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Supplemental Information

Structure-Based Design of Supercharged,

Highly Thermoresistant Antibodies

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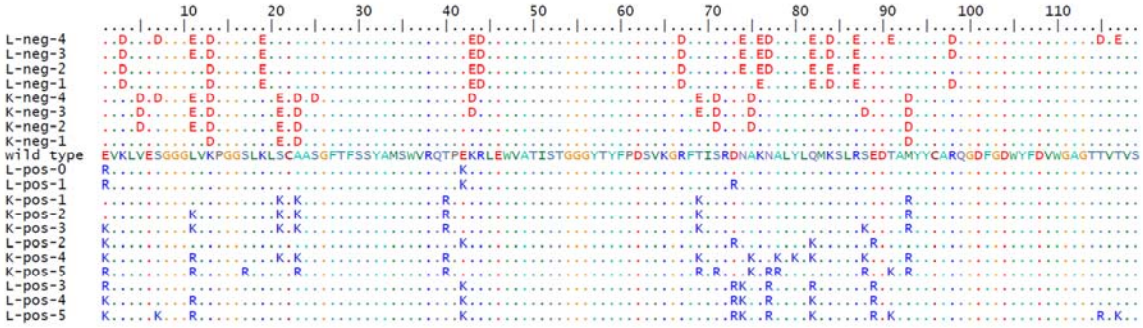
Inventory of Supplemental Information

- **Supplemental Figure 1, related to Table 1, is an alignment of all supercharged variants against wild-type.**
- **Supplemental Figure 2, related to Figure 3, shows SPR sensorgrams of K-pos-1 and wild-type binding characterization and function before and after incubation at 70°C for one hour.**
- **Supplemental Figure 3, related to Figure 3, shows characterization of K-pos-1 and wild-type by dynamic light scattering, size-exclusion chromatography, and differential scanning calorimetry.**
- **Supplemental Figure 4, related to Experimental Procedures, is the command line to run the Rosetta simulations we performed to obtain the designs in the manuscript.**
- **Supplemental Table 1, related to Figure 1, shows the results of additional 'supercharged' designs using AvNAPSA values and residue identities (as opposed to Rosetta).**
- **Supplemental Table 2, related to Figure 2, shows the reference energies used to bias the Rosetta potential function to include more charged residues.**
- **Supplemental File 1, (*"Supplementary File 1 - AvNAPSA values.xlsx")* is related to Table 1, and is an alignment of all designed sequences ranked by AvNAPSA value so that mutations may be compared based on solvent exposure.**
- **Supplemental File 2, (*"Supplementary File 2 – archive of supercharged scFv models.zip"*), related to Experimental Procedures, is a compressed archive containing PDB files of each model.**
- **Supplemental File 3, (*"Supplementary File 3 - resfile for positive supercharging.txt "*), related to Experimental Procedures, is the 'resfile' used to perform positive supercharging using Rosetta.**
- **Supplemental File 4, (*"Supplementary File 4 - resfile for negative supercharging.txt "*), related to Experimental Procedures, is the 'resfile' used to perform negative supercharging using Rosetta.**
- **Supplemental File 5. *"Supplementary File 5 – table of design energies.xlsx"*, related to Experimental Procedures, is a table containing a summary of the energy scores for each design to illustrate the scale of the changes effected by manipulating reference energies.**
- **The supplemental experimental procedures outline the process by which mutations were chosen to create the AvNAPSA-guided supercharged constructs and our protocol for harvesting scF_Vs from the periplasm using osmotic shock.**

