

Title: Synthetic nanobody–SARS-CoV-2 receptor-binding domain structures identify distinct epitopes

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10 **Abstract:** The worldwide spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) demands unprecedented attention. We report four X-ray crystal structures of three synthetic nanobodies (sybodies) (Sb16, Sb45 and Sb68) bind to the receptor-binding domain (RBD) of SARS-CoV-2: binary complexes of Sb16–RBD and Sb45–RBD; a ternary complex of Sb45–RBD–Sb68; and Sb16 unliganded. Sb16 and Sb45 bind the RBD at the ACE2 interface, positioning their CDR2 and CDR3 loops diametrically. Sb16 reveals a large CDR2 shift when
15 binding the RBD. Sb68 interacts peripherally at the ACE2 interface; steric clashes with glycans explain its mechanism of viral neutralization. Superposing these structures onto trimeric spike (S) protein models indicates these sybodies bind conformations of the mature S protein differently, which may aid therapeutic design.

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One Sentence Summary: X-ray structures of synthetic nanobodies complexed with the receptor-binding domain of the spike protein of SARS-CoV-2 reveal details of CDR loop interactions in recognition of distinct epitopic sites.

25 **Main Text:** SARS-CoV-2, a β -coronavirus, is remarkable for its high infectivity, rapid world-wide dissemination, and evolution of highly infectious new variants (1-4). The virus exploits its trimeric S glycoprotein to adsorb to the host cell-surface receptor, angiotensin converting enzyme (ACE) ACE2 (5) resulting in proteolytic processing and conformational changes required for membrane fusion and cell entry (6). Understanding the fundamental molecular and
30 cell biology and chemistry of the viral life cycle and the nature of the host immune response, offers rational avenues for developing diagnostics, therapeutics, and vaccines (7, 8). Exploring the detailed structures of anti-viral antibodies can provide critical understanding of the means to attenuate viral adsorption and entry, preventing or retarding ongoing infection and communal spread. An evolving database of X-ray and cryo-EM structures of the SARS-CoV-2 S and its
35 interactions with ACE2 or various antibodies contributes to the design of effective antibodies or immunogens (9). Recent studies indicate the value of single domain antibodies derived from camelids (nanobodies) (10) or camelid-inspired synthetic libraries (sybodies) (11), and the value of generating multivalent constructs (12) for effective treatment (11). Many properties of nanobodies make them well suited for structural studies and drug development (13).

40 Here, we take advantage of available sequences of three SARS-CoV-2 RBD-directed sybodies – Sb16, Sb45, and Sb68 (previously designated Sb#16, Sb#45, and Sb#68 (14)). We describe binding studies and X-ray structures of complexes of these with the RBD, and also the structure of Sb16 unliganded. The sybodies had been shown to be effective inhibitors of the

ACE2–RBD interaction (14), and neutralizers of viral infectivity (14). These sybodies (see
45 Supplementary Materials and Methods) behaved as monomers by size exclusion chromatography
(SEC) (15) (Figure S1), and we confirmed their activity in binding to the re-engineered RBD and
S using surface plasmon resonance (SPR) (Figure S2). All three sybodies bind to surface
immobilized RBD with K_D values of 0.038 to 0.77 μM (Figure S2A to S2D) – measurements that
are similar to those determined using RBD-YFP or RBD-Fc molecules by related techniques
50 (14). Binding of Sb16, Sb45, and Sb68 to S consistently revealed lower affinities, in the range of
0.07 to 2.6 μM (Figure S2E to S2H). Experiments using SEC of premixed solutions of sybodies
and RBD confirmed that all three sybodies bound the RBD (Figure S3). RBD consistently eluted
at 13 min. Mixtures of RBD with Sb16 or Sb45 eluted at ~ 11.3 min and with Sb68 at ~ 11.7 min
consistent with complex formation. For unliganded Sb16, the large change in elution time
55 suggests that its RBD binding site is that which interacts with the chromatographic column
matrix (Figure S3).

To gain insight into the precise topology of the interaction of each of the three sybodies
with the RBD, we determined crystal structures of these complexes. We obtained crystals of
several complexes: Sb16–RBD, Sb45–RBD, and the ternary Sb45–RBD–Sb68; and of Sb16
60 alone. These crystals diffracted X-rays to resolutions from 2.1 to 2.6 \AA (Table S1). After
molecular replacement, model building, and crystallographic refinement (see Materials and
Methods), we obtained structural models with $R_{\text{work}}/R_{\text{free}}$ (%) of 25.4/28.4, 18.6/21.6, 20.6/25.5
and 22.5/25.6, respectively, that satisfied standard criteria for fitting and geometry (Table S1).
Illustrations of the quality of the final models as compared with the electron density maps are
65 shown in Figure S4.

The structure of the RBD domain of these complexes (Figure 1A and 1B) revealed little difference between insect-expressed (16) and bacteria-expressed and refolded RBD. Each of the sybodies has a barrel of two β -sheets stabilized by a single disulfide-linked loop of 75 or 76 amino acids characteristic of an IgV fold (17, 18). The Sb16–RBD complex (Figure 1A and 2A) illustrates that CDR2 (residues 50-60) and CDR3 (residues 98-106) bestride the saddle-like region of the ACE2-binding surface of the RBD (see sequence alignment in Figure 1E). Sb16 angulates over the RBD by 83°. However, Sb45 (Figure 1B and 2B) straddles the RBD saddle in the opposite orientation, at an angle of -36°, and frames the interface with CDR2 (residues 50-59) and CDR3 (residues 97-111). CDR1s of both sybodies (residues 27-35) lie between the CDR2 and CDR3 loops. Superposition of the two structures, based on the RBD, emphasizes the diametrically opposite orientation of the two (Figure 1C), revealing that the CDR2 of Sb16 and CDR3 of Sb45 recognize the same epitopic regions.

Exploring conditions using mixtures of two or three sybodies and the RBD, we obtained crystals and solved the structure of a ternary complex consisting of Sb45–RBD–Sb68 at 2.6 Å (Table S1 and Figure 1D). The refined model revealed Sb45 and Sb68 interacting at two different faces of the RBD (Figure 1D and 2B). Here, Sb45 binds in an identical orientation to that observed in the binary Sb45–RBD structure (RMSD of superposition, 0.491 Å for 1981 atoms), but Sb68 addresses a completely different face of the RBD – similar to that bound by Fab of CR3022 on RBD of SARS-CoV-2 (19) and by V_{HH72} on RBD of SARS-CoV-1 (20). Of particular interest, whereas Sb45 CDR2 and CDR3 span the RBD saddle as noted above, the distinct contacts of Sb68 to the RBD are through the longer CDR3, with only minor contributions from CDR1 and CDR2. Walter et al visualized similar distinct interactions in cryo-

EM maps of two sybodies (Sb15 and Sb68) bound to S protein with local resolution of 6-7 Å (14).

90 Scrutiny of the different interfaces provides insights into the distinct ways each sybody exploits its unique CDR residues for interaction with epitopic residues of the RBD (Figure 2). Both Sb16 and Sb45 use longer CDR2 and CDR3 to straddle the RBD, positioning CDR1 residues over the central crest of the saddle (Figure 2A and 2B). Also, several non-CDR residues (Y37, E44, R45, E46, and W47 for Sb16; and W47 for Sb45), derived from framework 2 (21), 95 provide additional contacts to the RBD. The interface of Sb68 with RBD (Figure 2C) is quite different, predominantly exploiting eight CDR3, six CDR2, and four CDR1 residues, along with non-CDR residues at the interface. (Table S2 lists all individual contacts between each sybody and the RBD).

To evaluate the structural basis for the ability of these three sybodies to block the 100 interaction of RBD with ACE2, we superposed each of the three sybody–RBD structures onto the ACE2–RBD structure and examined the steric clashes (Figure 3A). Sb16 and Sb45 directly impinge on the ACE2 binding site, offering a structural rationale for their viral neutralization capacity (14). Sb68, which also blocks viral infectivity, binds to RBD at a site which appears to be noncompetitive for ACE2 binding. The carbohydrate at ACE2 residues N322 and N546 105 provides an explanation (Figure 3A, and below).

To compare the epitopic areas captured by these sybodies, we evaluated the buried surface area (BSA) interfaces between RBD and ACE2 or the sybodies. The BSA at the ACE2–RBD, Sb16–RBD, Sb45–RBD, and Sb68–RBD interfaces are 844 Å², 1,003 Å², 976 Å², and 640 Å², respectively (Figure 3B to 3E). Sb16 and Sb45 capture more surface area than ACE2 or other 110 published nanobody or sybody–RBD complexes (see Table S3). The interface with Sb68 is the

smallest (640 Å²) (Figure 3E). The total BSA captured by Sb45 and Sb68 in the ternary complex is 1,650 (1,010 plus 640) Å² (Table S4) and is consistent with the view that a linked bispecific antibody, as described by Walter et al (14), would exert strong avidity effects. Table S3 summarizes these BSA values and those of other nanobody–RBD interactions.

115 A reasonable explanation for the ability of Sb68 to block the ACE2–RBD interaction arises on inspection of the sites where Sb68, bound to the RBD, might clash with ACE2. Scrutiny of a superposition of Sb68–RBD with ACE2–RBD reveals several areas of steric interference. Sb68 loop 40-44 clashes with amino acid side chains of ACE2 (residues 318-320 and 548-552), loop 61-64 with ACE2 N322 carbohydrate, and loop 87-89 (a 3₁₀ helix) with
120 ACE2 N546 carbohydrate as well as residues 313 and 316-218 (Figure 3A). The ACE2 used in the crystallographic visualization of ACE2–RBD (22) was expressed in *Trichoplusia ni* insect cells, which produce biantennary N-glycans terminating with N-acetylglucosamine residues (23, 24). Electron density was observed only for the proximal N-glycans at residues N322 and N546, but larger, complex, non-sialylated, biantennary carbohydrates have been detected in
125 glycoproteomic analysis of ACE2 in mammalian cells (25). These are highly flexible carbohydrates adding greater than 1500 Da at each position, so are larger than the single carbohydrate residues visualized in the crystal structure. Additionally, molecular dynamics simulations of RBD–ACE2 implicated the direct interaction of carbohydrate with the RBD (26). Thus, the ability of Sb68 to impinge on ACE2 interaction with RBD likely involves the steric
130 clash of the N322- and N546-linked glycans.

