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Improving the biophysical properties of anti-ricin single-domain antibodies☆

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ABSTRACT

Single-domain antibodies (sdAbs) derived from heavy-chain only antibodies produced in camelids are attractive immunoreagents due to their small size, high affinity, and ability to refold and retain binding activity after denaturation. It has been observed that some sdAbs, however, exhibit undesirable properties including reduced solubility when subjected to heating or upon long-term storage at production-relevant concentrations, which can limit their usefulness. Using a multi-step, rational design approach that included consensus-sequence driven sequence repairs, the alteration of net protein charge, and the introduction of non-native disulfide bonds, augmented solubility and increased melting temperatures were achieved. The improved sdAbs tolerated storage in solution at high concentration (10 mg/mL) and were able to withstand multiple cycles of heating to high temperature (70 °C). This work demonstrates a pathway for improving the biophysical characteristics of sdAbs which is essential for expanding their utility for both diagnostic as well as therapeutic applications.

1. Introduction

There is an ongoing need for the development of robust recognition reagents for the sensitive detection of potential biothreat agents as well as new and emerging pathogens. Traditionally, biosensors utilizing antibody recognition elements have provided reliable detection capability for a broad range of targets; however, antibodies are prone to failure when exposed to harsh environments. Recombinantly-expressed binding domains derived from the remarkable heavy-chain-only antibodies produced by camelids (such as camels and llamas), termed single-domain antibodies (sdAbs), offer alternative binding elements. sdAbs provide the affinity and specificity of traditional antibodies, as well as additional desirable properties [1–4]. Importantly, many sdAbs have been demonstrated to refold into an active form able to bind antigen after heating to high temperatures [5–7]. Additionally, their small size (1/10 that of conventional antibodies), ability to bind "hidden" epitopes by virtue of an extended CDR3 region, and ability to be rationally-selected and produced in quantity by standard recombinant protein expression manufacturing methods make sdAbs attractive immunoreagents [1–4].

Unlike traditional antibodies, which normally aggregate and lose their ability to function after being heated above their melting temperature (T_m; the temperature at which half the protein is unfolded) [8], sdAbs are often found to recover their binding ability even after repeated thermal denaturation [5,7]. However, when heated above their melting temperature at high concentration and for extended periods of time, many sdAb clones are prone to aggregation [9]. As aggregation is also a problem with scFvs, recombinant binding domains derived from conventional antibodies, several strategies have been reported that led to more soluble and stable recombinant antibody-binding domains (rAbs). One successful strategy has been to produce sdAbs as fusions with a thermostable protein, as demonstrated by a fusion between a sdAb and a thermostable maltose binding protein that was able to withstand heating to 70 °C for an hour without loss of activity [10]. Increasing the net charge of the protein also can lead to more soluble rAbs. This can be accomplished by either appending a charged tail onto the rAb [9] or the substitution of charged amino acids in the protein sequence [11–16].

Researchers have also investigated schemes to increase the T_m of rAbs. For example, the T_m of a sdAb was increased through the process of random mutagenesis coupled with a stringent selection, which led to a ~7 °C increase in T_m to 90 °C [17]. Grafting the

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antigen binding loops (complementarity determining regions; CDRs) onto a more stable rAb framework has also provided increases in T_m and stability [18–21]. Finally, the introduction of a pair of cysteine residues in frameworks 2 and 3 of sdAbs is another method that has yielded sdAb variants with higher T_ms [22–25].

Although most sdAbs are already more soluble and thermally stable than conventional antibodies or their recombinantly expressed binding domains (i.e., scFv), enhancing the ability of sdAbs to retain binding activity after a heat challenge is a useful metric towards the goal of improving the performance of field portable detection devices in austere environments. To accomplish this metric, we started with previously described high-affinity sdAbs specific for three distinct epitopes on ricin [26,27], and implemented a multi-step process for obtaining binders with improved melting temperature and solubility. Ricin is a potent toxin listed as a select agent by the CDC, and the ability to detect ricin remains a high priority [28–31]. The potential offered by these thermal stabilized sdAb reagents to eliminate the cold-chain makes them a highly attractive alternative to conventional monoclonal antibody and scFv binding reagents. These sdAbs have the potential to provide more consistent function, while their ease of production could make their production less costly than conventional antibodies. Demonstrating excellent thermostability is the first step towards eliminating the need for refrigeration of these reagents – a big plus for forward-based troops or first-responders who could then keep their detection reagents at ambient temperature without negatively impacting shelf-life.