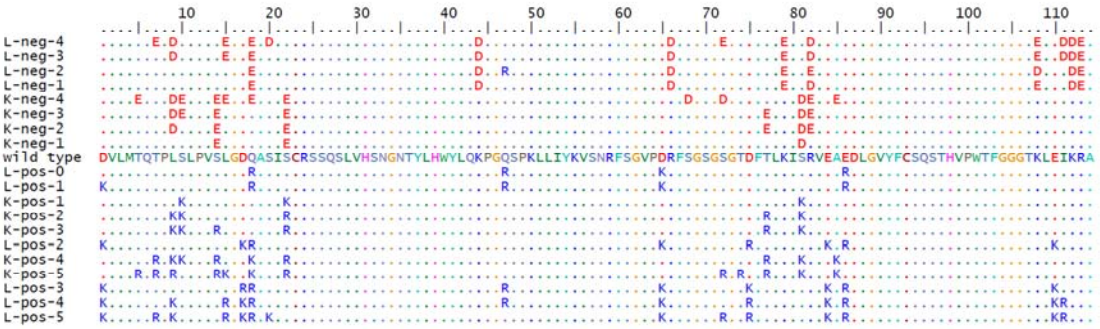
Supplemental Information

Supplemental Figure 1

V_H sequences

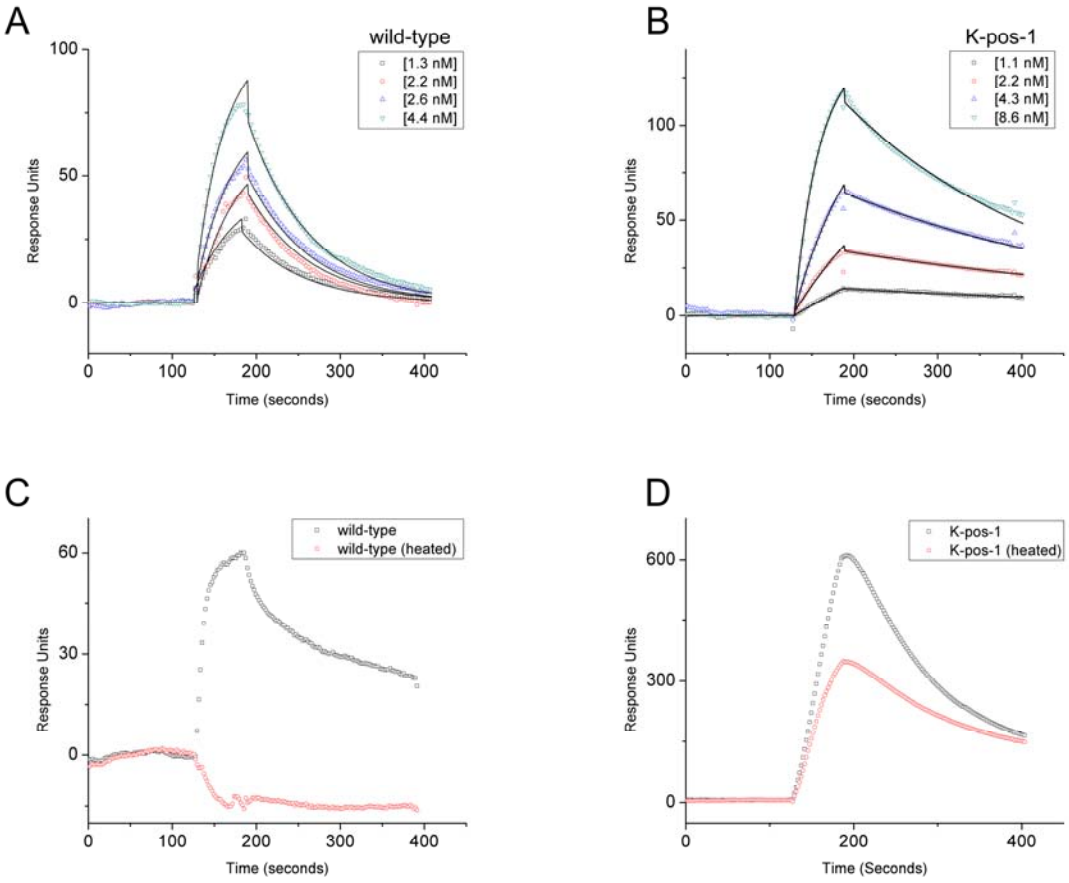


V_L sequences



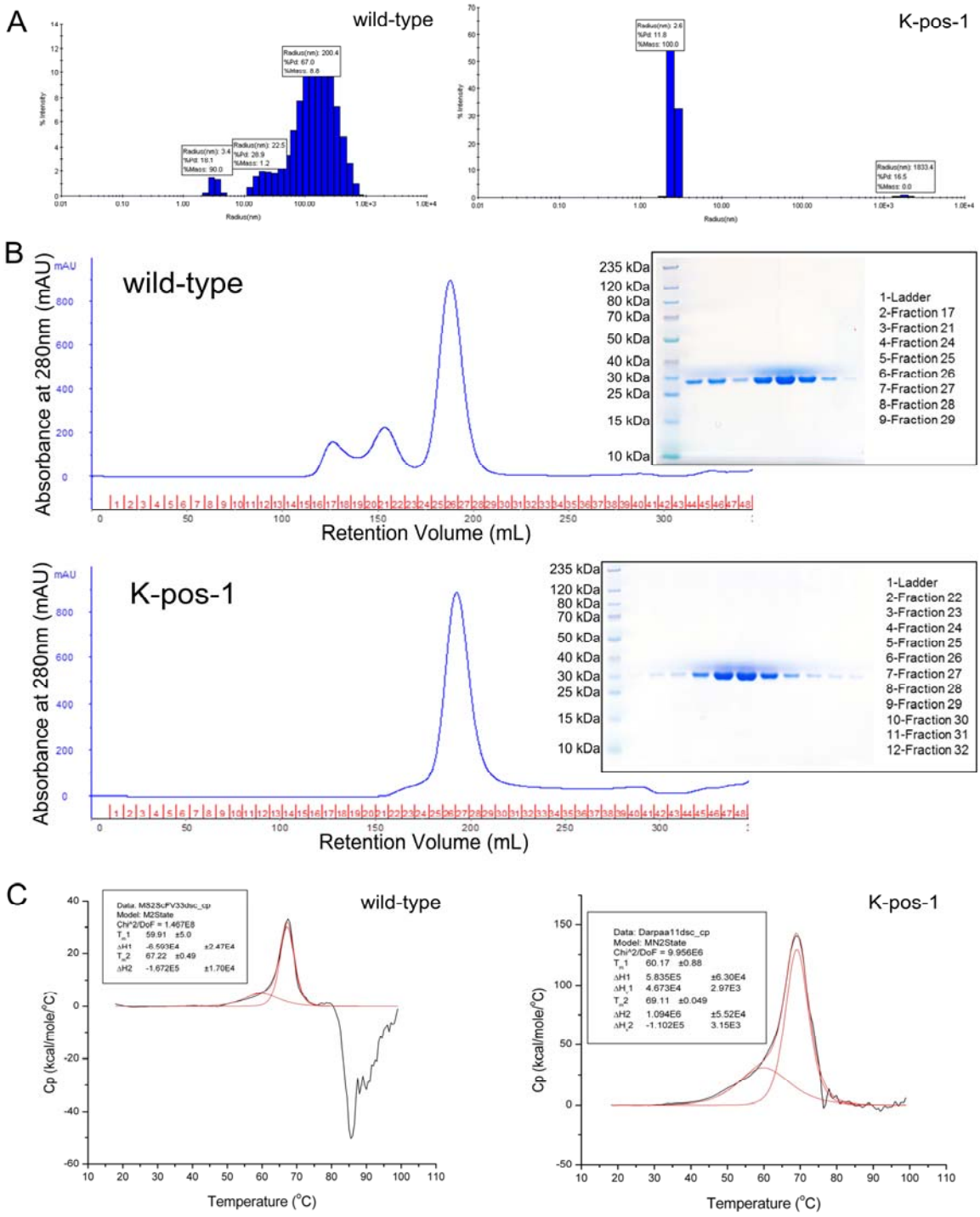
Supplemental Figure 1: Related to Table 1**.** Alignment of all supercharged variants against wild-type.

Supplemental Figure 2



Supplemental Figure 2. Related to Figure 3**.** Binding characterization of wild-type and K-pos-1 by SPR. **A,** variant K-pos-1 injected over immobilized MS2 coat protein dimer. Data shown as points, fits as lines, concentrations indicated in legend. **B**, wild-type injected over immobilized MS2 coat protein dimer. Data shown as points, fits as lines, concentrations indicated in legend. **C**, single injection of wild type over immobilized MS2 coat protein dimer before (black) and after (red) heating to 70°C for one hour. Sample was heated at 1 µM and diluted to 1 µg/mL before injection. **D**, single injection of K-pos-1 injected over immobilized MS2 coat protein dimer before (black) and after (red) heating to 70°C for one hour. Sample was heated at 1 µM and diluted to 1 µg/mL before injection.

Supplemental Figure 3



Supplemental Figure 3: Related to Figure 3. Characterization of wild-type and K-pos-1 by dynamic light scattering, size-exclusion chromatography, and differential scanning calorimetry. **A**, particle sizes and polydispersities of solutions of wild-type (left) and K-

pos-1 (right) determined by dynamic light scattering. K-pos-1 is appreciably more monodisperse and lacks the higher-molecular weight components found in solutions of the wild-type scF_V. **B**, chromatograms from preparative size-exclusion chromatography (S75 column) performed on wild-type (top) and K-pos-1 (bottom) with inset Coomassie-stained polyacrylamide gels. The samples injected onto the size-exclusion column were elutions from IMAC purifications. Again, larger multimers seen with the wild-type were not present in K-pos-1. **C**, melting temperatures of wild-type (left) and K-pos-1 (right) determined by differential scanning calorimetry. The mutations predicted by Rosetta in K-pos-1 yielded a mild increase in melting temperature.