We also obtained a 2.1 Å structure of free Sb16 (Figure S5). Remarkably, the CDR2 of Sb16 shows Y54 in starkly different positions in the unliganded structure as compared to the

complex: the C α carbon is displaced by 6.0 Å, while the O η oxygen of Y54 is 15.2 Å distant, indicative of dynamic flexibility.

135 To gain additional insight into the structural consequences of the interactions of each of these sybodies with a trimeric S protein, we superposed each of the individual sybody–RBD complexes on each of several cryo-EM-determined models of S, including examples of different combinations of RBD orientation: three-down (6XEY (27)), one-up, two-down (6Z43 (28)), two-up, one-down (7A29 (29)), and three-up (7JVC (30)) (see Figure 4). Both Sb16 and Sb45 may
140 dock on each of the three RBDs in the trimeric S in any of the four configurations, without any apparent clash (Figure 4A, 4B). However, Sb68 could not be superposed without clashes to any RBD of the three-down or to the one-up two down position. The only permissible superpositions were to two in the two-up, one-down (Figure 4C); and to all three in the three-up position (Figure 4C). For paired sybodies, either Sb16 and Sb68 or Sb45 and Sb68, superposition was possible
145 without clashes, with two or more RBDs in the up conformation (Figure 4D and 4E). Walter et al (14) suggested that a covalent bispecific Sb15–Sb68 reagent could bind S in both the two-up and three-up configurations, based on cryo-EM maps of complexes of S with Sb15 and Sb68, with local resolution in the range of 6-7 Å. It appears that Sb16 binds to S in an orientation similar to, but in detail distinct from that of Sb15. This analysis demonstrates an advantage of the small size
150 of sybodies or nanobodies in accessing epitopic regions of S.

Barnes et al (31) categorized a host of anti-S and anti-RBD Fabs into four classes (1-4) based on the location of the footprint, and whether the Fab has access to either the up only or up and down configuration of the RBD in the context of the full trimer (Figure S6A). Xiang et al (12) categorized anti-RBD nanobodies into five epitopic regions (I-V) (Figure S6A). By
155 superposition, Sb16 would clash with the light chain of the B38 Fab (7BZ5), as in class 1, but it

also clashes with the heavy chain of COVA2-39 (7JMP), as in class 2 (Figure S6B). Sb45 clashes effectively with the heavy chain of COVA2-39, and thus appears to be closer to a “true” class 2 sybody (Figure S6C). Both Sb16 and Sb45 are capable of binding the RBD of S in either the up or down position, a defining characteristic of class 2. By contrast, Sb68 competes mostly with the CR3022 heavy chain (6W41), V_{HH}72 (6WAQ) (20) and V_{HH}-U (7KN5) (32) placing it in class 4. Overall, our structural studies not only define the Sb16, Sb45, and Sb68 epitopes at high resolution, they suggest that a battery of sybodies or nanobodies have the potential to saturate the available RBD surface.

The significance of the ternary structure of Sb45–RBD–Sb68 (7KLW) is confirmed in a recent paper (32). Koenig et al determined a ternary nanobody structure of V_{HH}-E–RBD–V_{HH}-U (7KN5) which illustrates the binding to two distinct epitopic sites. Superposition of Sb45–RBD–Sb68 on V_{HH}-E–RBD–V_{HH}-U indicates that Sb45 and V_{HH}-E represent class 2 in recognizing the epitope region but do so in different orientations (Figure S7A, middle panel). Sb45 uses both long CDR2 and CDR3 loops riding along both sides of RBD surface, while V_{HH}-E uses a long CDR3 loop engaging one side of RBD surface.

Recently, several SARS-CoV-2 spike variants have been isolated and characterized with respect to their infectivity and severity of disease. The UK-SARS-CoV-2 variant has multiple substitutions including N501Y in the RBD (1). This is expected to impinge on the peripheral aspect of the footprint of Sb16 and Sb45 but would have no effect on the Sb68 site. Thus, precise mapping of anti-RBD antibody, nanobody, and sybody epitopes, especially for those that are developed for clinical trials, has implications not only for mechanistic understanding of the interactions of the RBD with ACE2, but also for evaluating the potential susceptibility of newly arising viral variants to currently administered vaccines and antibodies.

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J.A., J.J., K.N., and D.H.M. conceived the project; J.A., K.N., and L.F.B. engineered constructs,
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7KGK, 7KGJ, 7KLW and 7KGL for Sb16–RBD, Sb45–RBD, Sb45–RBD–Sb68, and Sb16
respectively.

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Supplementary Materials:

Materials and Methods

Figures S1-S8

Tables S1-S3

310 References (33-45)

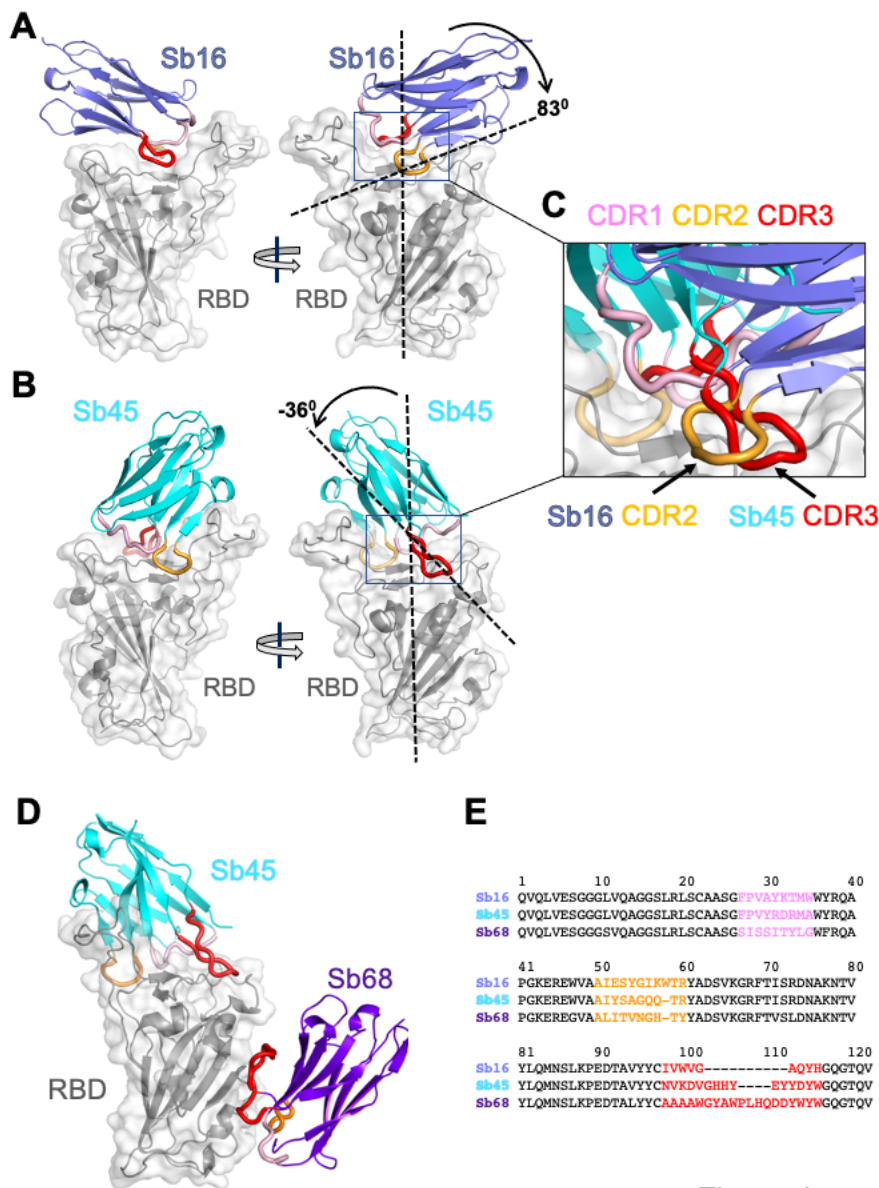


Figure 1

Fig. 1. Overall structures of Sb16, Sb45 and Sb68 complexes with SARS-CoV-2 RBD. Ribbons (sybodies) and ribbons plus surface (RBD) representations of the complex of (A) Sb16 (slate) with RBD (grey) (7KGK); (B) Sb45 (cyan) with RBD (7KGJ), and (C) Sb45 and Sb68 (purple) with RBD (7KLW). Sb16-RBD and Sb45-RBD, superimposed based on the RBD are shown in (D) to highlight CDR loops, which are color coded as indicated. The CDR2 of Sb16 and CDR3 of Sb45 interact similarly with the RBD surface.

