

## Communications

### Modulation of Single-Chain Antibody Affinity with Temperature-Responsive Elastin-Like Polypeptide Linkers

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Single-chain antibodies are genetically engineered constructs composed of a  $V_H$  and  $V_L$  domain of an antibody linked by a flexible peptide linker, commonly  $(GGGS)_3$ . We asked whether replacement of this flexible linker with peptides known to undergo environmentally induced structural transitions could lead to antibodies with controlled binding and release characteristics. To this end, we genetically modified and produced a series of anti-fluorescein single-chain antibodies with the general linker sequence  $(VPGXG)_n$ , where  $n$  is 1.2 to 3 and X is Val or His, to evaluate the effects of linker length and composition. Our results indicate that single-chain antibodies containing elastin-like polypeptide linkers have equilibrium affinity ( $K_D$ ) comparable to wild-type  $(GGGS)_3$  at room temperature but altered binding kinetics and faster ligand release as the temperature is raised. These results are consistent with the increased molecular order and contraction that elastin-like polypeptides are known to undergo with increased temperature. Modulation of antibody affinity using stimulus-responsive linkers may have applications in biosensors, drug delivery, and bioseparations.

#### Introduction

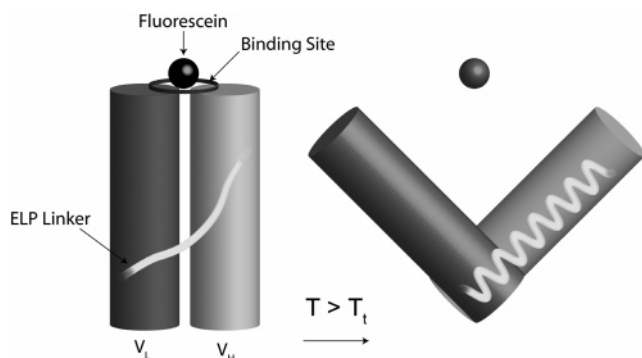
Single-chain antibodies (scFvs) are genetically engineered constructs composed of two immunoglobulin variable domains (light and heavy;  $V_L$  and  $V_H$ ) coupled by a polypeptide linker. This linker enables the expression of the construct as a single polypeptide chain and enhances the probability of  $V_L$ - $V_H$  association, thereby promoting formation of the antigen binding pocket by keeping the domains in close proximity. Typically, the linker is 10–25 residues and frequently composed of the repetitive polypeptide  $(GGGS)_3$ . The successful application of this linker to a variety of scFv constructs is thought to derive primarily from its relative inertness and flexibility. For example, Paci et al. have used computational techniques to demonstrate that the linker region is the most flexible portion of the scFv construct.<sup>1</sup> While diverse linker sequences have been explored, for example, by using phage display,<sup>2</sup> many linker modifications

have been antibody-specific, without regard for broad application to a variety of scFvs.

The abundance of proteins and peptides that undergo stimulus-responsive conformational transitions provides a rich resource from which elements can be selected that are responsive to a variety of molecular and environmental stimuli. These elements have the potential to serve as nanosensors and molecular transducers, changing conformation in an analyte- or environmentally dependent manner and propagating this change into a quantifiable signal by altering antibody binding.

Elastin-like polypeptides (ELPs) are a family of repetitive polypeptides derived from a portion of the primary sequence of mammalian elastin, VPGVG. To vary the physicochemical properties, the fourth residue can be substituted with any amino acid except proline. These polypeptides have the interesting property of undergoing a thermally induced phase transition, characterized by reversible intramolecular contraction and intermolecular coacervation, entropically driven by removal of ordered clathrates from the extended ELP.<sup>3</sup> ELPs and their derivatives have previously been used for a number of applications, including protein purification,<sup>4</sup> nanopatterning,<sup>5</sup> thermally

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**Figure 1.** Schematic depicting the principle behind using ELP linkers in scFvs. Left: The binding site is formed at the  $V_L$ - $V_H$  interface. Upon raising the temperature beyond the ELP transition temperature, the ELP linker undergoes a contraction (right) disrupting the interaction between the two variable domains and thus the binding site.

