De novo design and Rosetta-based assessment of high-affinity antibody variable regions (Fv) against the SARS-CoV-2 spike receptor binding domain (RBD)

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Abstract
The continued emergence of new SARS-CoV-2 variants has accentuated the growing need for fast and reliable methods for the design of potentially neutralizing antibodies (Abs) to counter immune evasion by the virus. Here, we report on the de novo computational design of high-affinity Ab variable regions (Fv) through the recombination of VDJ genes targeting the most solvent-exposed hACE2-binding residues of the SARS-CoV-2 spike receptor binding domain (RBD) protein using the software tool OptMAVEn-2.0. Subsequently, we carried out computational affinity maturation of the designed variable regions through amino acid substitutions for improved binding with the target epitope. Immunogenicity of designs was restricted by preferring designs that match sequences from a 9-mer library of “human Abs” based on a human string content score. We generated 106 different antibody designs and reported in detail on the top five that trade-off the greatest computational binding affinity for the RBD with human string content scores. We further describe computational evaluation of the top five designs produced by OptMAVEn-2.0 using a Rosetta-based approach. We used Rosetta SnugDock for local docking of the designs to evaluate their potential to bind the spike RBD and performed “forward folding” with DeepAb to assess their potential to fold into the designed structures. Ultimately, our results identified one designed Ab variable region, P1.D1, as a particularly promising candidate for experimental testing. This effort puts forth a computational workflow for the de novo design and evaluation of Abs that can quickly be adapted to target spike epitopes of emerging SARS-CoV-2 variants or other antigenic targets.

KEYWORDS
antibody design, computational protein design, Fv antibody fragments, Ig variable region, neutralizing antibodies

1 | INTRODUCTION

The COVID-19 pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) still poses a threat due to the continued emergence of variants.1,2 Early on in the pandemic, convalescent
plasma extracted from SARS-CoV-2-infected patients was used as a treatment. Characterization of the plasma samples of convalescent individuals showed that the majority of the neutralizing antibodies (nAbs) target the receptor binding domain (RBD) of the spike protein indicating its high immunodominance. Structural studies of nAbs in complex with the RBD indicated antigenic sites throughout the RBD. These varied antigenic sites suggest that nAbs can have different mechanisms of antiviral action ranging from completely blocking receptor binding to only disrupting some interactions with the receptor. Newly emerging variants contain an increasing fraction of amino acid changes in the RBD that confer immune evasion rather than increased affinity with the receptor protein hACE2 (human receptor angiotensin converting enzyme). Furthermore, as newer immune evading variants emerge, existing Abs for both therapeutic and serological testing appear to be less effective. For example, amino acid changes at the positions Phe456 and Glu484 on the RBD (present in Beta, Gamma, and Omicron variants) have been shown to prevent neutralization by monoclonal Abs and convalescent plasma. Moreover, a recent study showed that SARS-CoV-2 can completely escape from highly neutralizing COVID-19 convalescent plasma after several months of laboratory evolution. This escape can occur due to mutations and the presence of glycans in the N-terminal domain and the RBD of the virus. Therefore, the continued emergence of immune evading variants motivates the development of faster and cheaper methods for targeted design of nAbs.

Monoclonal Abs derived from both convalescent plasma and phage display libraries have shown excellent neutralizing properties against SARS-CoV-2 in both animal models and live-virus or pseudovirus assays. Some potential Ab-based therapeutics have also shown effectiveness in randomized clinical trials. Several hundred of these Abs have been isolated and characterized through detailed biochemical and structural studies (refer reviews for a detailed discussion of existing Abs targeting SARS-CoV-2 antigens). Several computational studies have focused on the redesign of existing Abs against other coronaviruses toward better binding with SARS-CoV-2 RBD using machine learning, structure guided mutagenesis, and CDR grafting approaches. In addition, computationally designed peptide inhibitors have shown promise in preventing SARS-CoV-2 infection in mammalian cells. However, studies that perform structure guided design of fully de novo high-affinity Abs against specific epitopes of SARS-CoV-2 spike protein are still lacking. Motivated by this shortcoming, here we explore the de novo design of Ab variable regions targeting the most solvent-exposed residues of the spike protein that are also part of the residue contact map involved in hACE2 binding, and trade-off binding energy against human sequence content in the variable region. We focus on hACE2 binding because Ab designs which may interfere with hACE2-binding regions on the RBD could be neutralizing by disrupting RBD–hACE2 interaction and hence block SARS-CoV-2 cell entry. Our goal is to exhaustively explore the sequence space of all possible variable region designs using the Ab design software OptMAVeN-2.0 and report a range of diverse solutions that can serve as potentially nAbs. We find that many different combinations of VDJ genes followed by affinity maturation can yield potentially high-affinity variable regions (scored using the Rosetta energy function) against an epitope of the spike protein RBD. Pareto optimal designs with respect to binding affinity versus human content were drawn and five affinity-matured designs are detailed in the results section. By grafting the designed heavy (VH) and light (VL) chain variable regions onto a human framework region, high-affinity and potentially neutralizing full-length monoclonal Abs can also be constructed. In addition, we describe a Rosetta-based computational evaluation of our top five designs where we compared the designs generated with OptMAVeN-2.0 to structural models generated by DeepAb and Rosetta to help identify the most promising designs prior to experimental testing.

