 68.2 pM. Similar experiments run using the original ECBC MS2 scFv are presented in Figure 8B. The K_D of the original scFv was determined to be 15.5 nM; therefore, the AnaptysBio staff provided an antibody that was well above the 100-fold improvement threshold. Finally, kinetics analysis was also performed on the thermostable-enhanced AnaptysBio MS2 IgG (APE 686), and the results are presented in Figure 8C; K_D was determined to be 235 pM.

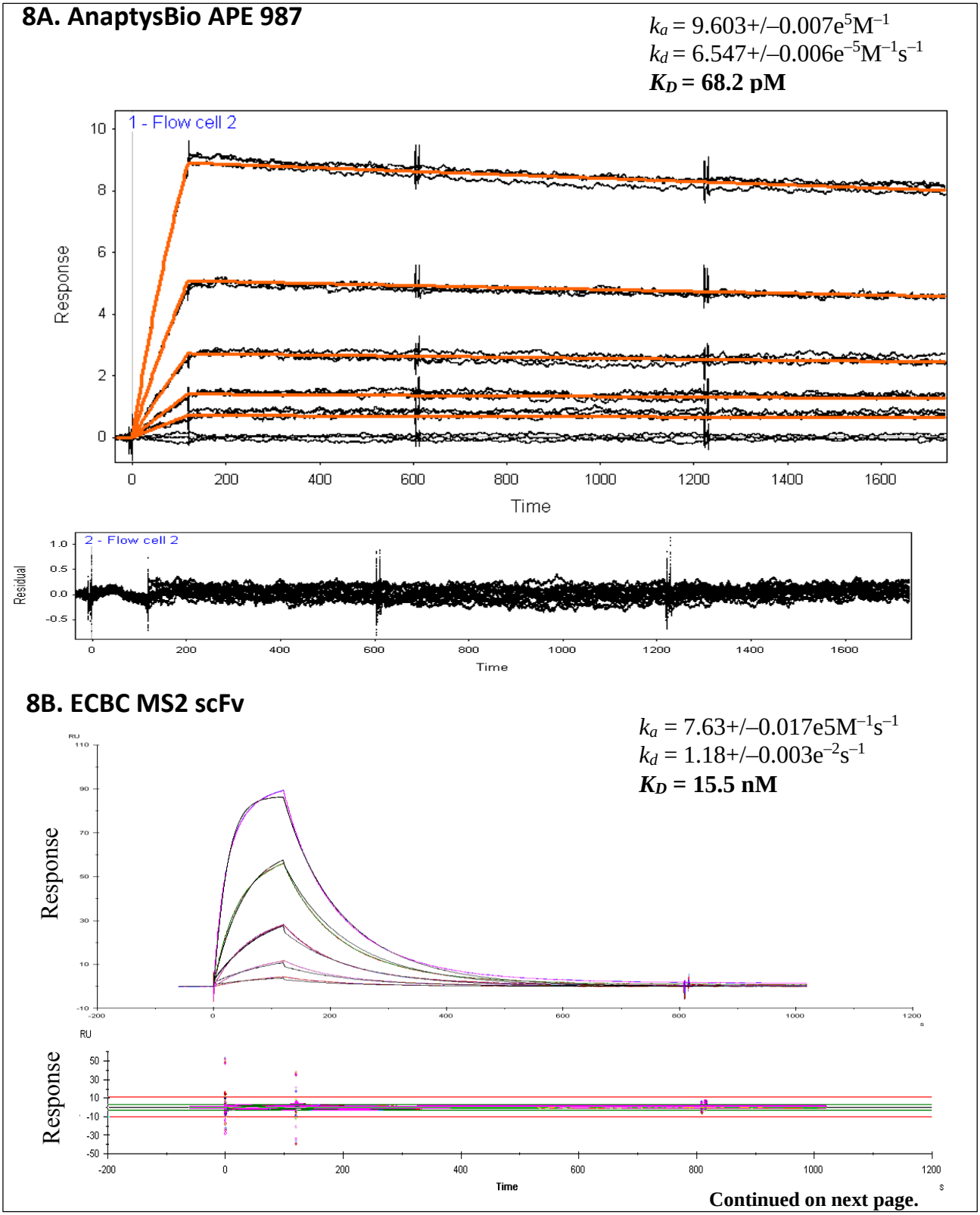

Figure 8. Comparison of the kinetic fits with residuals of the MS2 antibodies, determined using a Biacore T200 system: (A) kinetics of AnaptysBio affinity-enhanced MS2 IgG (APE 987, 68.2 pM) and (B) kinetics of original MS2 scFv (15.5 nM).

