# Supporting information

# Generation of multivalent nanobody-based proteins with improved neutralization of long α-neurotoxins from elapid snakes

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**Table S1: Molecular weight of nanobody and Quad proteins.** Molecular weights were calculated using the ProtParam tool from Expasy (https://web.expasy.org/protparam/).

| Molecule | Valence | MW monomer (kDa) | MW tetramer (kDa) |
|---|---|---|---|
| $V_HH$ | 1 | 15 | |
| Q187 | 4 | 19.6 | 78.3 |
| Q188 | 8 | 33.2 | 132.7 |
| Q189 | 8 | 33.4 | 133.6 |
| Q190 | 4 | 46.7 | 186.8 |
| Q191 | 8 | 60.3 | 241.2 |
| Q193 | 4 | 22.8 | 91.2 |
| Q194 | 8 | 36.4 | 145.6 |
| Q207/208 | 16 | 44.3 + 39.3 (LC) | 334.4 |

**Table S2: FIDA results for nanobody and Quad proteins binding to labeled long α-neurotoxins.** The excess indicator model from the FIDA One software was used to fit the measurements from a single experiment performed with technical duplicates.

| Molecule | Indicator | Indicator Rh (nm) | Complex Rh (nm) | $K_D$ (nM) | $R^2$ |
|---|---|---|---|---|---|
| $V_HH$ | α-cbtx | 1.7 | 2.7 | 25.2 | 0.99 |
| Q187 | α-cbtx | 1.4 | 4.8 | 2.5 | 0.98 |
| Q188 | α-cbtx | 1.5 | 5.4 | 1.9 | 0.99 |
| Q189 | α-cbtx | 1.4 | 5.1 | 1.6 | 0.98 |
| Q190 | α-cbtx | 1.4 | 7.8 | 1.3 | 0.98 |
| Q191 | α-cbtx | 1.3 | 7.4 | 0.8 | 0.98 |
| Q193 | α-cbtx | 1.7 | 5.1 | 3.3 | 0.99 |
| Q194 | α-cbtx | 1.8 | 6.8 | 2.2 | 0.99 |
| Q207/208 | α-cbtx | 1.6 | 9.1 | 0.7 | 0.99 |
| Q187 | Nm8 | 1.3 | 4.6 | 14.4 | 0.98 |

**Table S3: DLS measurements of select Quad molecules.** Measurements of the monodispersity were performed at a concentration of 1 mg/mL in triplicate using a Zetasizer Nano instrument.

| Molecule | Polydispersity (%) | Estimated Dh (nm) |
|---|---|---|
| Q189 | 3.6 | 13.00 ± 3.87 |
| Q193 | 2.1 | 13.50 ± 4.58 |
| Q194 | 9.5 | 17.12 ± 6.25 |

**Table S4: Indirect ELISA results of nanobody and Quad proteins binding to immobilized α-cobratoxin.** The titration-ELISA data are the results of two independent experiments and the equation described in Li et al. 2011 has been fitted to those data using GraphPad Prism.

| Molecule | $OD_{min}$ (405 nm) | $OD_{max}$ (405 nm) | $K_D$ (nM) | $R^2$ |
|---|---|---|---|---|
| $V_HH$ | 0.1134 | 2.648 | 0.03898 | 0.99 |
| Q187 | 0.2286 | 2.647 | 0.006322 | 0.99 |
| Q188 | 0.1345 | 2.654 | 0.008281 | 0.99 |
| Q189 | 0.2022 | 2.637 | 0.005865 | 0.99 |
| Q190 | 0.1511 | 2.657 | 0.00552 | 0.99 |
| Q191 | 0.1139 | 2.665 | 0.005007 | 0.99 |
| Q193 | 0.2988 | 2.647 | 0.005462 | 0.99 |
| Q194 | 0.2103 | 2.652 | 0.004387 | 0.99 |
| Q207/208 | 0.09299 | 2.67 | 0.007629 | 0.99 |

**Table S5: ITC binding parameters of nanobody and select Quad proteins.** Values of association constants ($K_A$), binding enthalpy ($\Delta H$), and binding stoichiometry (N) obtained in ITC analysis are expressed as means ± SD of duplicate experiments. Dissociation constants ($K_D$) were calculated from the reciprocal of $K_A$. The Gibbs free energy change ($\Delta G^0$) and the entropy change ($\Delta S^0$) were calculated from the equations $\Delta G^0 = -R \cdot T \cdot \ln(K_A)$ and $T \cdot \Delta S^0 = \Delta H - \Delta G^0$, respectively (R, gas constant; T, absolute temperature).

| Molecule | $K_A$ ($\times 10^7$ $M^{-1}$) | $K_D$ ($\times 10^{-9}$ M) | $\Delta G^0$ (kcal $mol^{-1}$) | $\Delta H$ (kcal $mol^{-1}$) | $\Delta S^0$ (cal $K^{-1}$ $mol^{-1}$) | N (sites) |
|---|---|---|---|---|---|---|
| $V_HH$ | 19.5 ± 4.6 | 5.1 | -11.3 | -18.83 ± 0.24 | -25.2 | 0.92 ± 0.22 |
| Q187 | 13.26 ± 9.7 | 7.5 | -11.1 | -18.08 ± 0.25 | -23.4 | 3.84 ± 0.07 |
| Q189 | 11.52 ± 4.9 | 8.68 | -11 | -16.65 ± 0.07 | -18.9 | 8.03 ± 1.05 |
| Q207/208 | 10.7 ± 0.7 | 9.35 | -10.9 | -17.65 ± 0.21 | -22.6 | 13.35 ± 1.20 |

**Table S6: SEC analysis of Quad molecules.** Quads were loaded on a HiLoad Superdex200 Increase 10/300 GL column with PBS as an eluent. Characteristics of the main elution peaks are in bold. The Molecular Mass (kDa) is the molecular weight determined for the observed elution volumes after calibration of the column with the Protein Standard mix 15-600 kDa from Sigma-Aldrich.

| Molecule | Peak number | Retention volume (mL) | Relative area (%) | Molecular Mass (kDa) |
|---|---|---|---|---|
| $V_HH$ | 1 | 17.56 | 100 | 15.2 |
| Q187 | **1** | **13.15** | **96.87** | **90.4** |
| | 2 | 17.31 | 3.01 | 15.3 |
| Q188 | **1** | **11.48** | **96.94** | **182** |
| | 2 | 16.30 | 2.39 | 25.4 |
| Q189 | **1** | **11.90** | **94.91** | **153.7** |
| | 2 | 18.18 | 4.34 | 11.8 |
| Q190 | 1 | 9.06 | 11.25 | 490.1 |
| | **2** | **10.09** | **88.51** | **321.1** |
| Q191 | 1 | 9.56 | 97.97 | 418.8 |
| | 2 | 16.29 | 1.43 | 23.7 |
| Q193 | 1 | 9.42 | 4.90 | 444.8 |
| | 2 | 10.16 | 11.54 | 324.4 |
| | **3** | **11.75** | **81.30** | **164.6** |
| | 4 | 17.57 | 1.22 | 13.7 |
| Q194 | 1 | 9.39 | 11.3 | 428.4 |
| | **2** | **10.65** | **86.26** | **255.6** |
| | 3 | 16.26 | 2.22 | 25.9 |
| Q207/208 | **1** | **9.17** | **100** | **496** |