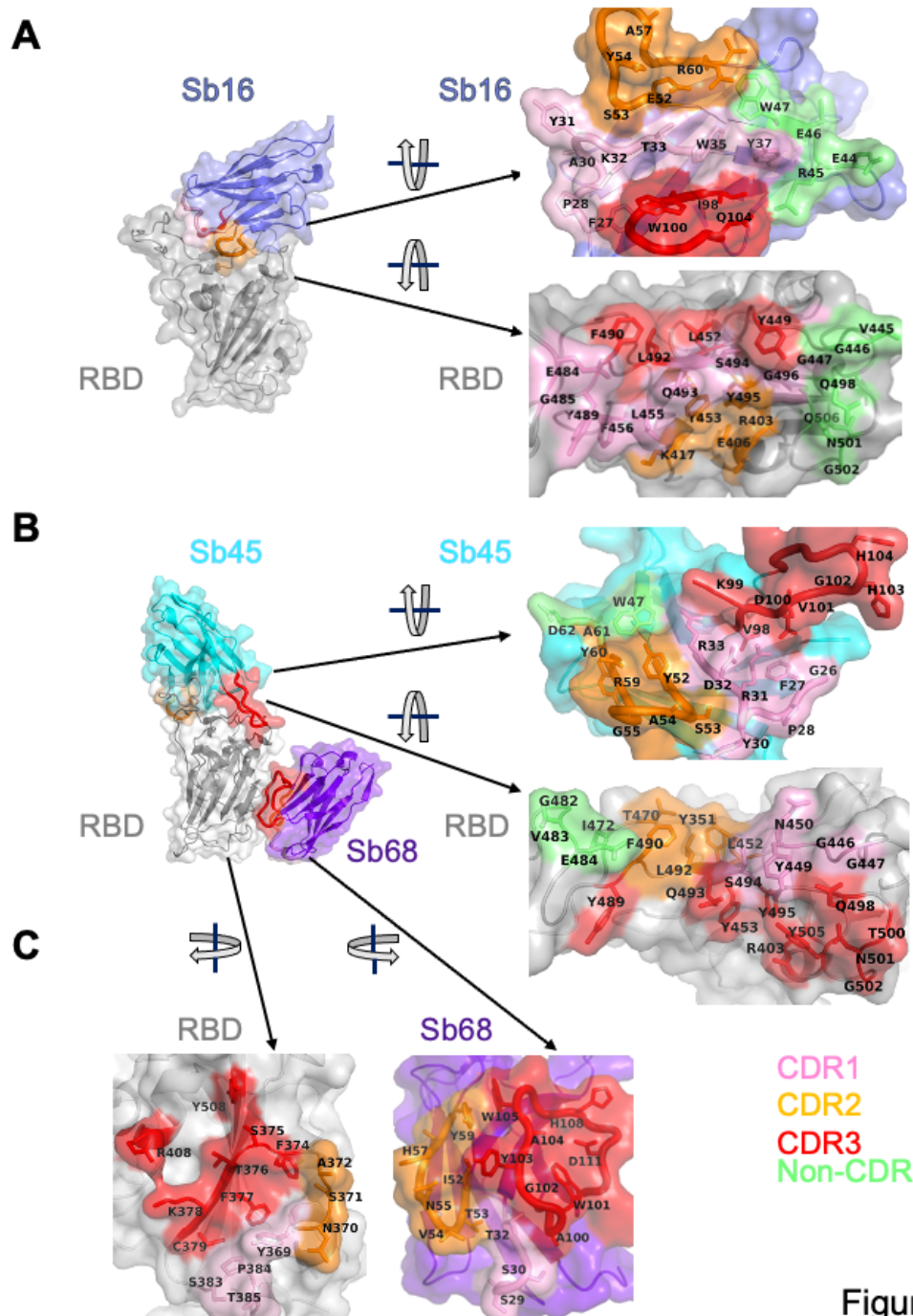


Figure 2

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Fig. 2. Interface and interaction of (A) Sb16-RBD, (B) Sb45-RBD and (C) Sb68-RBD. (Individual contacting residues are listed in Table S2 in Supplemental Materials). CDR1, CDR2, CDR3 regions are painted pink, orange and red respectively. Additional non-CDR region

contacting residues are colored lime. On the RBD surface, the epitopic residues that contact the sybodies are colored according to the sybody CDR.

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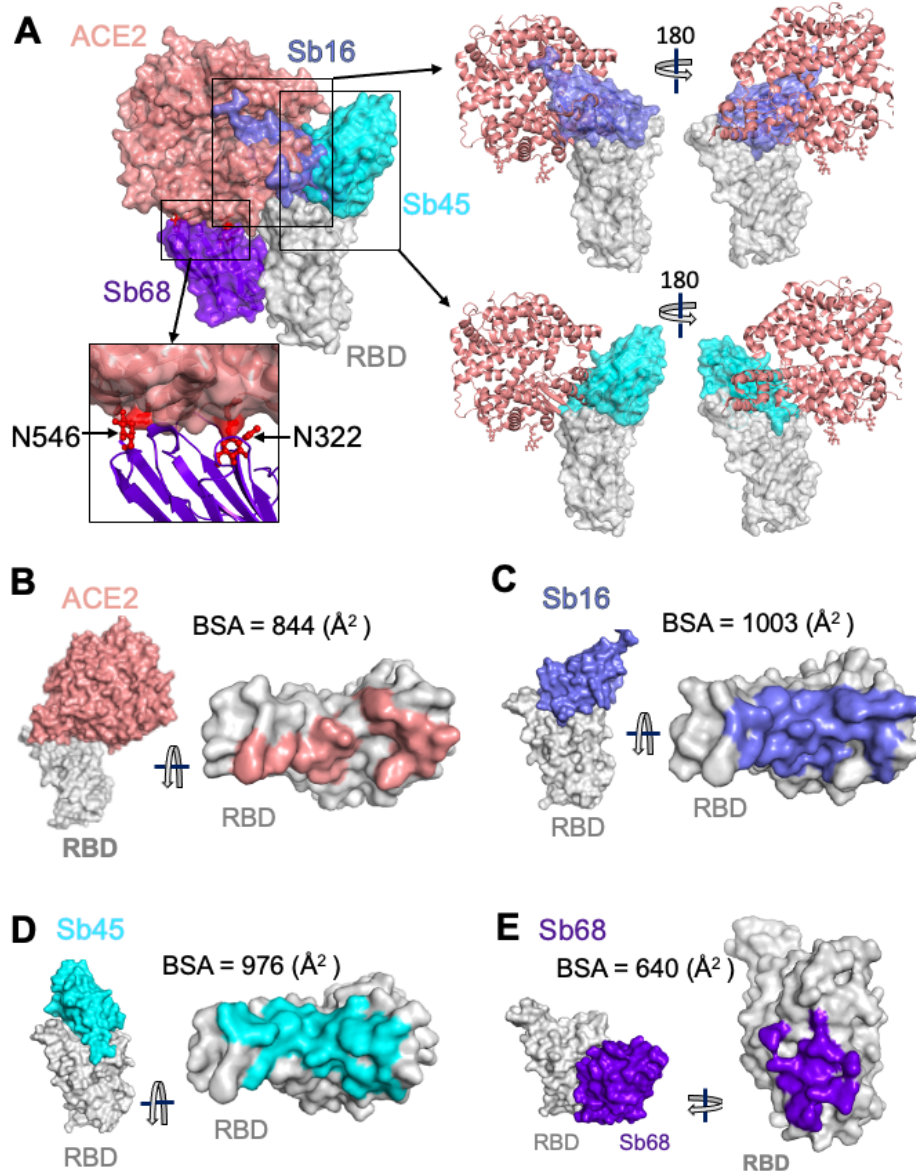


Figure 3

Fig. 3. Sybodies compete with ACE2 for RBD binding. (A) Sb16 (slate), Sb45 (cyan) and Sb68 (purple) – RBD complexes were superposed on the ACE2—RBD structure (salmon) (6M0J) based on the RBD. Sb16 is buried inside ACE2; Sb45 is partially buried in ACE2; and Sb68 has major clashes with two N-glycan sites (N322 and N546) of ACE2. (B) Epitopic areas (on RBD) captured by ACE2 (salmon), BSA = 844 (\AA^2); (C) by Sb16 (slate), BSA = 1003 (\AA^2); (D) by Sb45 (cyan), BSA = 976 (\AA^2); and (E) by Sb68 (purple), BSA = 640 (\AA^2).