### 2 | METHODS

#### 2.1 | Ab design in OptMAVeN-2.0

The initial Ab variable domain sequences were predicted using de novo Ab design software tool, OptMAVeN-2.0. Using an interatomic clash cut-off of 1.25 Å, 173 antigen poses were sampled, and each of which yielded a successful (not necessarily unique) Ab design targeted at the seven most solvent accessible hACE2-binding residues of SARS-CoV-2 spike RBD.

Before identifying Ab sequences complementary to the epitopes, OptMAVeN-2.0 first minimizes the z-coordinate of the epitopes, with their collective centroid set at origin, to allow the de novo designed Ab regions (see Supporting Information S1 for link to the entire MAPs fragment library) to bind from the bottom. Next, an ensemble of starting antigen poses is generated by a combination of discrete rotations (about the z-axis) and translations (in x, y, and z)—each of which are subsequently passed into the Ab design step. We started out with 3234 such antigen poses for the SARS-CoV-2 spike protein with the epitopes occupying the most negative z-axis coordinates.

#### 2.2 | Affinity maturation design in Rosetta

The affinity maturation protocol consisted of an initial refinement of the complex by RosettaDock followed by three iterations of backbone perturbation using RosettaBackrub, interface design using RosettaDesign, and rotamer repacking of the complex using a Monte Carlo based simulated annealing algorithm. During the Rosetta affinity maturation, only amino acids in the variable region within 5 Å from any epitope residue were allowed to mutate. Each affinity-matured designed complex was relaxed using FastRelax (with constraints) 10 times and energy minimized (using Minimize). For each of these relaxed poses, the binding energy (dG_separated) was calculated using the InterfaceAnalyzer application. The entire protocol was implemented in RosettaScripts using the REF2015 energy function (see Supporting Information S7). This computational protocol was executed for 8000 affinity-matured sequence-design cycles. The top five variable region designs which showed strongest interaction
energy scores with the viral spike and low immunogenicity (high H-scores) were further investigated to glean insight on the biophysics of interactions at the residue level.

2.3 | Ab modeling

Structural models of Abs were generated using DeepAb\(^{35}\) and ABODY-BUILDER.\(^{44}\) DeepAb is a machine learning-based method that utilizes a deep residual convolutional network (AbResNet) to predict Fv structure.\(^{35}\) The Ab models were generated using the heavy and light chain sequences from the top OptMAVEN-2.0\(^{33}\) designs as input. ABODY-BUILDER was similarly used to generate template-based models through the SAbPred webserver.\(^{45}\)

2.4 | Ab–antigen docking

Models of Abs in complex with the SARS-CoV-2 RBD were generated using SnugDock\(^{46,47}\) to predict the local energy landscape around the designed binding site. ref2015 was used as the default energy function for all Rosetta-based applications. The OptMAVEN-2.0 designs were minimized into the Rosetta energy function as described.\(^{48}\) The lowest scoring DeepAb model for each prototype was similarly docked using SnugDock. Docking consisted of a spin around the Ab–Ag center-of-mass axis with uniform sampling of degrees 0°–360°. Random perturbations of translations and rotations were sampled and applied from Gaussian distributions centered at 3 Å and 8°, respectively. Docking included refining the CDR H2 and H3 loops through kinematic loop closure and refining the VL–VH orientation through VL–VH docking in addition to Ab–antigen docking. One thousand models were generated for each docking run, of which the top 10 decoys were chosen based on low Rosetta energy. All structures are numbered using the Chothia numbering scheme.

3 | RESULTS

We first performed solvent accessibility analysis using the STRIDE\(^{49}\) program on the 21 hACE2-binding residues of the SARS-CoV-2 spike protein (S-protein) RBD to define our binding epitope. The top seven residues with the highest solvent accessibility scores (i.e., SAS) are (Arg346, Phe347, Ala348, Tyr351, Ala352, Asn354, and Arg355) comprising our binding epitope (see Figure 1). Furthermore, the epitope is accessible for binding to RBD in the open conformation of the full spike protein (see Figure S9).