mediated targeting of solid tumors,<sup>6</sup> cellular adhesion,<sup>7</sup> and nucleic acid delivery.<sup>8</sup>

We asked whether the linker domain in an scFv could be modified to introduce additional functionality. As a starting point, we elected to evaluate the influence of replacing the standard (GGGGS)<sub>3</sub> linker in the anti-fluorescein scFv 4D5Flu with stimuli-responsive peptide elements derived from ELPs (Figure 1). For proof-of-concept studies, we chose an anti-fluorescein scFv for two reasons: (i) The fluorescence of the ligand, which is quenched upon binding by the scFv, is a convenient assay by which to measure ligand binding, and (ii) the easily quantifiable nature of the fluorescein–anti-fluorescein scFv system can serve as a general platform on which to develop a variety of nanosensors.

Here, we present data on a series of scFvs with five ELP-based linkers. Our objective was to evaluate the feasibility of producing scFvs where the affinity can be disproportionately modulated with temperature, via a conformational transition in the ELP linker. Our results demonstrate that anti-fluorescein scFvs with ELP linkers generally exhibit affinity for fluorescein similar to the “wild type” (GGGGS)<sub>3</sub> linker at room temperature, but release bound fluorescein faster at elevated temperatures, before scFv denaturation. This result is consistent with a mechanism in which ELP intramolecular contraction and possibly steric interference destabilize the scFv at higher temperatures, thereby facilitating ligand unbinding.

## Materials and Methods

**Materials.** The pIG6 vector encoding the 4D5Flu scFv and JM83 *E. coli* were generously provided by Dr. Andreas Plückthun.<sup>9</sup> Chemicals were purchased from Sigma-Aldrich and restriction enzymes and T4 DNA ligase from New England Biolabs. Novablue *E. coli* were purchased from Novagen. Bacterial medium (LB) was purchased from Qbiogene. Reagents for site-directed mutagenesis (Quikchange) were purchased from Stratagene. Oligonucleotides encoding ELP linkers were purchased from Integrated DNA Technologies. Plasmid miniprep and purification kits were purchased from Qiagen. Ni–NTA-agarose was purchased from Invitrogen and SP Sepharose from Amersham Biosciences.

**Construction of the Mutants.** Site-directed mutagenesis was used to introduce a BseRI site 58 nucleotides downstream from the AflII site in the pIG6 vector containing the 4D5Flu DNA sequence. This mutation enabled us to excise the complete (GGGGS)<sub>3</sub> linker sequence and replace it with linkers of our choice. Briefly, the pIG6 vector was double-digested with AflII and BseRI and purified by agarose gel electrophoresis. Annealed oligonucleotides, with ends complementary

to the cut pIG6 overhangs, were then ligated to the vector using T4 DNA ligase. For production of vectors with shorter (<15-residue) linkers, site-directed mutagenesis was used to delete three or nine residues, starting from the N-terminus of the linker. Plasmids were transformed into Novablue *E. coli*, purified, and sequenced.

**Protein Expression and Purification.** For expression of the scFv constructs, plasmids were purified and transformed into JM83 *E. coli*. A 50-mL starter culture was grown overnight at 37 °C and 250 rpm in LB-containing ampicillin. Bacteria were isolated by centrifugation, resuspended in LB, and used to inoculate 4 L of LB-containing carbenicillin. This culture was grown at 37 °C and 250 rpm until an OD<sub>600</sub> of 0.5 was reached. At this point, the culture was transferred to a room-temperature incubator for 20 min prior to induction with a final concentration of 1 mM IPTG. To prevent periplasmic leakage, the agitation of the culture was decreased to 200 rpm, and bacteria were incubated overnight at room temperature.