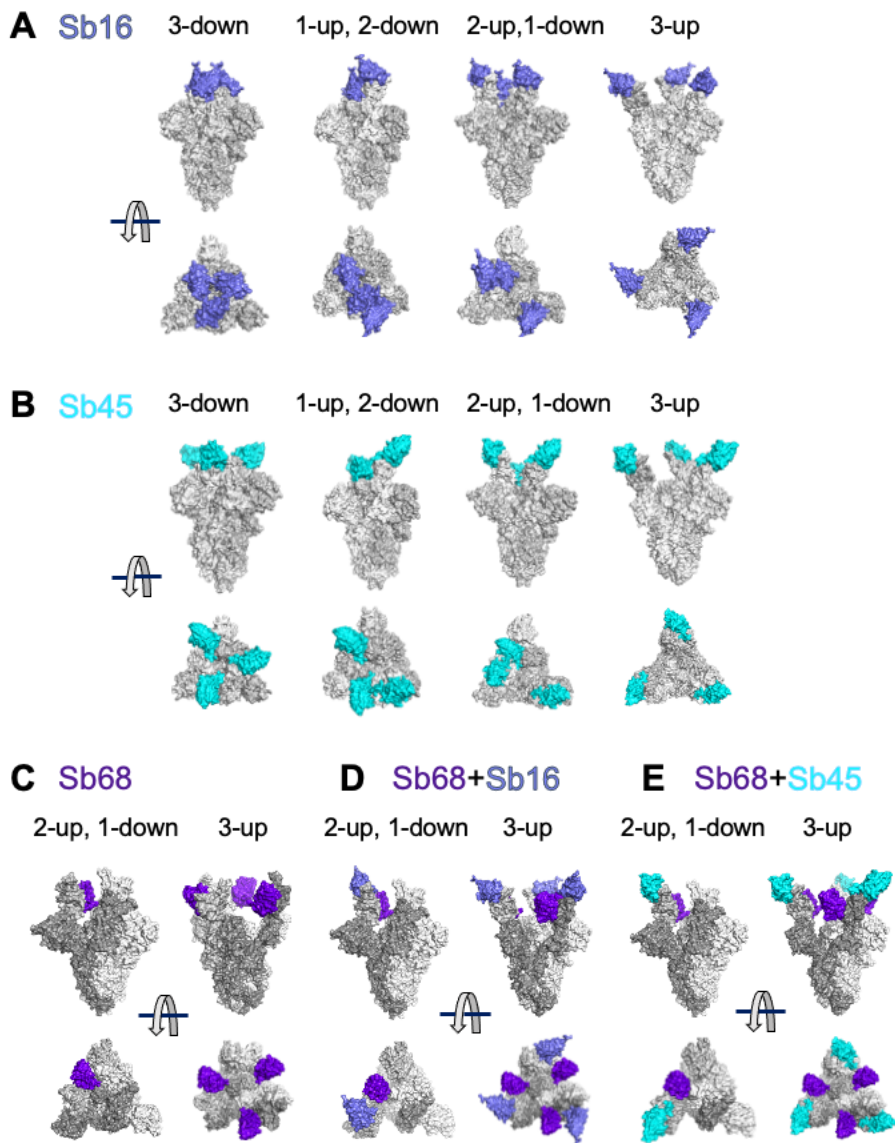


Figure 4

Fig. 4. Superposition of complexes on spike models reveals accessibility of sybodies. Superposition of (A) Sb16-RBD (slate) on spike (6XEY: 3-down; 6Z43: 1-up and 2-down; 7A29: 2-up and 1-down; 7JVC: 3-up); (B) Sb45-RBD (cyan); and (C) Sb68-RBD (purple), there

is no accessible surface for Sb68 on 3-down of spike; (D) Sb68 and Sb16 on RBD; (E) SB68 and Sb45 on RBD.

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Supplementary Materials for

Synthetic nanobody–SARS-CoV-2 receptor-binding domain structures identify distinct epitopes

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This PDF file includes:

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- Figs. S1 to S8
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22 **Materials and Methods**

23 *Subcloning, expression and purification of RBD, spike, and sybody proteins*

24 The sequences encoding the RBD of the SARS-CoV-2 spike protein (amino acids 333 to
25 529) were subcloned into pET21b(+), (Novagen) via *Nde*I and *Eco*RI restriction sites, using
26 pcDNA3-SARS-CoV-2-RBD-8his (Addgene #145145, (33)) as template. The primers used were
27 forward primer, 5'-TGCAGTCATATGAATCTTTGTCCGTTCCGGTGAG and reverse primer,
28 5'-TGCAGTGAATTCTCACCCCTTTTTGGGCCCAAACT. The RBD was expressed as
29 inclusion bodies in *E. coli* strain BL21(DE3) (Novagen). Expression, isolation of inclusion
30 bodies, denaturation and reduction was done in 6 M guanidine hydrochloride and 0.1 mM DTT
31 as described elsewhere (34). Briefly, refolding was carried out in a refolding buffer
32 supplemented with oxidized and reduced glutathione and arginine for 3 days at 4 °C followed by
33 dialysis against HEPES buffer (25 mM HEPES, pH 7.3, 150 mM NaCl). Concentrated and
34 filtered protein was analyzed by size-exclusion chromatography on a Superdex 200 10/300 GL
35 column (GE Healthcare) equilibrated with HEPES buffer. The peak corresponding to 24 kDa
36 (monomeric) protein was collected, concentrated and further purified by ion-exchange
37 chromatography on Mono-Q® (Cytiva).

38 Plasmids pSb-init encoding sybodies Sb16, Sb45. and Sb68 (Addgene #153524,
39 #153526, and #153527, respectively) were originally reported by Walter et al (35) and
40 generously made available. All plasmids were verified by DNA sequencing. Purification of the
41 recombinant proteins from the periplasm of *E. coli* MC1061 was based on a protocol described
42 elsewhere (36). Briefly, *E. coli* MC1061, transformed with a sybody-encoding plasmid, was
43 grown in Terrific Broth (TB) medium (Gibco) supplemented with 25 µg/ml chloramphenicol, at
44 37 °C with shaking at 160 rpm for 2 hrs. The temperature was then decreased to 22 °C until A_{600}

45 reached 0.5. Protein expression was induced by addition of L-(+)-arabinose (Sigma) to a final
46 concentration of 0.02% (w/v) and expression continued overnight at 22 °C and 160 rpm. The
47 next day cells were collected by centrifugation at 2000 x g for 15 minutes. The cell pellet was
48 then washed twice in PBS and resuspended in periplasmic extraction buffer (50 mM Tris/HCl pH
49 8.0, 0.5 mM EDTA, 0.5 µg/ml lysozyme, 20% w/v sucrose (Sigma)) at 4 °C for 30 min followed
50 by addition of TBS (pH 8.0) and 1 mM MgCl₂. Cells were then centrifuged at 10,000 rpm
51 (Fiberlite™ F21-8 x 507 Fixed Angle Rotor) for 30 min. Following transfer of the supernatant to
52 a fresh tube, imidazole was added to a final concentration of 10 mM. Ni-NTA resin (Qiagen)
53 equilibrated with TBS was added to the supernatant and incubated for 1 hr at RT with mild
54 agitation. The resin was collected, washed three times with buffer supplemented with 30 mM
55 imidazole and sybody proteins were eluted with 300 mM imidazole in TBS.

56 Plasmid encoding spike HexaPro (designated “S” throughout) was procured from
57 Addgene (#154754) and transfected into Expi293F™ cells (ThermoFisher Scientific) using
58 manufacturer’s protocol. Briefly, Expi293F™ cells were seeded to a final density of $2.5-3 \times 10^6$
59 viable cells/ml and grown overnight at 37 °C in Expi293™ Expression Medium (Gibco). The
60 following day, cell viability was determined, and cell density was adjusted to 3×10^6 viable
61 cells/ml with fresh, prewarmed Expi293™ Expression Medium. Transfection was then done as
62 per manufacturer’s instructions using 1 µg/ml plasmid DNA. Cultures were grown for 6 days
63 following transfection and supernatant was collected, filtered through a 0.22 µm filter and passed
64 over Ni-NTA resin for affinity purification. Further purification was accomplished by size-
65 exclusion chromatography using a Superose 6 10/300 GL column (Cytiva) in a buffer consisting
66 of 2 mM Tris pH 8.0, 200 mM NaCl.

67

68 *Preparative and analytical size-exclusion chromatography*

69 Sybodies purified by Ni-NTA affinity chromatography were concentrated using Amicon
70 10K MWCO concentrators and purified on a Sepax SRT-10C SEC100 column at a flow rate of 1
71 ml/min. Monomeric sybodies elute at a retention volume of 11–12.5 ml from the Sepax SRT-
72 10C SEC100 column. Monomeric peak fractions were collected and analyzed by SDS-PAGE.
73 Analytical SEC of RBD sybody complexes was performed on a Shodex KW-802.5 column at a
74 flow rate of 0.75 ml/min in TBS buffer (pH. 8.0). (The interaction of individual sybodies with
75 the column matrix is a well-documented phenomenon (36)).

76

77 *Surface Plasmon Resonance*

78 SPR experiments were performed on a Biacore T200 (Cytiva) at 25 °C in 10 mM HEPES
79 pH 7.2, 150 mM NaCl, 3 mM EDTA, 0.05% Tween-20. RBD was immobilized on a series S
80 CM5 sensor chip (Cytiva) by amine (NHS/EDC) coupling to flow cells. For background
81 subtraction a reference cell was mock coupled. Binding and kinetic studies were performed
82 multiple times for each sybody. Graded and increasing concentrations of SB16, SB45 and SB68
83 were injected over the RBD-immobilized surface at a flow rate of 30 µl/min with an association
84 time of 120 s and dissociation time of 2000 s. Binding data were analyzed by surface site affinity
85 distribution analysis by EVILFIT (37, 38). In general, these values were consistent with fits to
86 the Langmuir binding equation for a 1:1 interaction model using Biacore T200 Evaluation
87 Software v3.0, but revealed better statistics.

88

89 *Thermal stability*

90 Thermal melt analysis of the recombinant proteins was performed in triplicate in 96-well
91 plates in a QuantStudio 7 Flex real time PCR machine (Applied Biosystems). Each well
92 contained 2-4 μg protein in buffer (25mM TRIS pH 8, 150 mM NaCl) and 5x Sypro Orange
93 (Invitrogen, stock 5000x) in a total volume of 20 μl . Following an initial two-minute hold at 25
94 $^{\circ}\text{C}$, the plate was heated to 99 $^{\circ}\text{C}$ at a rate of 0.05 $^{\circ}\text{C}/\text{sec}$. Data were analyzed with Protein
95 Thermal Shift Software v1.3 (Invitrogen) to obtain T_m values for RBD, S, Sb16, Sb45, and Sb68
96 (Figure S8).

97

98 *Crystallization, data collection, structure determination and crystallographic refinement*

99 Purified sybodies (Sb16, Sb45 and Sb68) and RBD were mixed in approximate 1:1 molar
100 ratio to a final concentration of 8 mg/ml. The protein mixtures were incubated on ice for 1 hour
101 prior to screening. Initial screening for crystals was carried out using the hanging drop vapor
102 diffusion method using the Mosquito robotic system (sptlabtech.com). Crystals of SB16-RBD
103 and SB45-RBD complexes and Sb16 alone were observed within one week using Protein
104 Complex (Qiagen) and Wizard Classic 4 (Rigaku). Conditions for Sb16–RBD were either 0.1M
105 HEPES pH 7.0, 15% PEG 20000, or 0.1M HEPES pH 7.0, 18% PEG 12000; and for Sb45–RBD
106 was 18% PEG 12000 and 12% PEG 8000, 0.1 M HEPES pH 7.5, 0.2 M NaCl. Sb16 alone
107 crystallized in 20% PEG 4000, 0.1 M MES pH 6.0, 0.2 M LiSO_4 . We also screened mixtures of
108 two or three sybodies with RBD. Crystals of Sb45–RBD–Sb68 were obtained after one month
109 following mixing the three proteins in an equimolar ratio in 10% PEG 8000, 0.1M sodium
110 cacodylate pH 6.0.