We next used OptMAVEN-2.0 to computationally identify the combination of VDJ genes forming the variable region that best binds the desired epitope. Previously, OptMAVEN\(^{52}\) has been used successfully to design five high-affinity CDRs against a FLAG tetrapeptide,\(^{53}\) three thermally and conformationally stable Ab variable regions (sharing less than 75% sequence similarity to any naturally occurring Ab sequence) against a dodecapeptide mimic of carbohydrates\(^{54}\) and two thermostable, high-affinity variable heavy chain domains (V\(_H\)) against α-synuclein peptide responsible for Parkinson’s disease.\(^{55}\) All these designs were experimentally constructed and nanomolar affinities for their respective target antigens was demonstrated.

Through a combination of rotations and translations, OptMAVEN-2.0 identified 3,234 unique antigen poses that presented the epitope to the Ab differently. The combinatorial space of different VDJ genes

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**FIGURE 1** (A) The SARS-CoV-2 spike trimer structure in RBD-open conformation (PDB-id: 6VYB) superposed with human-ACE2-RBD complex (PDB-id: 6LZG). One of the trimer units is shown in cartoon representation color coded as per the schematic in (B). NTD, N-terminal domain; RBD, receptor binding domain; S1/S2, ends of S1/S2 cleavage site; FP, ends of fusion peptide. (C) Zoomed-in view of the human-ACE2-RBD complex from (A). ACE2’s active-site residues and the target epitope residues on the RBD are shown as viewed from the front in inset boxes (top and bottom, respectively). The numbering scheme for the ACE2 and RBD residues is the same as that in the crystal structure 6LZG (rcsb.org/structure/6LZG or 6WV1\(^{29}\) and 6MOJ\(^{31}\)).
that upon recombination form the variable region of the prototype Ab was informed by the MAPs database of Ab parts. MAPs (see Supporting Information S1 for link to full database) contains 929 modular Ab (i.e., variable—V*, complementarity determining—CDR3, and joining—J*) parts from 1168 human, humanized, chimeric, and mouse Ab structures (last updated in 2013). MAPs follows the Ab parts residue numbering convention as listed in the International ImmuNoGeneTics (IMGT) database. IMGT catalogs Ab parts as variable (V), diversity (D) and joining (J) structure libraries. MAPs stores complete CDR3 parts, C-terminus-shortened V parts (i.e., *V* parts), and N-terminus-shortened J parts (*J* parts). Note that CDR3 includes the entire D gene and also up to the C-terminus of the V gene and up to the N-terminus of the J gene. In the remainder of the manuscript, the list of parts used to design the variable region are referred to as CDR3, *V*, and *J* parts.

For each one of the 3234 spike poses, OptMAVEn-2.0 identified a variable region combination composed of end-to-end joined V*, CDR3, and J* region parts that minimized the Rosetta-binding energy between the variable region and spike epitope formed by the seven residues. As part of OptMAVEn-2.0, the combinatorial optimization problem was posed and solved as a mixed-integer linear programming problem using the cplex solver. The solution of this problem identifies, for each one of the spike poses, the complete design of the variable region using parts denoted as HV*, HCDR3, HJ* for the heavy chain H and L/KV*, L/KCDR3 and L/KJ* for the light chain L/K. Only 173 antigen-presenting poses out of 3234 explored yielded nonclashing antigen–Ab complexes. These 173 poses were ranked based on their Rosetta-binding energies with the spike epitope and classified into 27 clusters (using k-means) in a 19-dimensional space defined by quantiative descriptors of sequence similarity, three-dimensional spatial pose, and the angle at which they bind to the target epitope (see details in original paper). The top five prototype designs with the highest Rosetta-binding energies were present in four clusters and spanned a highly diverse set of choices of MAPs parts (see Table 1) with minimal conservation of the same part among the five prototype designs. The number entries in Table 1 correspond to the id of the gene in the MAPs database (which are identical to the ids used in IMGT). Note that the design P5 uses a lambda (L) light chain instead of a kappa (K). Figure 2A plots the pairwise sequence similarity scores of the five Ab variable domains that were used in the top five designs. As expected, the top five prototype designs P1, P2, P3, P4, and P5 are the most dissimilar in their respective CDR3 domains in both light L, heavy H, and HV* domain (but not LV*). They are the most similar in the choice of parts for the J* domains (see Figure 2A) reflecting the lack of diversity among possible choices for the J* domains in the MAPs database.