2. Materials and methods

2.1. Reagents

Ricin was purchased from Vector Laboratories. The reagents 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide (sulfo-NHS), and NHS-LC-LC-biotin were purchased from Pierce. Anti-ricin sdAbs were described previously [26,27]. Enzymes used for cloning were from New England Biolabs. DNA sequencing services were provided by Eurofins Genomics. Oligos were also purchased from Eurofins Genomics. Mutagenesis was carried out using the QuickChange Site Directed Mutagenesis Kit or QuikChange Multi Site-Directed Mutagenesis Kit from Agilent Technologies. Sequence alignments were performed using MultAlin [32]. Unless otherwise specified, chemical reagents were from Sigma Aldrich, Fisher Scientific, or VWR International.

2.2. Selection

Additional ricin clones were selected from our previously described immune library using essentially the same protocol previously employed [26]. Two rounds of panning were performed, and then binding phage were identified by monoclonal phage ELISA as described previously [26].

2.3. Introduction of negatively charged amino acids

Negatively charged amino acids were introduced into the sequences for C10, D12f, and H1W by multisite-directed mutagenesis using the following primers (5′–3′): GGAGGATTGGTGCAGGATGGGGGCTCTCTGAGA, AGGATATCGTATGCGGATTCCGTGAAGGGCCGA, and AGCGACAACTGGAAGAACACGGTGTATCTGCAA (C10); GTCACAGACTATGCAGATTCCGTGAAGGGTCGA and GCTAGTAGAAACTCGGATGACTATGGTTACTGG (D12f); and GGAGGATTGGCGCAGGATGGGGGTTCTCTCCGA and CAGGCTCCAGGGAAGGAACGTGAGTTTGTGGCT (H1W). Mutagenesis reactions were carried out as described in the product protocol and mutants were identified and confirmed by DNA sequencing.

2.4. Introduction of additional disulfide bond

The introduction of an additional disulfide bond in each of the sdAbs was accomplished by site-directed mutagenesis using the following primers and their reverse complements: GAGCGTGAATTTGTCTGCGTTATTAGTGGTTCT and AAGGGTCGATTCACCTGCTCCAGAGACGTCGCC (D12neg), GAACGTGAGTTTGTGTGCGCAATTAGGGCGAGA and AAGGGGCGATTCACCTGCTCCAGAGACAACGGC (H1Wneg), and GAGCGTGAGTTTGTTTGCGCACTTCGGTGGACT and AAGGGCCGATTCACCTGCTCCAGCGACAACTGG (C10neg). Following mutagenesis, the presence of an additional pair of cysteines was confirmed by sequencing.

2.5. Protein production

A PCR strategy was utilized to remove the upper hinge sequence; the sdAbs were cloned into pet22b for expression in the periplasm [33]. Bacteria were grown and protein purified as described [9,17]. Briefly, protein was purified through immobilized metal affinity chromatography and size exclusion. Protein concentration was determined by absorbance at 280 nm on a NanoDrop spectrophotometer.

2.6. Fluorescence based melting assay

The fluorescence based melting assay was conducted using an Applied Biosystems StepOne Real-Time PCR system and Sypro Orange dye (Sigma) [33]. A total of 10 μg of each purified sdAb was added to a 20 μL volume of PBS buffer. The Sypro Orange dye was diluted 1000-fold into each reaction solution. The temperature was increased from 25 to 99 °C at a rate of 1.2 °C/min. All measurements were done in triplicate and the values agreed within 0.6 °C.

2.7. T_m determination by circular dichroism

Refolding and melting temperatures were assessed using a Jasco J-815 circular dichroism (CD) spectrometer [33,34]. Samples were diluted with deionized water to a final concentration of 40 μg/mL. The differential absorbance of the protein sample was measured at 205 nm to monitor the secondary structure of the protein as the temperature was increased from 25 to 90 °C incrementally at a rate of 2.5 °C/min. The melting point correlated to the temperature at the inflection point between the folded and unfolded state. Ellipticity was recorded as samples were cooled to determine the refolding ability. The error on the T_m determinations was within ±1 °C.

2.8. Evaluation of aggregation

To evaluate the degree of aggregation, sdAbs were prepared at a concentration of 1.0 mg/mL (as measured by OD_{280} using a NanoDrop spectrophotometer) in PBS and incubated for 1 h at a range of temperatures (25, 57, 72, and 87 °C). A second experiment to determine the degree of aggregation resulting from thermal cycling was performed by preparing sdAbs at a concentration of 1.0 mg/mL and then subjecting them to 10 cycles of heating to 70 °C for 30 min followed by cooling to 25 °C for 30 min. Following incubation or thermal cycling, samples were centrifuged to pellet any precipitated protein and the concentration was measured again on the NanoDrop to quantify remaining soluble protein.

2.9. Surface plasmon resonance (SPR)

A BioRad ProteOn XPR36 system and standard GLC sensor chip was used to assess binding kinetics. Ricin was immobilized to the sensor chip surface on four rows at a saturating concentration of