The following day, bacteria were collected by centrifugation and lysed in binding buffer (0.1 M Tris, pH 8.0, 1 M NaCl) by sonication on ice, at a moderate intensity. The lysate was centrifuged at 15 000 g for 30 min at 4 °C to pelletize insoluble debris, and the clarified lysate was gently mixed with Ni–NTA-agarose resin for 1 h at 4 °C. After loading, the resin was washed with 20 volumes of binding buffer, followed by 10 volumes of binding buffer containing 20 mM imidazole. The scFvs were eluted by applying binding buffer containing 100 mM imidazole. To obtain a highly pure product for affinity measurements and mitigate potential problems associated with protease activity, we subjected the eluent from the Ni–NTA column to a second round of ion-exchange purification using the cation-exchange resin SP–Sepharose. The theoretical pI's for the scFvs under study were estimated to be approximately 8.5.<sup>10–12</sup> We therefore dialyzed the eluent from the Ni–NTA column into 20 mM MES buffer, pH 6.0 prior to loading on the cation exchange column. The column was washed with MES buffer with 100 mM sodium chloride and eluted in MES buffer with 500 mM sodium chloride. A final buffer exchange, into phosphate buffered saline (PBS, pH 7.2, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 2.7 mM Na<sub>2</sub>HPO<sub>4</sub>, 155 mM NaCl) was then performed by dialysis. This procedure consistently yielded highly pure scFv where no contaminating proteins were visible on SDS–PAGE gels after staining with Coomassie Blue.

**Equilibrium Dissociation Constant ( $K_D$ ) Measurements.** Equilibrium dissociation constants were determined at room temperature (~25 °C) by titrating 500 nM fluorescein in PBS containing 100  $\mu$ g/mL bovine serum albumin (BSA), to block surface adsorption, with various concentrations of scFv and measuring emission intensity at 510 nm, with excitation at 483 nm, in a 96-well microplate (*fmax*, Molecular Devices). We verified that inclusion of 50–500  $\mu$ g/mL BSA did not destabilize scFv binding to fluorescein. The maximum quenching of fluorescein was found to be close to 100% with excess antibody. Consequently,  $K_D$  was determined by a two-parameter fit ( $p$ ,  $K_D$ ) to eq 1

$$r = \frac{pC_s}{K_D + C_s} \quad (1)$$

where  $r$  is specific binding,  $p$  is the number of binding sites per scFv,  $C_s$  is the concentration of free ligand (fluorescein), and  $K_D$  is the equilibrium dissociation constant.<sup>13</sup>

**On-Rate Measurements.** On-rates for scFvs were measured at room temperature by stopped-flow fluorimetry using a Cary Eclipse fluorimeter (Varian) with rapid mix accessory (Applied Photophysics) at an excitation wavelength of 483 nm and emission of 510 nm. Fluorescein and scFv were mixed in a volume ratio of 1:1 and final volume of 300  $\mu$ L for each measurement. For determination of  $k_{on}$ , four to five different molar excess (at least tenfold) scFv concentrations were rapidly mixed with fluorescein (50 nM), at least three times each. Apparent first-order association rate constants ( $k_{obs}$ ) were obtained by fitting the stopped flow data to the equation for single-

**Table 1.** Single-Chain Antibodies and Their Characteristics

construct	linker sequence	approximate linker length <sup>a</sup> (Å)	linker MW (Da)	$K_D^b$ (nM)	$p^c$	$k_{on}^d$ ( $10^6 M^{-1} s^{-1}$ )	$k_{off,calc.}^e$ ( $s^{-1}$ )
WT	(GGGGS) <sub>3</sub>		964	$43.8 \pm 14.4$	$1.2 \pm 0.12$	$1.15 \pm 0.05$	0.050
V1-15	GGGGS-VPGVG-GGGGS		1058	$49.2 \pm 7.6$	$1.4 \pm 0.07$	$0.86 \pm 0.14^f$	0.042
V3-15	(VPGVG) <sub>3</sub>	44	1246	$44.7 \pm 15.2$	$0.55 \pm 0.05^f$	$1.37 \pm 0.13^f$	0.061
H3-15	(VPGHG) <sub>3</sub>	44	1361	$24.8 \pm 4.9$	$0.81 \pm 0.11^f$	$3.22 \pm 0.31^f$	0.080
V2-12	VG-(VPGVG) <sub>2</sub>	35	993	$23.6 \pm 2.1$	$0.95 \pm 0.08^g$	$4.25 \pm 0.82^g$	0.100
V1-6	(G-VPGVG)	18	485	$40.1 \pm 7.8$	$1.3 \pm 0.02^g$	$1.98 \pm 0.53$	0.079