Inspection of the noncovalent interactions formed by the design P1 with the spike epitope revealed (Figure 3A) both electrostatic (22 hydrogen bonds) and hydrophobic contacts at the interface (Supporting Information S3a). Lys356 and Asn354, Thr353, Tyr351, Arg454, Arg457 of RBD form two important clusters of interactions with light chain and heavy chain residues of P1, respectively (see Figure 3B,C). Notably, RBD residues Thr353 and Asn354 have been deemed important for hACE2 binding. Similarly, the designs P2 through P5 capture the RBD by multiple hydrogen bonds, hydrophobic interactions, and salt-bridges (Supporting Information S3b–e).

We next applied Rosetta-based in silico affinity maturation (see Section 2) for each one of the five prototype designs shown in Table 1 to further improve noncovalent binding interactions between the Ab variable domains and the SARS-CoV-2 spike RBD. This computationally mimics the process of somatic hypermutation leading to eventual affinity maturation of naive Abs in B cells. This procedure identified a total of 124 unique variable designs by introducing mutations in the five prototypes (see Figure 4A). We retained 106 designs of these which achieved both an improvement in the Rosetta-binding energy over their respective prototype sequences and further stabilization (i.e., lower total Rosetta energy) of the antigen–Ab complex (see upper right quadrant of Figure 4A). On average, upon affinity maturation, the binding energy was improved by ~14 kcal/mol and the total energy of the complex was improved by ~37 kcal/mol. Supporting Information File S2 lists first the starting prototype design (i.e., P1, P2, P3, P4, or P5) followed by the 106 affinity-matured designs (labeled as P1.D1, P1.D2, etc). On average, there were 4.5 mutations (Supporting Information S4) between computational affinity-matured and prototype variable region designs.

We then assessed the departure of the 106 affinity-matured variable regions from fully human Ab sequences using H-Score. H-score is defined as the sum of the minimum edit distance between all possible 9-mer fragments in the designed variable region from a library of all 9-mer sequences (prepared in T Li et al. observed in human Abs. The value of H-score is scaled to 100 and normalized by the length of the sequence. Between two sequences, the sequence with greater human Ab sequence content will have a higher value of H-score and vice versa. Figure 4B illustrates the trade-off between the Rosetta-binding energy versus H-score for these affinity-matured variable region designs. For comparison, we calculated the H-score for the human Abs CR3022, 80R, 62R, and M396 which are known to be neutralizing against SARS-CoV-1. These Abs (including only Fv regions) have an average H-score of 62.98 (st dev: 4.9) which are in the same range as our most human designs (Figure 4B,C). Note that one could use a 9-mer sequence library prepared using Ab sequences relevant to a particular application (here, anti-RBD Abs), and hence tune the H-score calculation to obtain perfect scores (close to 100) for known anti-RBD human Abs.

We selected five designs that were on the Pareto optimum curve shown in Figure 4B. The Pareto optimum curve is defined as the collection of designs for which no other design exists that can simultaneously improve upon both criteria (i.e., Rosetta-binding energy and H-score). Designs P1.D1 and P1.D2 shown in blue (in Figure 4B) have the lowest Rosetta-binding energies whereas P3.D1 and P3.D3 shown in yellow correspond to the ones with the highest H-scores. Design P4.D1 is an intermediate design that balances both binding energy and H-score. The lowest binding energy designs (P1.D1, P1.D2), irrespective of H-score, would be relevant in ELISA-based in vitro detection assays whereas the lowest H-score designs (P3.D1, P3.D3) may offer the highest potential as therapeutic Abs. In addition, we
calculated the Rosetta-binding energy between the human CR3022\textsuperscript{65} (anti-SARS-CoV-1 Ab) and the SARS-CoV-2 spike protein RBD using complex structure (PDB-id: 6W41) to be \(-56.4\) kcal/mol which is very close to the Rosetta-binding energy of designs P1.D1 and P1.D2. However, P1.D1 and P1.D2 bind a different epitope on the spike RBD than the one that CR3022 targets (see Figure S8). Supporting Information File S2 contains the list of Rosetta energies and H-scores for all 106 affinity-matured designs.

Figure 5 shows the sequence alignment of the five selected affinity-matured sequences (i.e., P1.D1, P1.D2, P4.D2, P3.D1, and P3.D3). A total of 156 out of 226 aligned positions are conserved among all designs. Table 2 lists the most important (strongest) contacts with the spike protein as informed by an in silico alanine scanning (Table S5) on the spike-binding residues of the variable region designs. In essence, the alanine scanning analysis identifies the loss in binding energy that is incurred upon mutating each residue (one at a time) to alanine.