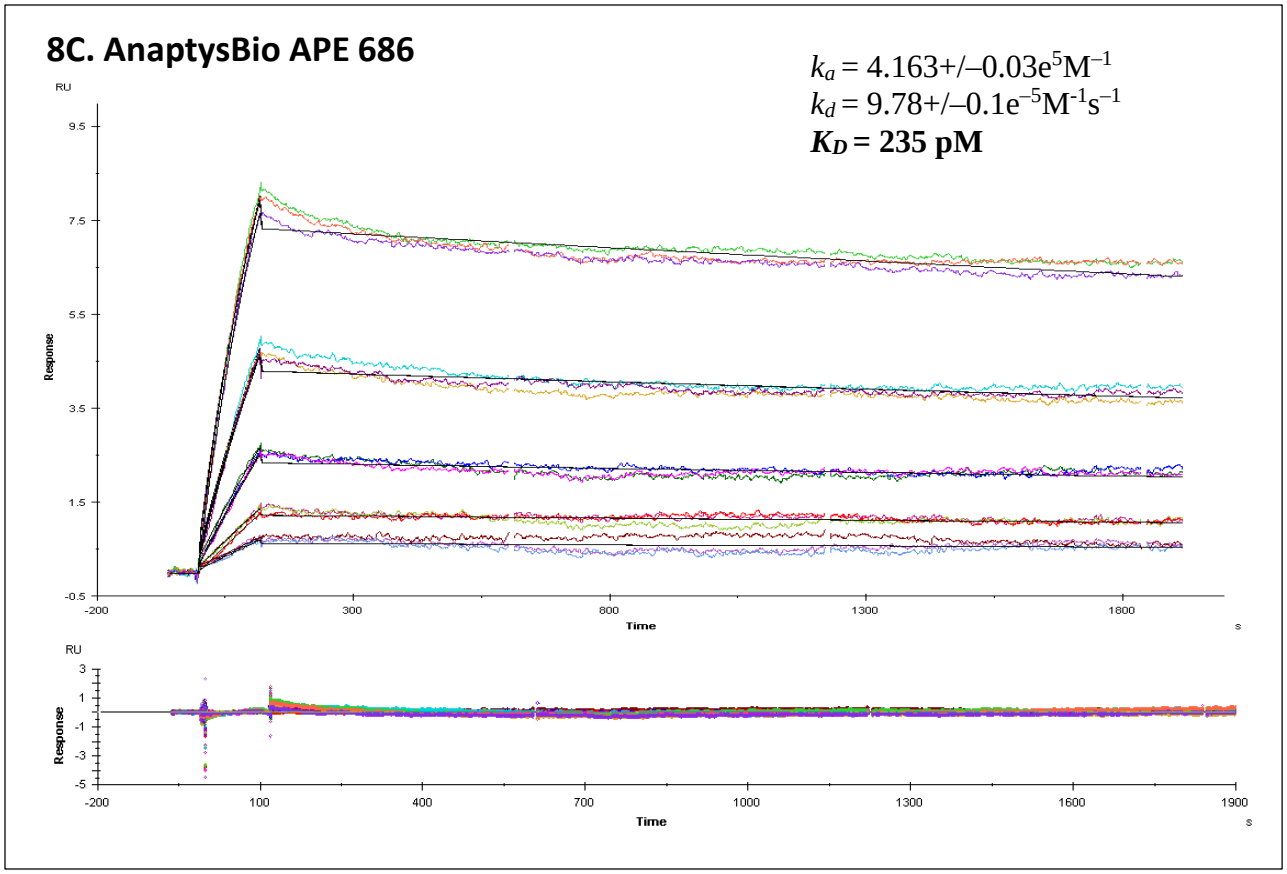

Figure 8 (continued). Comparison of the kinetic fits with residuals of the MS2 antibodies, determined using a Biacore T200 system: (C) kinetics of AnaptysBio thermostable-enhanced MS2 IgG (APE 686; 235 pM).

4. DISCUSSION

This study used the established and standardized parametric tests on the MS2 scFv antibody, which was selected by the DARPA ATP as the initial substrate to be used to demonstrate molecular schemes for improving the thermal stability or affinity of an antibody for its target antigen. Profiles of the MS2 scFv's physical characteristics were obtained using the NanoDrop, Experion, and DLS measurement platforms. These characteristics were compared to those of the improved antibodies that were submitted by AnaptysBio, Inc. The AnaptysBio MS2 IgG functional characteristics for assessing the effect of molecular engineering on thermal stability or affinity were obtained using the DSC, ELISA, and SPR analytical platforms.

An accurate assessment of protein concentration is critical for all of the test procedures described in this report. We applied the standard technique of spectrophotometry with the NanoDrop ND-1000. This instrument was used to provide the A_{280} of the sample, which is influenced by the number of tryptophan and tyrosine residues in a given protein. For this reason, the extinction coefficient was used in conjunction with A_{280} to determine an accurate concentration.

After protein concentration was determined with the NanoDrop system, molecular weight and purity data were collected with the Experion automated electrophoresis system. This system employs microfluidic technology to automate electrophoresis for protein analysis. The results of Experion analysis of the AnaptysBio MS2 IgG protein fell within the acceptable range of purity for use in assay development, and the molecular weight determined by the software was typical for an IgG (Figure 1).

DLS was used in conjunction with Experion and NanoDrop systems to evaluate how the protein behaved in solution. DLS data indicate the physical state and potential aggregation of a protein in solution by measuring the polydispersity, hydrodynamic radius, and molecular weight of a sample. The DLS data established whether the MS2 IgGs provided by AnaptysBio were monomeric and monodisperse. Less than 1% of the sample mass appeared to be aggregating (Figure 2) in solution. To make sure all testing would be consistent and to mitigate the exacerbating effect of freeze-thawing on future sample aggregation, the AnaptyBio MS2 IgGs were aliquoted into single-use vials and centrifuged before use.