<sup>a</sup> Estimated from atomic force microscopy characterization in ref 17. <sup>b</sup> Equilibrium dissociation constant at room temperature. <sup>c</sup> Apparent number of binding sites per scFv. <sup>d</sup> On-rate for scFv binding to antigen at room temperature. <sup>e</sup> Calculated off-rate from mean  $K_D$  and  $k_{on}$ . <sup>f</sup> Different from WT. <sup>g</sup> Different from V3-15. All reported values are mean  $\pm$  standard deviation and were determined in triplicate or greater, with Student's *t*-test and  $\alpha = 0.05$  as the determinant of significance.

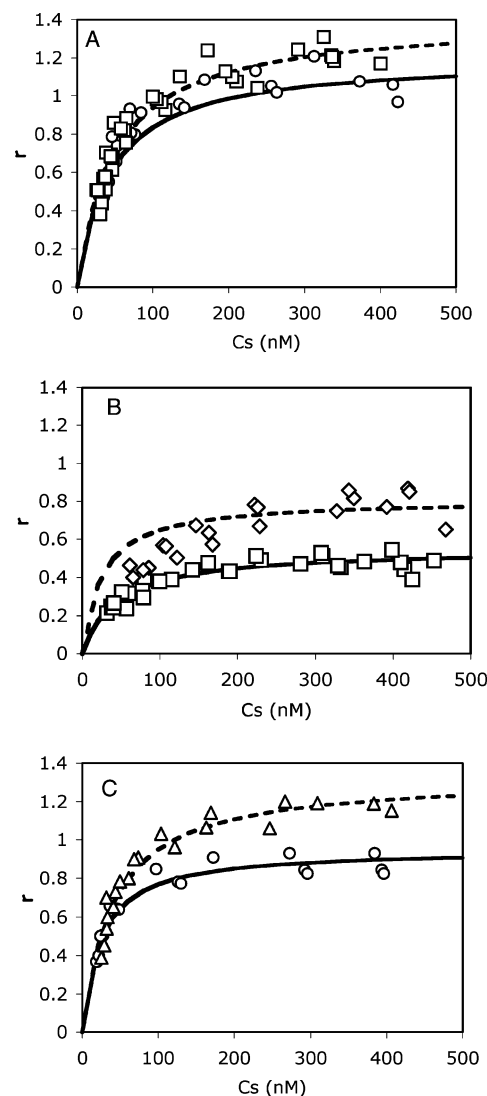
exponential decay. This parameter was then plotted as a function of antibody concentration, and  $k_{on}$  was estimated by linear regression of these plots.

**Thermally Induced Ligand Unbinding.** To evaluate bound ligand release as a function of temperature, 500 nM scFv was mixed with 100 nM fluorescein and allowed to equilibrate for 10 min at room temperature. This mixture resulted in a  $89.9 \pm 2.5$  nM ( $n = 18$ ) of bound fluorescein, as determined by fluorescence. Aliquots of the scFv-bound fluorescein mixture were then subjected to heating (25 to 55 °C, 0.5 °C/min) in an iCycler thermal cycler (Bio-Rad) with fluorescence measured on the green channel, and the release of fluorescein quantified by an increase in fluorescence with heating, corrected for temperature effects on fluorescence. The loss of scFv activity after heating to 55 °C was determined by cooling to 25 °C for 10 min, remeasuring fluorescence quenching, and comparing to the initial quenching at 25 °C.

**Statistical Analysis.** All statistical comparisons were performed using Student's *t*-test, with  $\alpha = 0.05$  as a cutoff for significance. All reported values were determined in triplicate or greater.