Abs that strongly bind to the RBD but do not inhibit hACE2 binding have been shown to be neutralizing for SARS-CoV-2 \(47\text{D11}\textsuperscript{66}\) and for SARS-CoV-1 (CR3022 in combination with CR3014).\textsuperscript{61} The mechanisms of neutralization of such Abs are not completely known.\textsuperscript{66,67} It is possible that upon binding, these Abs perturb the interaction network of the RBD with hACE2 thereby rendering RBD-hACE2 binding less effective. Using the cryo-EM structure of 47D11 (PDB-id: 7AKD\textsuperscript{67}) in complex with RBD, we found that the Rosetta-binding energy is \(-52.56\) kcal/mol which is close to the energy of our best-binding designs (Figure 3C). In addition, Wrapp et al.\textsuperscript{68} showed that nanobody VHH-72 raised against SARS-CoV-1 had a neutralizing effect despite binding to an epitope that does not overlap with the hACE2 residue binding domain. By fusing VHH-72 with a human IgG1 they demonstrated SARS-CoV-2 neutralizing activity. They hypothesized that binding of the nanobody with the trimeric spike protein may disrupt conformational dynamics and consequently prevent binding to hACE2.

**Table 1** V\*, CDR3, J\* part numbers chosen from the MAPs\textsuperscript{36} database for the top five prototype variable region designs and their corresponding Rosetta-binding energies\textsuperscript{34,42}

| Prototype design | Modular antibody parts number chosen in each design | Antigen pose (rotation of epitope about vertical axis) | Rosetta-binding energy (kcal/mol) |
|------------------|-----------------------------------------------------|-------------------------------------------------|----------------------------------|
| P1               | HV* 82, HCDR3 315, HJ* 5, L/ KKV* 61 (K), L/ KCDR3 4 (K), L/ KJ* 3 (K) | 0° | \(-36.77\) |
| P2               | HV* 52, HCDR3 94, HJ* 1, L/ KKV* 61 (K), L/ KCDR3 17 (K), L/ KJ* 3 (K) | 0° | \(-27.57\) |
| P3               | HV* 105, HCDR3 12, HJ* 5, L/ KKV* 6 (K), L/ KCDR3 23 (K), L/ KJ* 4 (K) | 300° | \(-29.20\) |
| P4               | HV* 79, HCDR3 204, HJ* 1, L/ KKV* 2 (K), L/ KCDR3 1 (K), L/ KJ* 4 (K) | 240° | \(-19.78\) |
| P5               | HV* 108, HCDR3 212, HJ* 1, L/ KKV* 37 (L), L/ KCDR3 5 (L), L/ KJ* 5 (L) | 360° | \(-38.62\) |

**Note:** Antigen poses are described with the angle that the vertical axis through the epitope (shown in pink) centroid and the C\beta carbon of the residue with greatest z-axis coordinate forms.
In comparison, our design P1.D1 forms strong contacts (see Table 2) with many residues of the RBD which in turn also indirectly interact with hACE2 (see Figure 6). For example, residues L455 and T470 of the RBD are in contact with both hACE2 contacting RBD residues Y449, F490 and P1.D1 contacting RBD residues Y351, I465. By perturbing the inter-residue interaction network of RBD-hACE2 a neutralizing effect can be achieved.

Next, we used Rosetta’s SnugDock application to computationally predict if the designed binding complexes can be recovered through Rosetta’s Monte Carlo approach to local docking. Predicted binding energy was evaluated by plotting the Rosetta interface score—an estimate for binding energy—against the interface RMSD when calculated from the designed OptMAVEn-2.033 structure. We considered an Ab to be a more likely binder if these plots show that models similar to the design (low-RMSD) have distinctly lower interface scores than the bulk of the models (Figure 7A,D). To assess whether SnugDock was able to find energy minima, we calculated the interface RMSD from the input structure. We evaluated the top five lowest binding energy designs overall and the top two lowest binding energy designs for each prototype based on Rosetta-binding energy. Figure 2 (A) Pairwise sequence similarity percentages between the members of the six parts that were used to construct the top five prototype variable regions with the lowest Rosetta-binding energies with the viral spike epitope. (B) The amino acid sequence of prototype design P1 with the different domain parts of heavy chain (HV, HCDR2, HJ) and light chain (KV, KCDR3, KJ) labeled below.

Figure 3 (A) The SARS-CoV-2 spike trimer structure in RBD-open conformation superposed with human-ACE2-RBD complex and the antibody design P1.D1; RBD and NTD are shown in orange and pink cartoons, antibody is shown in green cartoon and ACE2 is shown in blue surface. (B) Structural view of the interface between the RBD (shown in pink) and antibody design P1 with amino acid residues forming representative noncovalent interactions shown as stick representations. Both light (shown in pale green) and heavy chains (shown in green) of the antibody contribute to the interface with the RBD. The insets show zoomed-in views of the interactions formed at the heavy chain (top) and light chain interfaces, respectively.
The top five designs produced the most promising energy funnels. These designs were all derived from the P1 prototype, of which P1.D1 was the top design overall and produced the deepest energy funnels (Figures 7A and S10). To quantify the quality of the funnel, we use the N5 metric, which is the number of top five lowest scoring decoys within 4.0 Å from the designed structure. We consider an N5 of 3 or above a good funnel. For P1.D1 we observed an N5 of 3 (Figure 7A). Structural visualization of the lowest scoring models and the designed structure further confirmed highly similar binding modes for the design P1.D1. Seven of the top 10 lowest scoring decoys appear to agree in orientation with each other and with the designed structure (see Figure 7B).