111 Crystals of protein complexes were optimized with slight adjustments of the
112 concentration of PEG components. Crystals were cryoprotected in mother liquor containing 5%

113 ethylene glycol and 5% glycerol and flash frozen in liquid nitrogen for data collection.

114 Diffraction data were collected at the Southeast Regional Collaborative Access Team (SER-

115 CAT) beamline 22ID-D at the Advanced Photon Source, Argonne National Laboratory and data

116 were processed with XDS (39). Multiple data sets were collected for the protein complexes from

117 2.3-3.2 Å resolution. The initial model of Sb16 and Sb45 for the molecular replacement search

118 were built by the MMM server (manaslu.fiserlab.org/MMM (40)), using the heavy chain V

119 domain and the RBD of the Fab B38–RBD complex (PDBid: 7BZ5) (41). The initial model of

120 Sb68 for molecular replacement was built based on the V_H domain of 7BZ5. Molecular

121 replacement solutions were found using Phaser (42, 43). Subsequent refinements were carried

122 out with PHENIX (44). CDR loops were manually rebuilt by fitting to the electron density maps

123 with Coot (45). In particular, Sb68 CDR loops were deleted before refinement and built in

124 manually based on electron density maps. Illustrations and calculations of superpositioned

125 models were prepared in PyMOL (The PyMOL Molecular Graphics System, Version 2.4.0

126 Schrödinger, LLC). Calculation of hinge relationships of domains was accomplished with

127 HINGE (<https://niaidsis.niaid.nih.gov/hinge.html>), provided courtesy of Peter Sun, NIAID.

128 Buried surface area (BSA) calculations were performed with PISA

129 (<https://www.ebi.ac.uk/pdbe/pisa/>).

130 The final structures for the RBD-SB16 and RBD-SB45 complexes showed $R_{\text{work}}/R_{\text{free}}$ (%)

131 25.4/27.7 and 18.6/21.6 respectively, and for SB16 alone with $R_{\text{work}}/R_{\text{free}}$ 22.4/25.9. Data

132 collection and structure refinement statistics are provided in Table S1.

133

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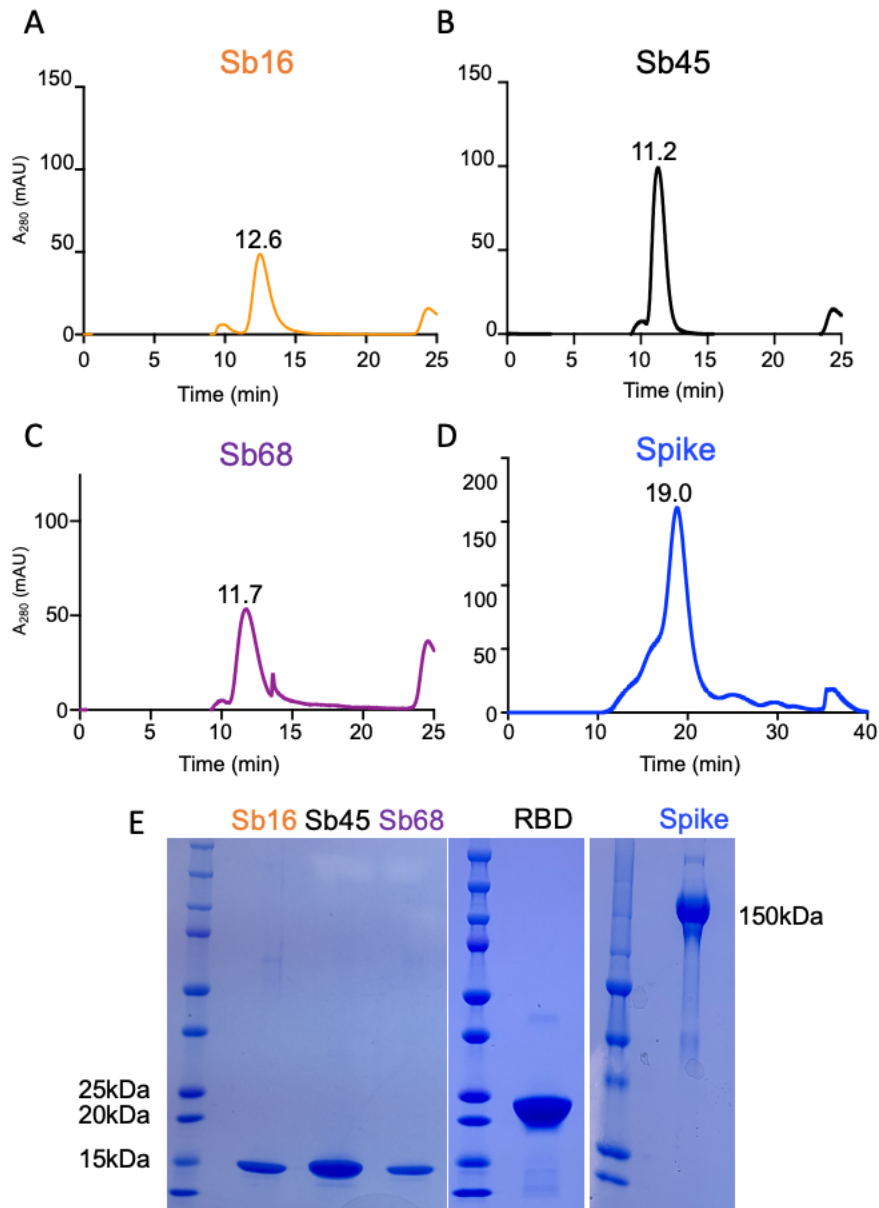


Figure S1.

135

136 **Fig. S1.** SEC profiles and purification of sybodies, RBD, and spike. (A, B, C) Monomeric
137 sybodies, as indicated, were purified on SRT-10C-SEC100 columns. Elution time of each
138 sybody is indicated above each peak. The y axis represents A₂₈₀ nm absorbance units (mAu). (D)
139 SEC profile of trimeric spike protein (Superose™ 6 10/300 GL). (E) SDS-PAGE image of
140 purified sybodies, RBD and spike protein.

141

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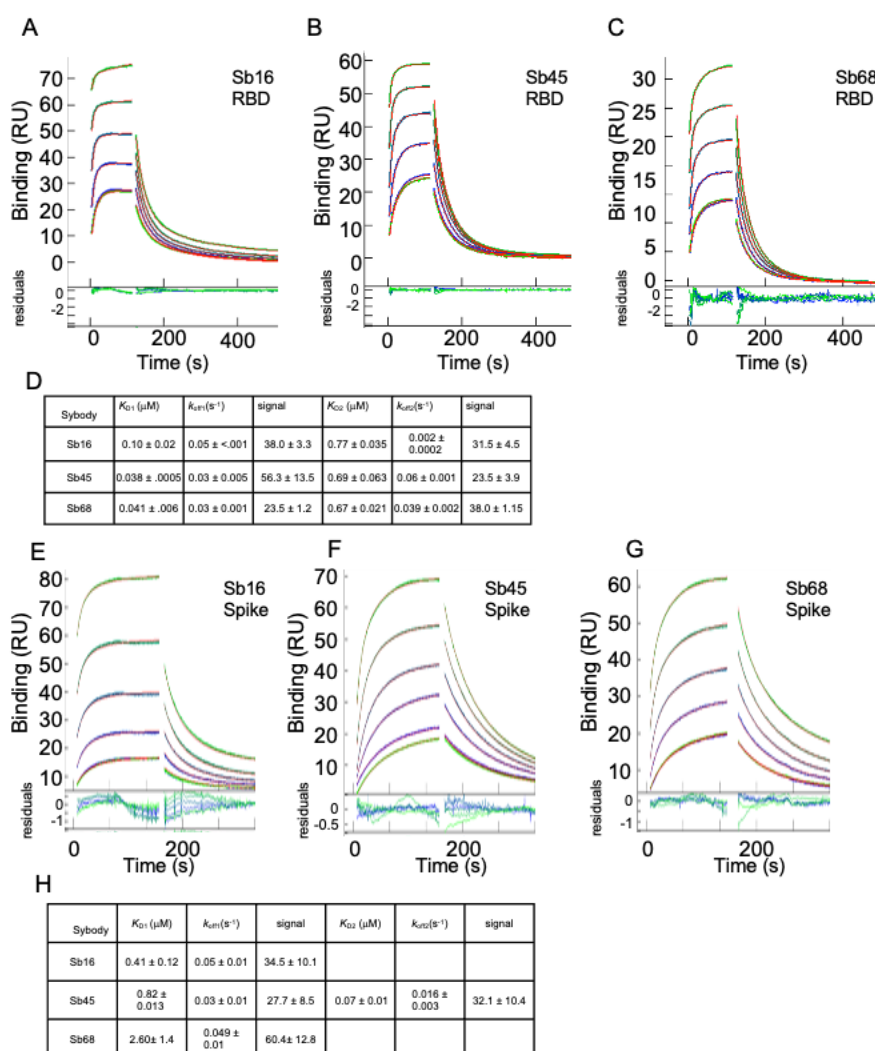


Figure S2.