## Results

**Construction of Mutant scFvs.** To evaluate the effect of substituting the wild-type (GGGGS)<sub>3</sub> linker with ELP sequences with different properties, we used cassette mutagenesis to replace (GGGGS)<sub>3</sub> with the ELP sequence (VPGXG)<sub>3</sub>, where X is V or H (Table 1). On the basis of the inverse temperature transition ( $T_t$ ) characterization by Urry et al., we reasoned that changing the guest residue from valine ( $T_t = 24$  °C) to histidine ( $T_t = -10$  °C) may lower the range of temperature responsiveness.<sup>3</sup> Urry defined  $T_t$  as the onset temperature for the hydrophobic folding and assembly transition, as determined by light scattering.<sup>3</sup> These values were determined for high molecular weight ELPs, making it unlikely that they apply quantitatively in this case. We also asked whether decreasing the length of linkers entirely composed of ELP might enhance the effect of the ELP linker contraction on ligand unbinding. To evaluate this, we constructed two additional analogues of the V3-15 scFv, namely, V1-6 (G-VPGVG) and V2-12 (VG-VPGVG-VPGVG) (Table 1). We hypothesized that shortening the linker would lead to more steric stress being exerted on the regions surrounding the linker during ELP contraction, which would enable ligand release to occur at lower temperatures than those observed with the 15-residue linkers. Single-chain antibodies with shorter linkers may exist as inter- rather than intramolecular pairs, a characteristic that could also influence ligand binding and release.<sup>14</sup> However, SDS-PAGE analysis in the absence of disulfide reducing agents did not yield any bands that appeared to be composed of intermolecular aggregates, indicating that intermolecular disulfide bond formation does not occur with high frequency in these scFvs.



**Figure 2.** Specific binding ( $r$ ) versus concentration of free fluorescein ( $C_s$ ) for scFvs at room temperature. Antibodies are grouped by thermal response categorization, as described in the Discussion section of the paper. Lines indicate fits used to derive  $K_D$  and  $p$ . (A) V1-15 (□, dotted) and WT (○, solid). (B) H3-15 (◇, dotted) and V3-15 (□, solid). (C) V1-6 (△, dotted) and V2-12 (○, solid).

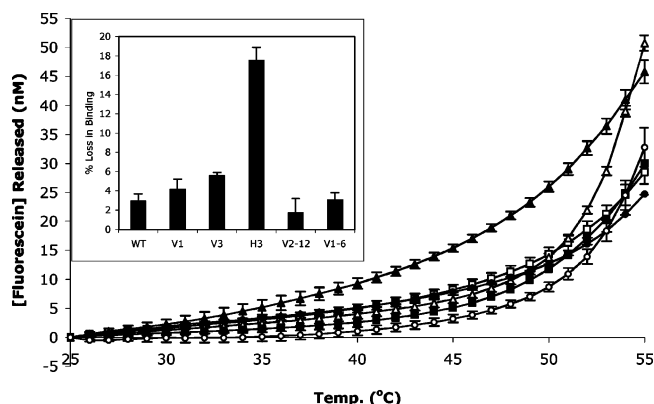
**Equilibrium Binding Properties.** The equilibrium dissociation constants ( $K_D$ ) of the antibodies were measured at room temperature and compared to WT (for 15-residue linkers V3-15 and H3-15) and to V3-15 (for shortened linkers V2-12, V1-6). The wild-type 4D5Flu construct had a  $K_D$  of 43.8 nM (Table 1, Figure 2A), in good agreement with previously published data (9.7–34 nM).<sup>9,15,16</sup> The V1-15

construct had a  $K_D$  similar to WT. Mutant antibodies with 15-residue linkers entirely composed of ELP had  $K_D$  values that were similar to wild-type and ranged from 24.8 nM (H3–15) to 44.7 nM (V3–15) (Table 1, Figure 2B). Shortening the linker to 12 residues (V2–12) slightly but insignificantly lowered  $K_D$  to 23.6 nM, while further decreasing the length of the linker to 6 residues (V1–6) also resulted in no significant change in  $K_D$  (40.1 nM) in comparison to WT (Table 1, Figure 2C).