By contrast, P3.D1 shows less promising energy funnels (Figure 7C) with an N5 of 0, which is consistent with the fact that the
initial OptMAVEn-2.0 P3 designs had a less favorable Rosetta-binding energy than the top P1 designs (Table 1). The structures revealed vastly different binding modes between the low energy SnugDock models and the designed structure, with only partly overlapping footprints, and only moderate agreement among the low scoring models (Figure 7D). Therefore, this design is unlikely to bind in the location designed for by OptMAVEn-2.0.

Analysis of polar interactions for P1.D1 showed that all the top 10 lowest scoring SnugDock structures include at least one hydrogen bond between the desired RBD epitope and Ab, while eight of these structures include at least three hydrogen bonds. Although lower than the number of hydrogen bonds found in the MD simulations involving P1.D1, this is still a promising number of bonds since it exceeds the average number of hydrogen bonds between hACE2 and the RBD.50 A hydrogen bond involving Tyr351 sidechain of the desired RBD epitope and Leu68 backbone of the Ab is conserved between the OptMAVEn-2.0 structure and six of the top 10 lowest scoring SnugDock structures (Figures 7C and S11). One additional SnugDock structure engages Tyr351 with Gly65 instead of Asn354 (Figure S11F). Similarly, a hydrogen bond between the Arg346 sidechain of the epitope and the Asp110 backbone of the Ab is conserved in seven out of the top 10 lowest scoring SnugDock structures (Figures 7F and S11). Two additional SnugDock structures predict an H-bond between Arg346 and the Phe111 backbone or the Gln27 sidechain instead (Figure S11E,I). In addition, many of the SnugDock lowest scoring structures include hydrogen bonds not found in the OptMAVEn-2.0 structure, some of which involve Ab sidechains. The notable ones are a hydrogen bond involving the sidechain of Ab light chain Trp135 and the sidechain of epitope Asn354 (Figure S11B), and a bond involving the sidechain of Ab heavy chain Tyr69 with the sidechain of epitope Ala352 (Figure S11A,D,H,I).

We also carried out an all-atom molecular dynamics (MD) simulation of the best-binding design P1.D1 in complex with the RBD of the SARS-CoV-2 spike protein to assess the stability of the complex. Preliminary results for a 50 ns trajectory counted an average of approximately four hydrogen bonds (sd dev: 1.5) present at the Ab–antigen interface (Figure S6). This is quite encouraging, as in an

| Matured antibody design id | Interface residue from antibody | Interacting spike residue(s) | Loss in binding energy upon mutation to alanine (kcal/mol) | Corresponding variable region |
|---------------------------|---------------------------------|-----------------------------|----------------------------------------------------------|-----------------------------|
| P1.D1                     | G62                             | A352                        | 2.13                                                     | HCDR3                       |
|                           | G63                             | Y351                        | 2.13                                                     | HV*                         |
|                           | L65                             | Y489                        | 1.26                                                     | HV*                         |
|                           | Y66                             | S349                        | 0.53                                                     | HV*                         |
|                           | I56                             | C488                        | 0.94                                                     | HV*                         |
| P1.D2                     | F109                            | K356                        | 2.77                                                     | KCDR3                       |
|                           | T65                             | Y351                        | 1.42                                                     | HV*                         |
|                           | Y66                             | A348, S349                  | 1.06                                                     | HV*                         |
|                           | I56                             | C488                        | 0.94                                                     | HV*                         |
|                           | S57                             | A352                        | 0.71                                                     | HV*                         |
| P4.D1                     | A56                             | L452                        | 0.573                                                    | KCDR3                       |
|                           | D35                             | R357                        | 0.08                                                     | KV*                         |
|                           | G28                             | T478, G482                  | 0.01                                                     | HV*                         |
|                           | T85                             | N481                        | 0.00                                                     | KV*                         |
|                           | L67                             | V445                        | 0.00                                                     | KV*                         |
| P3.D1                     | W64                             | A352                        | 2.30                                                     | HV*                         |
|                           | N57                             | N354                        | 0.65                                                     | HV*                         |
|                           | F107                            | T345                        | 0.57                                                     | KCDR3                       |
|                           | S29                             | E340                        | 0.14                                                     | HV*                         |
|                           | S108                            | R346                        | 0.12                                                     | KCDR3                       |
| P3.D3                     | N57                             | N354                        | 0.99                                                     | KV*                         |
|                           | F107                            | T345                        | 0.33                                                     | KCDR3                       |
|                           | D38                             | R346                        | 0.18                                                     | KV*                         |
|                           | S29                             | E340                        | 0.22                                                     | HV*                         |
|                           | Q106                            | R346                        | 0.03                                                     | KCDR3                       |