143

144 **Fig. S2.** Sybodies bind RBD with K_D values in the nanomolar range. RBD (A, B, C) or S protein
 145 (E, F, G) was coupled to a biosensor chip as described in Materials and Methods. Graded
 146 concentrations (31 to 500 nM) were flowed over the coupled surfaces (from $t=0$ to $t=120$ s,
 147 followed by buffer washout) and net RU signal (compared to mock-coupled surface) was
 148 measured by SPR for Sb16 (A, E); Sb45 (B, F); and Sb68 (C, G). Summary of triplicate mean \pm
 149 SD determinations is shown in Tables (D, H). Global analysis (curve fits in red, residuals below
 150 the curves) was accomplished using EVILFIT (37, 38), and the major components of binding are
 151 shown.

152

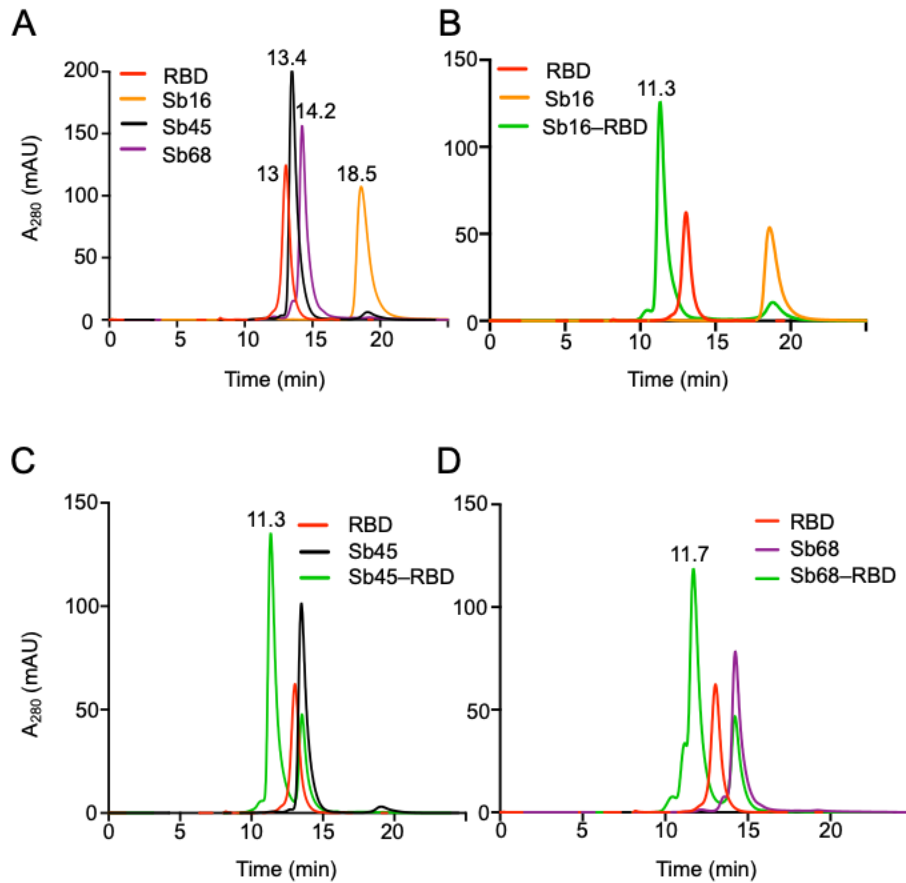


Figure S3.

153

154 **Fig. S3.** SEC profiles reveal direct interaction of sybodies with RBD. (A) Sybodies (50 μ g) and
155 RBD (50 μ g) were injected onto a Shodex-KW-802.5 column (0.75 ml/min) in 100 μ l
156 individually and elution times are shown. Sb16 and RBD (B), Sb45 and RBD (C), or Sb68 and
157 RBD (D) were mixed in equal concentrations (50 μ g in 100 μ l), incubated at 4 $^{\circ}$ C overnight and
158 then analyzed on the Shodex column.

159

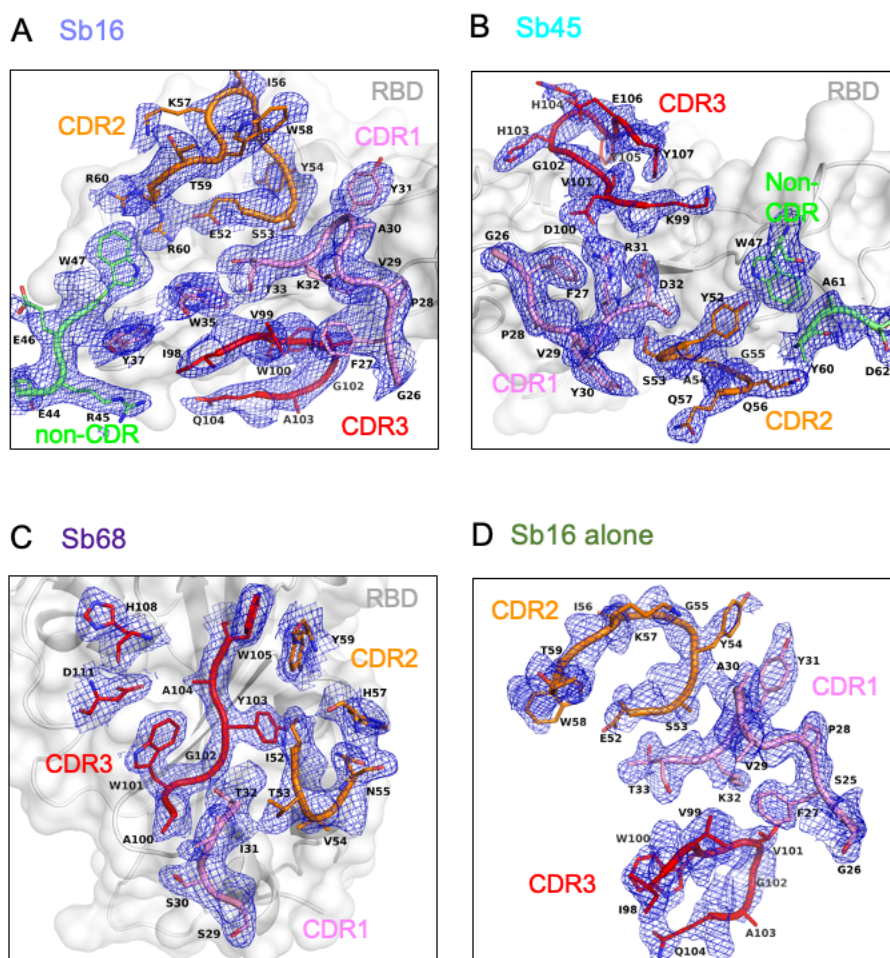


Figure S4.

160

161 **Fig. S4.** Electron density (as blue) maps (2mFo-DFc) for CDR loops and those residues in
162 contact with RBD: (A) Sb16 on RBD surface, resolution=2.6 Å, $R_{\text{free}}=0.277$; (B) Sb45 on RBD
163 surface, resolution=2.3 Å, $R_{\text{free}}=0.216$; (C) Sb68 on RBD surface, resolution=2.6 Å, $R_{\text{free}}=0.255$;
164 (D) Sb16 alone, resolution=2.1 Å, $R_{\text{free}}=0.259$. Contour at 1.0 σ , CDR1 loop as pink, CDR2 loop
165 as orange, CDR3 loop as red, non-CDR residues as lime, and RBD is in background as gray.

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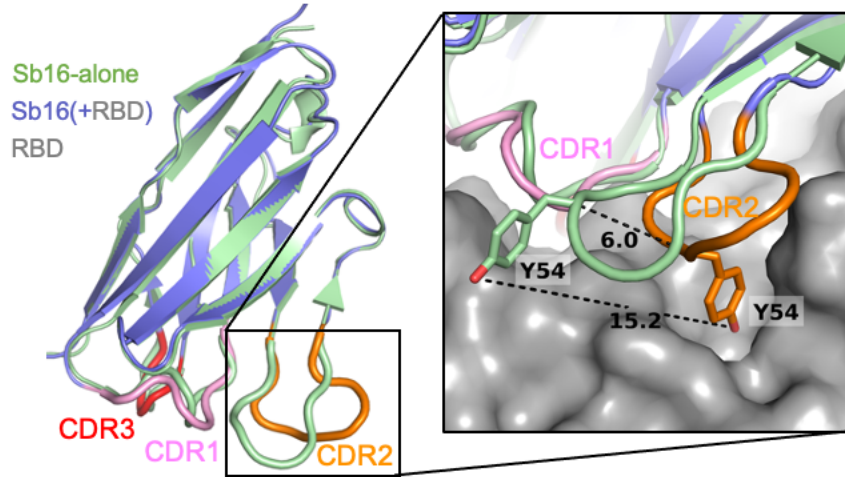


Figure S5.

168

169 **Fig. S5.** A superimposition of Sb16 alone (“unliganded”, free, green) and Sb16–RBD
170 (“liganded”, complexed, slate) reveals the large movement of CDR2 loop (about 6 Å).
171 Particularly, Y54 moved about 15 angstroms and dipped into a binding pocket which surrounded
172 by epitopic residues of Q409, E406, D405, R403, G416, K417, I418, N422, L455, Y453, Y495.
173 (RBD surface as gray).