The number of binding sites per scFv was estimated from the binding data (Figure 2A–C) and eq 1. If the scFv molecules fold as intended, each should have 1 binding site for fluorescein. However, the hydrophobic nature of the ELP linkers (which becomes more pronounced at  $T > T_t$ ) may lead to misfolding due to interactions with the heavy and/or light chains or interactions between mutant scFvs that do not readily occur with the wild-type linker. As expected, the WT scFv had approximately 1 binding site ( $1.2 \pm 0.12$ ) per scFv (Table 1). This was in contrast to the V3–15 mutant, which showed a significant ( $p < 0.001$ ) decrease in the number of binding sites ( $0.55 \pm 0.05$ ) that may result from interactions between scFv molecules that block binding sites and/or the presence of a fraction of inactive scFv in the preparation. The H3–15 ( $p < 0.001$ ) construct exhibited a small but significant decrease in the number of binding sites per scFv (Table 1). There was no significant difference between WT and the V1–15 mutant. Shortening the linkers from 15 to 12 or 6 residues (V2–12 and V1–6, respectively) restored the number of binding sites to approximately 1.

**Analysis of Kinetic Binding Parameters.** While the equilibrium dissociation constants remained similar to wild-type for most mutants, we sought to determine whether the kinetics of the interaction were also similar. Stopped flow fluorescence experiments indicated that the on-rates ( $k_{on}$ ) for all antibodies were within the range of  $10^6 \text{ M}^{-1} \text{ s}^{-1}$  (Table 1), in good agreement with previously published data, also obtained by stopped flow fluorimetry, on the WT 4D5Flu construct ( $k_{on} = 2.7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ,  $k_{off} = 0.062$ ).<sup>15</sup> The V1–15 construct exhibited an on-rate approximately 25% less than WT ( $p = 0.02$ ), indicating slower ligand binding. scFvs with mutant 15-residue linkers that were entirely composed of ELP (V3–15 and H3–15) exhibited a faster on-rate. For V3–15,  $k_{on}$  was 1.2 times that of WT ( $p = 0.042$ ), and for H3–15,  $k_{on}$  was 2.8 times that of WT ( $p < 0.001$ ). The 12-residue mutant (V2–12) exhibited a 3.1-fold increase ( $p = 0.002$ ) in the  $k_{on}$  versus V3–15, while  $k_{on}$  for the 6-residue mutant (V1–6) was approximately equivalent ( $p = 0.11$ ) to that for V3–15 (Table 1).

**Effect of Temperature on the Release of Bound Fluorescein.** Since ELPs are known to undergo thermally induced conformational changes, we evaluated the binding of scFv to fluorescein under conditions of increasing temperature (Figure 3). The data indicate a differential fluorescein release profile, in comparison to wild-type. In general, mutants exhibited an increase in fluorescein release rate that appeared to be triggered at approximately 50–52 °C, though the H3–15 mutant exhibited a greater rate of release at lower temperatures. The temperature for 50% release of the bound ligand was decreased by 0.5–4 °C in the mutant antibodies, with mutants releasing 15–105% more fluorescein than WT at 55 °C (Figure 3). Shortening the linker resulted in a dramatic sharpening of the release curve at approximately 49 °C (Figure 3), though this effect was more pronounced in the scFv with a 6-residue linker (V1–6). At 55 °C, the V1–6 scFv released approximately 105% more bound fluorescein than WT and 70% more than V3–15. The V2–12



**Figure 3.** Amount of bound fluorescein released from scFvs as a function of temperature (0.5 °C/min scanning rate). H3–15 ( $\blacktriangle$ ), V1–6 ( $\triangle$ ), V2–12 ( $\circ$ ), V3–15 ( $\blacksquare$ ), V1–15 ( $\square$ ), WT ( $\bullet$ ). Error bars are standard deviation ( $n = 3$ ). Inset: Percent loss in fluorescein binding after cooling at 25 °C for 10 min.

construct released 32% more bound fluorescein than WT and 10% more than V3–15. Interestingly, some scFvs (V3–15, V2–12, and V1–6) exhibited slightly enhanced binding at lower temperatures, in comparison to WT. With the exception of H3–15, all mutant scFvs exhibited a similar loss in binding activity after heating to 55 °C and cooling to 25 °C for 10 min (Figure 3, inset).