Note: For each contact, the corresponding amino acid residues from the antibody and RBD are listed along with the loss in binding energy upon mutating the antibody amino acid to alanine.
earlier study, MD simulation of the hACE2 receptor in complex with the spike protein RBD reported an average of only 2.7 hydrogen bonds at the interface. This implies that this design has the potential to competitively bind the RBD of the SARS-CoV-2 spike protein potentially sequestering it from hACE2. This is also corroborated by the Rosetta-binding energy value of around \(-48.3\) kcal/mol calculated for the spike protein RBD with hACE2 (from PDB-id: 6lzg) which is weaker by over 7 kcal/mol compared to designs P1.D1 and P1.D2. Finally, it is important to stress that our designs rely on the accuracy of the Rosetta energy function to recapitulate experimental affinities and that carrying out experimental binding assays are needed to confirm or refute these findings. However, since protein folding requires MD simulations of microsecond timescales, they are unsuitable for validating if the Ab sequences fold as predicted by OptMAVEn-2.0 in a high-throughput manner.

We hence used DeepAb, a deep learning method for predicting Ab structures from sequence and SAbPred, which uses the ABody-Builder algorithm to generate structural models of the designed sequences. The models produced by these methods were aligned to the H3 loop of the OptMAVEn design using PyMOL (Figure 8). For the P1.D1 design, there was a poor alignment among all three structures and the H3 loop RMSD between the OptMAVEn-2.0 structure and the DeepAb structure was 2.6 Å. In contrast, the results for the P3.D1 design showed an excellent alignment between the DeepAb and OptMAVEn models, with an H3 RMSD of 0.5 Å, and a decent agreement with the SAbPred model. Thus, the designed sequence for P3.D1 is likely to fold into the intended structure, whereas the P1 designs may fold into alternative conformations, particularly in the CDR H3 loop.

In addition, we redocked the Abs from DeepAb models to the RBD using SnugDock and generated funnel plots. None of the DeepAb docking models resulted in converging energy funnel plots (Figures 8B,D and S13). Furthermore, all the DeepAb docking models had higher interface binding energies compared to the corresponding OptMAVEn-2.0 structures (Figure S14). Visual inspection of the low scoring models showed a variety of binding sites and orientations for the low energy models, suggesting that no clear energy minimum was found (see Figure 8C,F).

4 | DISCUSSION

In summary, the goal of this computational analysis was to assess the range of possible Ab designs that can affect binding with the viral spike protein by interacting with residues involved in hACE2 binding. We reported on de novo prototype variable regions targeting the most solvent accessible seven-residue epitope in the spike RBD and their (computationally) affinity-matured sequences with the lowest Rosetta-binding energies. Designs were rank ordered not only in terms of their Rosetta-binding energy but also their humanness score metric (H-score). We reported complete amino acid sequences for the 106 affinity-matured designs as well as the five prototype sequences and V*, CDR3, and J* parts used. Importantly, we would like to note that high affinities of designed Abs, as modeled using the Rosetta-binding energy function, need not necessarily translate to therapeutic effectiveness. De novo Ab design methods require considerable screening of variants to isolate a high-affinity binder. Therefore, in addition to the OptMAVEn-2.0 protocol, we described a
workflow for computational quality control and validation of designed sequences to be used before experimental testing. This workflow includes “forward folding” of the designed Ab sequences and docking of the Abs to their antigen. These steps seek an energetic minimum of the Ab structure and the Ab–antigen complex. Designed structures and binding sites that agree with the predicted energetic minima are more promising candidates for experimental testing. Other successful computational protein design studies targeting antigen epitopes have included forward folding and docking tests to prioritize candidates for testing. This approach allows us to optimize the Ab design process by efficiently identifying promising Ab designs in silico. However, the evaluation method depends on the accuracy of the Rosetta energy function but this limitation in accuracy is compensated for by the speed and low cost allowing for the evaluation of many potential binders simultaneously. Furthermore, we compared the RBD residues that form noncovalent interactions with the Ab chains for five of our designs P1.D1, P2.D1, P3.D1, P4.D1, and P5.D1 with those of a set of 70 nonredundant anti-RBD Abs taken from the CoV3D database. Of these, we found only four Abs with PDB-ids 6XKP, 7K8T, 7JX3, and 7CDJ that had around 35% common contacting RBD residues compared to our designs. All other Abs had lesser overlap of contacting residues indicating the novelty of our designs.