Supplemental Figure 4. Command line for Rosetta simulations (Rosetta svn revision 36730).

```
./fixbb.linuxgccrelease –database
/home/minirosetta_database -ex1 -ex2 -use_input_sc -
minimize_sidechains -resfile input_resfile.txt -l
ten_homology_models.txt -nstruct 15
```

Supplemental Table 1

Name	Charge	Rosetta Score (Δ wild-type)	Mutation Count	Expression	Binding	Thermal Resistance
L-neg-4	-41.5	16.7	33	+++	*No*	
L-neg-3	-34.5	11.6	24	++	*No*	
L-neg-2	-28.5	10.8	19	++++	*No*	
L-neg-1	-27.5	12.3	20	+	**Yes (weak)**	*No*
wt	7.5	0	0	+++	**Yes (strong)**	*Yes (trace)*
L-pos-0	15.5	3.3	6	++ (cytosol)	**Yes (strong)**	*Yes (trace)*
L-pos-1	21.5	4.8	8	*+ (cytosol)*	**Yes (weak)**	*No*
L-pos-2	31.5	7.6	14	+	*No*	
L-pos-3	34.5	8.9	16	(cytosol)	**Yes (medium)**	No
L-pos-4	38.5	7.8	20	*No Expression*		
L-pos-5	44.5	9.5	27	*No Expression*		

Supplemental Table 1: Related to Figure 1. Screening of supercharged single-chain F_V variants designed semi-rationally using AvNAPSA values and residue identities.

Supplemental Table 2.

	Arg	Lys		Glu	Asp
	-0.98	**-0.65**		**-0.81**	**-0.67**
K-pos-1	-0.40	-0.27	K-neg-1	-0.40	-0.33
	-0.50	-0.33	K-neg-2	-0.50	-0.41
	-0.60	-0.40		-0.60	-0.50
K-pos-2	-0.70	-0.46	K-neg-3	-0.70	-0.58
K-pos-3	-0.80	-0.53		-0.80	-0.66
	-0.90	-0.60		-0.90	-0.74
	-1.00	-0.66		-1.00	-0.83
K-pos-4	-1.10	-0.73	K-neg-4	-1.10	-0.91
	-1.20	-0.80		-1.20	-0.99
	-1.30	-0.86		-1.30	-1.08
K-pos-5	-1.40	-0.93		-1.40	-1.16
	-1.50	-0.99		-1.50	-1.24
	-1.60	-1.06		-1.60	-1.32
	-1.70	-1.13		-1.70	-1.41
	-1.80	-1.19		-1.80	-1.49
	-1.90	-1.26		-1.90	-1.57
	-2.00	-1.33		-2.00	-1.65

Supplemental Table 2: Related to Figure 2. Reference energy scanning to vary the extent of supercharging.

Supplementary File 1. *"Supplementary File 1 - AvNAPSA values.xlsx"* Related to Table 1. Alignment of all supercharged variants ordered by AvNAPSA values. CDR regions (forbidden from mutating) are highlighted in grey. K-series designs are included on this chart to indicate the AvNAPSA values of the residues mutated by Rosetta. AvNAPSA values were used to design the L-series constructs as described in the supplemental methods.

Supplemental File 2. *"Supplementary File 2 – archive of supercharged scFv models.zip" Compressed archive containing PDB files of each model.*

Supplemental File 3. *"Supplementary File 3 - resfile for positive supercharging.txt "* 'resfile' for positive supercharging.

Supplemental File 4. *"Supplementary File 4 - resfile for negative supercharging.txt "* **'**resfile' for negative supercharging.

Supplemental File 5. *"Supplementary File 5 – table of design energies.xlsx "* Table containing a summary of the energy scores for each design to illustrate the scale of the changes effected by manipulating reference energies.

Supplemental Experimental Procedures

Designing supercharged antibodies: Supercharging by identity and exposure

The AvNAPSA algorithm was used to calculate the average number of neighboring atoms per sidechain atom for each residue of the lowest-energy Rosetta Antibody model of the anti-MS2 scF_V. Residues were ranked by AvNAPSA value and mutations were selected by inspection based on wild-type residue identity and AvNAPSA value. Preference was given to "flipping" the charge of exposed residues, for example, in positive supercharging, exposed aspartates or glutamates would be changed to lysines or arginines. Upward limits for the AvNAPSA values were guided by the values from the GFP supercharging experiments performed by the Liu group. No mutations were made to the CDR loops, and generally no residues were considered that had AvNAPSA values >144 (with the exception of two constructs in which values of up to 160 were considered to create variants with higher charges). To produce negative and positive series of increasing charge magnitude, the AvNAPSA cutoff was moved (thus permitting mutations to less-buried residues) and the pool of residue identities to which mutations were made was expanded (thus permitting mutations to highly-exposed residues that were not polar or charged).