## Discussion

The ability to modulate the interaction of an antibody with its ligand via analyte concentration or environmental conditions has many potential applications, including sensing, delivery, and purification. As a first step toward truly “tunable” scFvs, we used an approach where the randomly structured  $(GGGG)_3$  linker was substituted with derivatives of ELPs. This approach was motivated by the fact that ELPs are thought to undergo a significant contraction when they assume  $\beta$ -spiral formation at  $T_t$ .<sup>3</sup> On the basis of X-ray crystal structure data, we estimated the linear distance between the linked ends of the  $V_H$  and  $V_L$  domains to be approximately 36 Å. Atomic force spectroscopy experiments conducted by Urry and colleagues enabled us to estimate the length of the  $(VPGVG)_3$  linker at approximately 44 Å.<sup>17</sup> With a large reduction in length at  $T_t$ , we hypothesized that the linker would become shorter than required to bridge the distance between the two variable domains, thereby creating tension that could facilitate unbinding at the  $V_L$ – $V_H$  interface and hence loss of the antigen binding pocket (Figure 1). To investigate the ability to tailor the temperature at which this unbinding occurs, we characterized ELP linkers with two different guest residues, valine and histidine, and three lengths, 15-, 12-, and 6-residue (Table 1).

All mutant scFvs had equilibrium dissociation constants equal to or lower than WT at room temperature, indicating that the flexible  $(GGGG)_3$  linker could be substituted with  $(VPGXG)_n$  derivatives without losing binding activity (Table 1, Figure 2). As might be expected from scFvs containing hydrophobic ELP linkers, some mutant scFvs with 15-residue linkers exhibited a reduction in the apparent number of binding sites, possibly due to aggregation. This reduction was apparently ameliorated by shortening the linker to 6 or 12 residues (Table 1). These data indicate that there is probably an optimum ELP linker length that preserves affinity and promotes monomolecular scFv assembly without exposing hydrophobic linker or framework residues in a manner that promotes aggregation. Interestingly, the H3–15 scFv showed less reduction in the apparent number

of binding sites. This may be due to partial ionization of histidine residues in the H3–15 linker in pH 7.2 phosphate buffer. Such ionization would be expected to decrease hydrophobic interaction of the linker with other hydrophobic moieties.

Although the equilibrium dissociation constants were similar, we noted differences in the binding kinetics of scFvs containing ELP linkers when compared to WT or V3–15 (Table 1). With the exception of V1–15, all scFvs with ELP linkers had faster on-rates, with the H3–15 construct being exceptionally faster with a 2.8-fold increase in on-rate. Shortening the V3–15-based linker to 12 or 6 residues increased  $k_{on}$  substantially in the former case without changing it significantly in the latter (Table 1). These results suggest a faster rate of ligand exchange for the mutant scFvs, where an increase in  $k_{on}$  is slightly attenuated by a lesser increase in  $k_{off}$ . The faster rate constants may result, in part, from the relative structure ( $\beta$ -turn-like) of ELP versus the random (GGGS)<sub>3</sub> linker, which may decrease the number of possible intermediate states that can form during binding and unbinding. Shortening the linker may further decrease the number of these intermediate states, though it is speculative to interpret the interplay between linker length and  $k_{on}$  at this time.

To evaluate the change in ligand binding with temperature, we quenched fluorescein with scFvs and subjected the complexes to a temperature ramp. Our results indicate that the all mutant scFvs release bound ligand at a rate that is significantly different from wild-type (Figure 3). The peak in this differential release was at approximately 55 °C, though H3–15 exhibited greater release throughout the temperature range. Interestingly, some mutants released less fluorescein than wild-type at lower temperatures. On the basis of the fact that these constructs exhibited faster calculated off-rates than wild-type, one may expect a different result. However, given the thermally responsive nature of the linker, it is possible that the kinetics of binding and unbinding may change substantially at elevated temperatures. With the exception of H3–15, which lost 17.6% of binding activity after exposure to 55 °C, all mutant scFvs lost less than 6% of binding activity (Figure 3, inset). This indicates that the differential rate of release is not simply due to denaturation and the antibodies are reusable after at least one temperature cycle.