By analyzing our designed SARS-CoV2-RBD binders, we found that P1.D1 design binding sites were most efficiently recovered during local docking. More specifically, the P1.D1 design yielded the highest number of low energy structures which were within 1 Å of the original design. Similarly, the P1.D3 and P1.D4 models both yielded low energy docking structures within 1 Å of their respective designs. This suggests that the P1.D1, P1.D3, and P1.D4 designs are most likely to have an energetic minimum at their spike protein binding site and are the most promising for testing in vitro.

To probe the structural predictions of the OptMAVEn-2.0 designs, we generated Ab Fv structural models with DeepAb and ABodyBuilder. Since Ab structure prediction is generally reliable for non-H3 CDR loops and framework regions we focused on the CDR H3 loop conformations. The P3 models produced with DeepAb had a CDR H3 loop conformation similar to the ones originally predicted by OptMAVEn-2.0. We were not, however, able to recover CDR H3 conformations of any other OptMAVEn-2.0 designs within 1 Å when using DeepAb (Figure 7D). These results suggest that the P3 models are most likely to fold into the intended structure among the prototype designs.
tested. Conversely, we were unable to recover H3 conformations of the P3 models or any of the other OptMAVEn-2.0 designs with ABodyBuilder. The difference in the CDR H3 loop structures may arise from the uncertainty of the DeepAb prediction.35 Alternately, since OptMAVEn-2.0 incorporates the target antigen before relaxing whereas DeepAb and SAbPred are blind to the antigen, the conformation difference could simply reflect the conformational change upon binding. Finally, the OptMAVEn-2.0 designed structures may deviate because it is over-weighting the interfacial interactions relative to the Ab structure and stability, as most CDR H3 loops change little upon binding.76

Among the top five prototype designs presented in this work, we have not identified a design that is predicted to both fold into the designed structure and bind at the designed location when stratifying our results with ABodyBuilder, DeepAb, and Rosetta SnugDock. In agreement with this observation, we were not able to recover the designed complex structure for any of the top five prototype designs tested. These results suggest that additional designs may need to be screened to identify successful high-affinity RBD binding Abs. The fact that we were able to isolate designs predicted to bind as intended and other designs predicted to fold as intended, suggests that our workflow for Ab analysis may be an effective screening tool for in silico assessment of computational Ab designs. In summary, our computational pipeline for design and validation of de novo Ab variable regions provides a time-efficient approach to shortlist promising Ab designs against choice epitopes which can be further tested experimentally. Our work also contributes to the ongoing development and improvement of Ab design algorithms and computational validation approaches.77

FIGURE 8  Computational evaluation of best-binding designs by prediction of Fv structures. (A) P1.D1 H3 loop structures generated by OptMAVEn-2.0 (red), DeepAb (pink) and SAbPred (blue). (B) Plot of interface score versus interface RMSD (with respect to OptMAVEn-2.0 structure) from docking the DeepAb Fv model with the RBD. (C) P1.D1-RBD complex generated with OptMAVEn-2.0 along with the top 10 lowest scoring decoys (yellow light chain, pink heavy chain) obtained from docking model (antibody chains of the docked decoys and OptMAVEn-2.0 complex are shown in transparent and solid cartoon representations respectively whereas the RBD is shown in surface representation). (D) Similar to A, featuring design P3.D1 (E) Similar to B, featuring design P3.D1 (F) Similar to C, featuring design P3.D1

AUTHOR CONTRIBUTIONS
Ratul Chowdhury designed and performed the OptMAVEn experiments. Veda Sheersh Boorla designed and performed the affinity maturation experiments, MD simulations, and analyses of designs. Ranjani Ramasubramanian, Brandon Ameglio, and Rahel Frick performed the structure prediction and docking methods. Veda Sheersh Boorla, Ratul Chowdhury, Costas D. Maranas, Ranjani Ramasubramanian, Brandon Ameglio, Rahel Frick and Jeffrey J. Gray wrote the manuscript.

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CONFLICTS OF INTERESTS
Jeffrey J. Gray is an unpaid board member of the Rosetta Commons. Under institutional participation agreements between the University of Washington, acting on behalf of the Rosetta Commons, Johns Hopkins University may be entitled to a portion of revenue received on licensing Rosetta software including applications mentioned in this
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**DATA AVAILABILITY STATEMENT**
The data that supports the findings of this study are available in the supplementary material of this article.

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