Based on these considerations, six positively charged designs with net charges varying from +15.5 to +44.5 (named L-pos-0 – L-pos-5) and four negatively charged designs with net charges varying from -27.5 to -41.5 (named L-neg-1 – L-neg-4) were designed from the wild-type scF_V (with a charge of +7.5) (**Supplemental Table 2**). These variants contained from 6 to 33 substitutions, closely following previously successful efforts to supercharge GFP. However, L-pos-0 and L-pos-1 were explicitly designed to contain a smaller number of substitutions (6 and 8, respectively) so as to provide a direct comparison between AvNAPSA and Rosetta approaches. These designs were expressed in the cytosol (L-pos-1 did not express via periplasmic export), but neither performed as well as K-pos-1, 2, or 3, despite having comparable charges and mutational loads. This reinforces our hypothesis that an energetics-based approach to selecting mutations is more effective in increasing protein charge to achieve thermal resistance. The sequences designed are aligned against wild-type in **Supplemental Figure 1** and ordered by AvNAPSA values in **Supplemental File 1**.

Purification of scF_V constructs from the periplasm (osmotic shock)

Anti-MS2 scF_V constructs in the pAK400 vector were transformed into electrocompetent Jude-1 cells and plated on LB agar plates. All culture steps were performed in the presence of 34 μg/mL chloramphenicol to maintain the pAK400 plasmid. Individual colonies were picked, inoculated into 5 mL LB, and grown overnight at 37°C. Overnight cultures were diluted 1:1000 into 1 L LB media and grown for 18 hours at 30°C in a shaking incubator. Cells were isolated by centrifugation at 4000xg for 20 min. at 4°C, and resuspended in 1 L Superior Broth (Athena ES). These cultures were placed in a shaking incubator at 18°C for 1 hour prior to induction by the addition of IPTG (Cellgro) to a final concentration of 1 mM. Induction was allowed to proceed for 18 hours at 18°C, after which cells were harvested by centrifugation at 4000xg for 20 min. at 4°C.

Harvested cells were gently resuspended in 25 mL ($1/40^{th}$ culture volume) of ice-cold Spheroplasting Buffer (0.75 M sucrose, 100 mM TRIS pH 7.5) and transferred to an ice bath on a rotary shaking table for gentle agitation, where all subsequent steps were performed up to the separation of the spheroplasts and the shock fluid. 2.5 mL ($1/10^{th}$ the resuspension volume) of 10 mg/mL lysozyme (Sigma) in Spheroplasting Buffer was added. After 10 min., 50 mL (2 resuspension volumes) of 1 mM ice-cold EDTA was added dropwise. After an additional 15 min., 3.5 mL (0.14 resuspension volume) of 500 mM $MgCl_2$ was added and incubated for an additional 15 min. Spheroplasts were then separated from the shock fluid by centrifugation at 15,000xg for 20 min. at 4°C. 1000 units of benzonase (Novagen) were added to the shock fluid before dialysis against 4°C IMAC buffer (20 mM MOPS, 500 mM NaCl, 20 mM imidazole, pH 7.4) for 1 hour using a 10 kD cutoff dialysis membrane (Snakeskin tubing, Pierce).

Following dialysis the shock fluid was filtered through a 0.45 micron Supor filter (Pall) and applied to a 1 mL column of nickel-charged chelating sepharose resin (GE) which had been equilibrated with IMAC buffer. The column was washed with 10 mL of IMAC buffer and 5 mL of IMAC buffer supplemented to 100 mM imidazole. Bound scF_Vs were then eluted with 5 mL of IMAC buffer supplemented to 400 mM imidazole. Expression and subsequent purification were verified by SDS-PAGE (Novex NuPAGE 4-12% Bis-Tris gels, Invitrogen). The eluates were further purified by size exclusion chromatography on an S75 HiLoad 26/60 column (GE) in 10 mM sodium phosphate, 150 mM NaCl, pH 7.4. Fractions corresponding the monomer scF_V were pooled and concentrated using Amicon Ultra-15 centrifugal spin columns with a 10 kD cutoff (Millipore).