In general, we believe that the mutant scFvs presented here can be broadly broken into 3 categories: First, V1–15, with 1 ELP pentamer flanked by a GGGS on either side, behaves most similarly to WT. This indicates that 1 ELP pentamer perturbs the 15-residue linker region less than 3 pentamers. The second category includes V3–15 and H3–15, where an increase in  $k_{on}$  is partially offset by an increase in  $k_{off}$ . These scFvs exhibited a faster rate of ligand release than WT, with more gradual slopes than the shortened linkers. The third category includes those scFvs with shortened linkers, V2–12 and V1–6. These scFvs had a higher affinity at lower temperatures and a sharper release transition than that observed for any of the scFvs containing 15-residue linkers (Figure 3). At this point, we also cannot exclude the possibility of noncovalent intermolecular association or steric effects, which may influence ligand binding and release, particularly with the V1–6 construct.

In selecting an scFv for our proof-of-concept studies, we sought a construct that would provide a simple binding assay and where we could use it as a platform for general biosensing capabilities. We also desired an scFv where the V<sub>L</sub> and V<sub>H</sub> domains were highly stable, thus allowing domain dissociation, and not unfolding, to be the primary factor driving ligand unbinding. On the basis of these requirements, we selected the 4D5Flu scFv, which binds fluorescein and remains folded up

to 6 M urea, thus being one of the most stable scFvs ever produced. In retrospect, we recognize that the efficacy of our approach may have been compromised by selecting such a stable scFv framework that binds to a hydrophobic antigen. The modulation of binding reported in this work may therefore be less than what could be expected using an scFv with more typical framework stability and/or which binds to a more hydrophilic antigen. The combination of temperature with other elution strategies (e.g., changing buffer conditions) may also yield antibodies with improved responsiveness.<sup>18</sup> As we have reported, the peak differences in ligand release between the wild-type 4D5Flu and mutants occurred at approximately 55 °C. To make this technology useful for most biomedical applications, it would be necessary to lower the temperature at which this peak occurs. This might be accomplished by site-directed mutagenesis of framework residues to destabilize the scFv. Another possibility is to utilize polypeptides that undergo larger conformational changes (e.g., bacterial periplasmic binding proteins) as linkers.

There are at least 2 groups that have published attempts to use ELPs as molecular switches in proteins. Reiersen and Rees have substituted a native type I  $\beta$ -turn in the IgG-binding minidomains derived from protein A with variants of the GVGVPVGV sequence, which also forms  $\beta$ -turns.<sup>19,20</sup> Their characterization revealed an improvement of affinity with increased temperature as the  $\beta$ -turn became more structured. Li et al. used computational techniques to evaluate the effect of substituting a native turn in chymotrypsin inhibitor 2 with VPGVG.<sup>21</sup> Their results indicated that the ELP-containing mutant was more stable at 40 °C than the wild-type. In contrast to both of these studies, here we have substituted a randomly structured peptide domain with ELP pentamers with the intention of disrupting, rather than stabilizing, the protein structure. It is feasible, however, that stabilization of binding may occur at lower temperatures, where the ELP linker may possess or gain structure without undergoing full contraction. According to the CD results of Reiersen, et al., short ELPs (8 residues) are less disordered at low temperatures than longer ELPs (18 residues), exhibiting mean residual ellipticity values within the range expected from an equimolar mixture of random coil and type II  $\beta$ -turns.<sup>22</sup>

## Conclusions

We have demonstrated a proof-of-concept of the incorporation of ELP switches into scFvs. Our results demonstrate that these linkers can modulate scFv affinity, though they are located relatively far from the binding pocket, consistent with an intramolecularly induced unbinding as the ELP linker undergoes an inverse temperature transition. Future work can focus on elucidating the role of the guest residue in this responsiveness and evaluating the secondary structure of the linker region as a function of temperature. We also believe that this concept provides a promising platform for the development of biosensors where the linker undergoes a conformational change in response to the concentration of an analyte or environmental conditions.

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