174

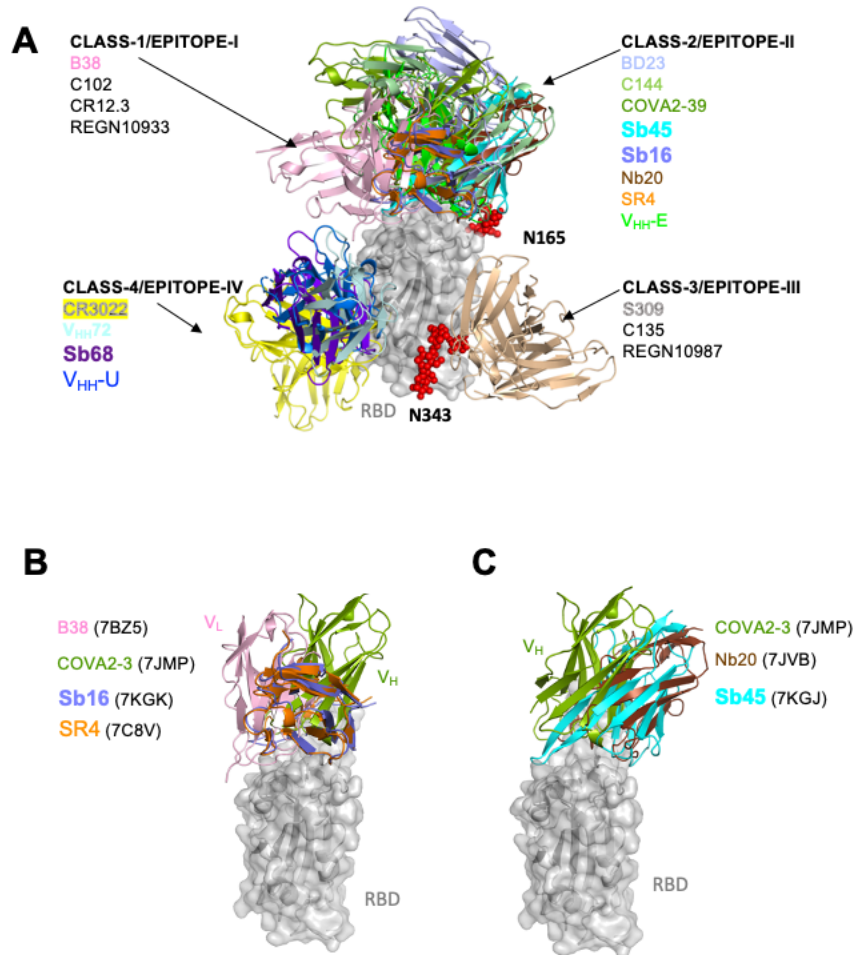


Figure S6

175

176 **Fig. S6.** Sybodies, nanobodies, and antibodies bind different epitopic regions of the RBD. (A)
 177 Definition of Classes according to Barnes et al. (31) and Epitope area according to Xiang et
 178 al.(12). Epitope I to IV are almost the same as Class 1 to 4, except for Epitope V which spans
 179 Class 3 and Class 4 (not shown). Color codes are for representative Fab (only shown are the
 180 variable domains) or sybody/nanobody. Sb68 falls into Class-4/Epitope-IV overlapping with
 181 V_{HH}72 and HH-E, and CR3022. RBD is in gray and two N-glycans (N165 and N343 in red) are
 182 shown. (B) Sb16 clashes with L-chain of B38 (7BZ5) of Class-1 and H-chain of COVA2-39
 183 (7JMP) H-chain Class-2. It falls between Class-1/Epitope-I and Class-2/Epitope-II but almost

184 covers SR4. (C) Sb45 overlaps with H-chain of COVA2-39 (7JMP) of Class-2 and lies in the
185 same orientation as Nb20 (7JVB) - Epitope-II.
186

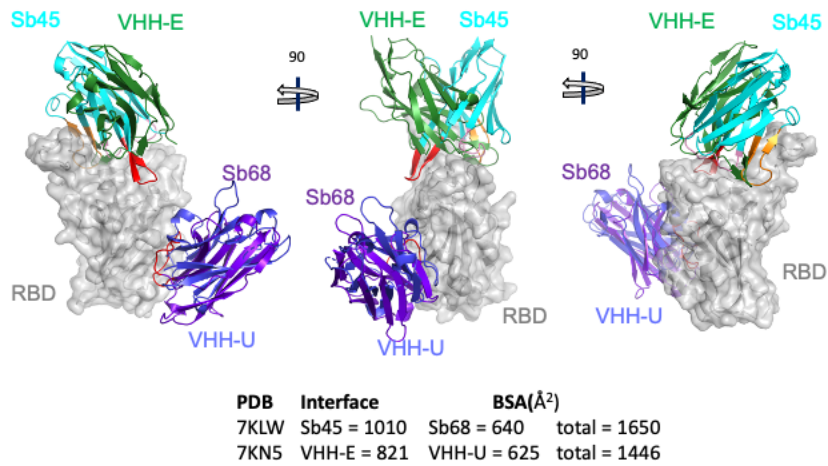
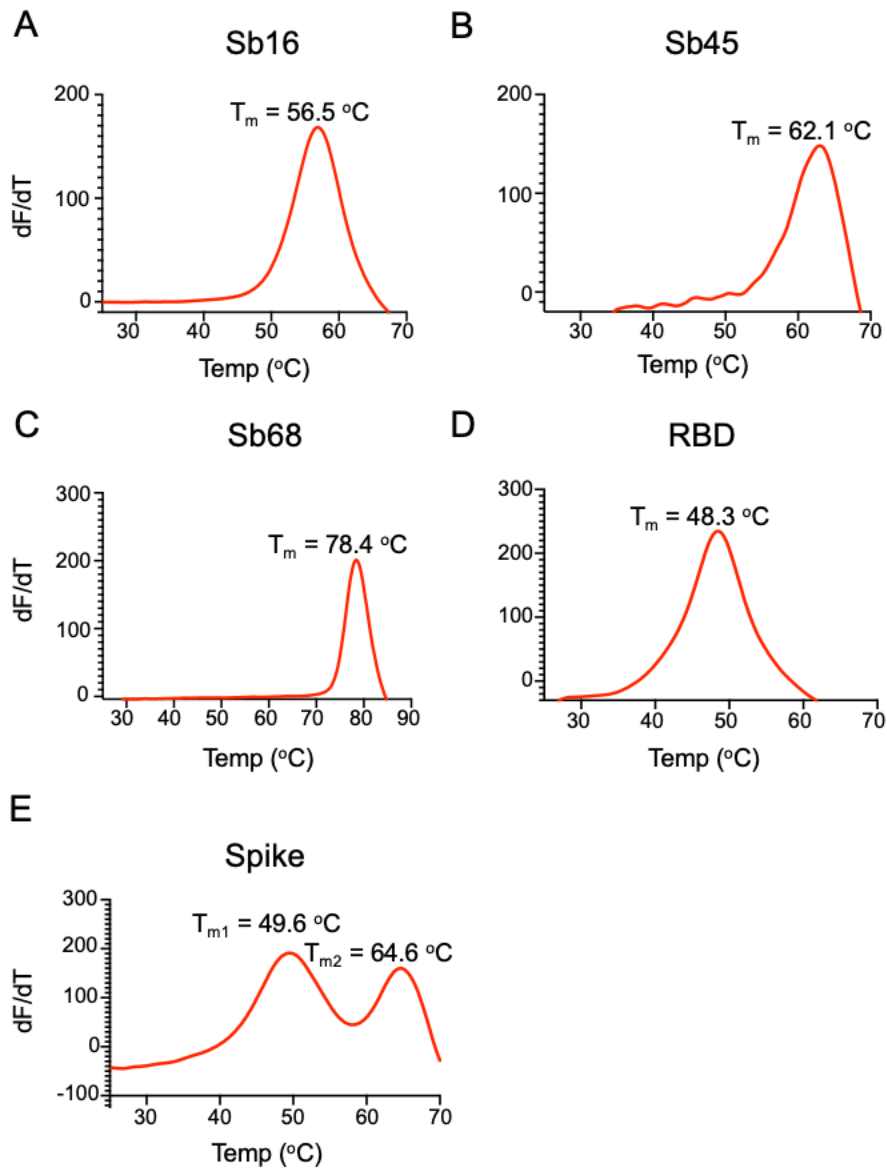


Figure S7

187

188 **Fig. S7.** Comparison of the ternary sybody structure (Sb45–RBD–Sb68, 7KLW) with the ternary
189 nanobody structure (V_{HH}-E–RBD–V_{HH}-U, 7KN5), Koenig et al. (32). Sb45 in cyan, Sb68 in
190 purpleblue, RBD in gray, V_{HH}-E in green, V_{HH}-U in blue. CDR1, CDR2, and CDR3 of Sb45,
191 CDR3 of Sb68 are highlighted in pink, orange, and red. Three views are each rotated by 90°.
192 CDR3 and CDR2 loops of Sb45 ride along both sides of the RBD, while V_{HH}-E uses only an
193 extended CDR3 loop on the side. Sb68 is slightly lower than V_{HH}-U while V_{HH}-E is similar to
194 V_{HH}72.

195



196

197 **Fig. S8.** Sybodies, RBD, and spike protein reveal unique thermal stability. T_m of each of the
198 indicated purified proteins was determined by thermal melt analysis as described in Materials
199 and Methods. Note the biphasic behavior of the trimeric S protein.