The next round of testing evaluated the thermostability of the AnaptysBio MS2 IgGs using DSC, ELISA and SPR systems. DSC was used to get a quantitative T_m. The T_m should predict the results of ELISA and SPR thermostability testing. A T_m above 70 °C predicts that the percent activity of the MS2 IgGs after thermal stress should remain above 50%. A T_m below 70 °C predicts at least a 50% decrease in MS2 IgG activity after thermal stress. The AnaptysBio MS2 IgG antibody that was optimized for thermostability (APE 686) and described herein, exhibited a T_m of 82.76 °C (Figure 4). This was compared with a T_m from the original MS2 scFv of 67.5 °C. Therefore, it was expected that heating this sample above 70 °C would not cause the sample to unfold and lose at least 50% of its activity when evaluated using ELISA and SPR systems.

The results of the thermal stress test demonstrated that the AnaptyBio MS2 IgG remained active for over 60 min of heating at 70 °C. The ELISA and SPR data confirmed that the AnaptysBio IgG (APE 686) was able to bind the MS2CP, even after 60 min of heating, unlike the original MS2 scFv reference antibody, which was unable to bind the MS2CP after only 15 min of heating at 70 °C (Figures 6 and 7).

SPR was also used to obtain a kinetic analysis of the affinity-enhanced AnaptysBio MS2 IgG (APE 987) binding to its target antigen MS2CP to compare binding parameters with the original antibody. Kinetic data for APE 987 MS2 IgG binding to the MS2CP were obtained using the Biacore SPR platform, which yielded a K_D of 68.2 pM, whereas the original MS2 scFv yielded a K_D of 15.5 nM on the Biacore platform (Figure 8). These K_Ds clearly show that AnaptysBio well exceeded the 100-fold improvement requested by DARPA.

5. CONCLUSION

The DARPA ATP sought to establish methods for rapidly engineering a given antibody reagent that would exhibit physical and functional properties far exceeding those of its native state, thereby expanding user confidence in fielding antibody-based detection and

diagnostic platforms in environments or operational scenarios that would degrade or interfere with the currently available reagents. By optimizing the thermal stability or binding affinity of an antibody for its biological target, the DARPA ATP sought to yield antibody reagents that can reliably function in harsh environmental conditions and increase the sensitivity of a sensor platform to detect lower levels of a threat agent.

This report documents the testing of an improved thermostable antibody (APE 686) and an affinity-improved antibody (APE 987), which were both produced by AnaptysBio, Inc. staff. This study evaluated the physical and functional characteristics of these IgGs in ECBC testing. The results were compared to the baseline characteristics of the original antibody's physical properties to include: concentration, molecular weight, purity, state of aggregation in solution, and functional measures such as binding affinity and thermal stability. Both AnaptysBio antibodies exhibited enhanced thermal stability and affinity for binding to the MS2CP antigen.

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LITERATURE REFERENCES

1. O'Connell, K.; Khan, A.S.; Anderson, P.E.; James, J.; Cork, S. *Recombinant Antibodies for the Detection of Bacteriophage MS2 and Ovalbumin*; ECBC-TR-231; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2002; UNCLASSIFIED Report (ADA400 677).

2. Byrne, N.; Wang, L.M.; Belieres, J.P., Angell, C.A. Reversible Folding-Unfolding, Aggregation Protection, and Multi-Year Stabilization, in High Concentration Protein Solutions, Using Ionic Liquids. *Chem. Commun. (Camb)* **2007**, *26*, 2714–2716.

3. Bio-Rad Experion Pro260 Analysis Kit Instruction Manual, Version 10000975, Rev B, Catalog #700-7101; Bio-Rad Laboratories, Inc.: Hercules, CA, 2010.

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ACRONYMS AND ABBREVIATIONS

A_{280}	absorbance at 280 nm
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ATP	Antibody Technology Program
BGG	bovine gamma globulin
BSA	bovine serum albumin
CRP	Critical Reagents Program
DARPA	Defense Advanced Research Projects Agency
DLS	dynamic light scattering
DSC	differential scanning calorimetry
ECBC	U.S. Army Edgewood Chemical Biological Center
ELISA	enzyme-linked immunosorbent assay
H+L	heavy and light chains
HRP	horseradish peroxidase
Ig	immunoglobulin
JPMO BSV	Joint Project Management Office Biosurveillance
k_a	association rate constant
k_d	dissociation rate constant
K_D	overall affinity constant
MDB	milk diluent block
MS2CP	MS2 coat protein
NRL	U.S. Naval Research Laboratory
OD	optical density
PBS	phosphate buffer solution
R_{Max}	maximum analyte-binding capacity of the surface in RU
RU	response units
PBS	phosphate buffered saline
PBS-T	phosphate-buffered saline with Tween 20
scFv	single-chain fragment variable
SPR	surface plasmon resonance
T_m	melting temperature

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