200

	Sb16-RBD	Sb45-RBD	Sb45-RBD-Sb68	Sb16
PDBID	7KGK	7KGJ	7KLW	7KGL
Data collection				
Space group	P6 ₅ 22	P3 ₂ 21	C222 ₁	P6 ₃ 22
Cell dimensions				
<i>a</i> , <i>b</i> , <i>c</i> (Å)*	65.64, 65.64, 344.69	62.55, 62.55, 168.82	74.50, 102.40, 138.97	69.32, 69.32, 105.57
α , β , γ (°)□	90.0, 90.0, 120.0	90.0, 90.0, 120.0	90.0, 90.0, 90.0	90.0, 90.0, 120.0
Resolution (Å)	57.34-2.60 (2.69-2.60)	45.59-2.30 (2.38-2.30)	44.12-2.60 (2.69-2.60)	60.0-2.10 (2.18-2.10)
<i>R</i> _{sym} or <i>R</i> _{merge}	0.080 (0.455)	0.101 (0.849)	0.095 (0.739)	0.055 (0.714)
<i>I</i> / σ (<i>I</i>)	18.0 (3.3)	14.9 (3.4)	13.1 (2.1)	17.8 (2.7)
Completeness (%)	98.8 (99.1)	99.3 (98.3)	98.8 (98.7)	98.9 (98.4)
Redundancy	10.3 (10.9)	7.9 (8.2)	7.2 (7.4)	6.1 (6.5)
<i>R</i> _{pim}	0.024 (0.134)	0.038 (0.293)	0.038 (0.287)	0.025 (0.311)
CC _{1/2}	0.999 (0.987)	0.997 (0.919)	0.998 (0.895)	0.999 (0.891)
Estimated twin fraction	0.0 (none)	0.06 (-h, -k, l)	0.0 (none)	0.0 (none)
Refinement				
Resolution (Å)	56.09-2.60 (2.69-2.60)	45.59-2.30 (2.38-2.30)	36.72-2.60 (2.69-2.60)	32.9-2.10 (2.18-2.10)
No. reflections	13219 (1185)	17592 (1687)	16508 (1627)	9278 (823)
<i>R</i> _{work} / <i>R</i> _{free} (%)	25.8/27.7 (36.3/44.2)	18.6/21.6 (24.1/29.8)	20.6/25.5 (29.3/34.5)	22.4/25.9 (31.9/31.4)
No. atoms	2486	2641	3552	980
Protein	2486	2500	3456	913
Water + ligands	0	141	96	67
B-factor Wilson/Average	39.3/59.8	26.9/32.9	33.9/31.5	23.2/36.9
Protein	59.8	32.8	31.5	37.0
Water + ligands	0	34.7	29.5	35.0
R.m.s. deviations				
bond length (Å)	0.002	0.005	0.003	0.003
bond angle (°)	0.54	0.74	0.64	0.57
Ramachandran				

avored (%)	92.9	97.4	96.3	93.0
allowed (%)	7.1	2.6	3.7	6.1
outliers (%)	0.0	0.0	0.0	0.9

201 **Table S1.** X-ray data collection and refinement statistics

202 *Values in parenthesis are for highest resolution shell.

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ACE2+RBD (6M0J)

RBD	ACE2	DIST	ACE2	DIST
K417	D30	2.90		
Y449	Q42	2.79	D38	2.70
Y453	H34	2.86		
L455	H34	3.62		
F456	T27	3.49		
A475	Q24	3.65	T27	3.97
G476	Q24	4.43		
F486	Y83	3.52	M82	3.64
N487	Y83	2.79	Q24	2.69
Y489	F28	3.55	Y83	3.55
Q493	K31	2.93	E35	3.13
G496	K31	3.08		
Q498	Q42	2.93	Y41	3.59
T500	R357	3.51	N330	3.64
N501	K353	3.61		
G502	K353	2.78		
Y505	E37	3.46	K353	3.61

Sb16+RBD (7KGK)

RBD	Sb16	DIST	Sb16	DIST	CDR
R403	Y54	3.00			CDR2
E406	Y54	2.70			CDR2
K417	Y54	3.30			CDR2
V445	E44	3.00			
G446	Y37	3.30			CDR1
G447	Y37	3.20			CDR1
Y449	I98	3.60	Q104	3.70	CDR3
L452	W100	3.29			CDR3
Y453	S53	2.95	Y54	3.50	CDR2
L455	Y31	3.49	S53	3.44	CDR1
F456	Y31	3.37			CDR1
E484	K32	2.39	F27	3.30	CDR1
G485	P28	3.38			CDR1
Y489	Y31	3.41	A30	3.34	CDR1
F490	W100	3.38			CDR3
L492	W100	3.85			CDR3
Q493	K32	3.51	T33	3.70	CDR1
S494	T33	3.73			CDR1
Y495	W35	3.13	Y54	4.30	CDR2
G496	W35	3.71			CDR1
Q498	Y37	2.91	W47	3.48	
N501	R60	2.38			
G502	R60	3.60			
Y505	A57	3.50	E52	3.80	

Sb45+RBD (7KGJ)

RBD	Sb45	DIST	Sb45	DIST	CDR
Y351	A54	3.80			CDR2
R403	H103	3.00	G102	3.30	CDR3
G446	F27	3.70	G26	3.90	CDR1
G447	P28	3.50			CDR1
Y449	D100	3.80	R31	3.20	CDR3
N450	Y30	3.50			CDR1
L452	S53	3.90			CDR2
Y453	V101	3.40			CDR3
T470	G55	3.80	R59	2.95	CDR2
I472	R59	3.90			
G482	R59	3.30			
V483	Y60	3.60	D62	4.10	
E484	R33	2.50	Y52	2.60	CDR1
Y489	K99	4.20			CDR3
F490	A54	3.70			CDR2
L492	A54	3.70			CDR2
Q493	K99	3.10	D32	3.41	CDR3
S494	D32	2.80			CDR1
Y495	V101	3.80			CDR3
Q498	G26	3.30			CDR1
N501	H103	3.50			CDR3
G502	H103	3.30			CDR3
Y505	V101	2.86	H103	3.80	CDR3

Sb68+RBD (7KLW)

RBD	Sb68	DIST	Sb68	DIST	CDR
Y369	T32	3.30	Y103	3.50	CDR1
N370	N55	3.30	V54	3.90	CDR2
S371	N55	4.40			CDR2
A372	H57	3.20			CDR2
F374	Y103	2.90	Y59	3.40	CDR3
S375	A104	3.40	W105	2.80	CDR3
T376	A104	3.40	Y103		CDR3
F377	G102	3.40	Y103	2.90	CDR3
K378	D111	3.30	W101	3.40	CDR3
C379	W101	2.80			CDR3
V382	S29	4.30			CDR1
S383	A100	4.40			CDR3
P384	G101	3.60	A100	3.60	CDR3
T385	T32	3.70			CDR1
R408	H108	3.20	D111	2.60	CDR3

224 **Table S2A.** Interactions between RBD Residues and either ACE2 or sybody residues. In each
 225 rectangle (first column) is listed the identification of an RBD amino acid in contact with one or
 226 two residues of the indicted chain. Distance between residues is given in Å. For the sybodies, the
 227 particular CDR designation is also indicated.
 228

	370	380	390	400	410	420	
RBD	YNSASFSTFKCYGVSP	TKLNDLCFTNVYADSFVIRGDEV	RQIAPGQTGKIADYNYKLPDDF				
ACE2						*	
Sb16				*	*		*
Sb45							
Sb68	****	*****	***		*		
	445	450	460	470	480	490	500
RBD	VGGNYNYLYRLFRKSNL	KPFERDISTEIQAGSTPC	NGVEGFNCYFP	LQSYGFQPT	NGVGY		
ACE2	*	*	*	**	*	*	*
Sb16	***	*	**	*	**	**	**
Sb45	**	**	**	*	*	**	**

229 **Table S2B.** Tabulation of residues of RBD that contact each of the indicated chains, based on
 230 6M0J for ACE2–RBD, 7KGK for Sb16–RBD, 7KGJ for Sb45–RBD, and 7KLW for the Sb68–
 231 RBD interface. Note that ACE2 contacts broadly overlap those for Sb16 and Sb45, but that Sb68–
 232 contacts are distinct.
 233

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Complex (A + B)	BSA(\AA^2)	$N_{\text{res}}^{\text{A}}$	$N_{\text{res}}^{\text{B}}$	N_{HB}	N_{SB}	PDB	Res. \AA
ACE2+RBD	844	26	26	13	2	6M0J	2.45
Sb16+RBD	1003	26	29	8	1	7KGK	2.60
Sb45+RBD	976	27	33	15	4	7KGJ	2.10
*(Sb45)+RBD	1010	26	35	14	4	7KLW_B	2.60
*(Sb68)+RBD	640	21	17	9	4	7KLW_C	2.60
*(Sb45+Sb68)+RBD	1650	47	52	23	8	7KLW	2.60
*(VHH-E)+RBD	821	29	27	13	1	7KN5_C	1.87
*(VHH-U)+RBD	625	16	20	17	0	7KN5_E	1.87
*(VHH-E+VHH-U)+RBD	1446	45	47	30	1	7KN5	1.87
H11D4+RBD	599	20	20	11	4	6YZ5	1.80
H11H4+RBD	637	17	19	5	4	6ZBP	1.85
(CR3022)+RBD	991	19	20	7	4	6XC7_HL	2.88
VHH72+RBD	796	21	25	9	2	6WAQ_A	2.20
Nb20+RBD	705	22	21	9	4	7JVB	3.29
Nb6+Spike	788	24	21	9	1	7KKK_D	3.03
Sb23+Spike	772	21	22	5	0	7A25_D	3.06
Sb23+Spike	585	18	21	5	0	7A29_D	2.94
Ty1+Spike	795	23	26	3	0	6ZXX_D	2.93
C144+Spike	689	22	24	7	0	7K90_H	3.24
C002+Spike	728	22	23	7	1	7K8S_H	3.40
*(REGN10933)+RBD	935	28	30	9	1	6XDG_BD	3.90
*(REGN10987)+RBD	607	21	19	5	0	6XDG_AC	3.90

*(REGN10933+	1542	49	49	5	0	6XDG	3.90
REGN10987)+RBD							

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239 **Table S3.** Variety of Buried Surface Area (BSA) at interfaces of Sybodies, Nanobodies, and
240 Fabs with RBD or Spike. BSA of each of indicated interface was calculated from the relevant
241 chains using PISA (<https://www.ebi.ac.uk/pdbe/pisa/>), to account for the non-overlapping
242 surface area. BSA in Å². N_{res} indicates the number of residues in contact; N_{HB}, the number of
243 potential hydrogen bonds; N_{SB}, the number of potential salt bridges.

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