Affinity maturation of cross-reactive CR3022 antibody against the receptor binding domain of SARS-CoV-2 via *in silico* site-directed mutagenesis

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Abstract

Introduction: Computational antibody engineering, affinity maturation, and screening greatly aid in vaccine and therapeutic antibody development by increasing the speed and accuracy of predictions. This study presents a protocol for designing affinity enhancing mutants of antibodies through *in silico* mutagenesis. A SARS-CoV-2 cross-reactive neutralizing antibody, CR3022, is considered as a case study.

Methods: Our study aimed at generating antibody candidates from the human antibody CR3022 (derived from convalescent SARS patient) against the RBD of SARS-CoV-2 via *in silico* affinity maturation using site-directed mutagenesis in mutation hotspots. We optimized the paratope of the CR3022 antibody towards the RBD of SARS-CoV-2 for better binding affinity and stability, employing molecular modeling, docking, dynamics simulations, and molecular mechanics energies combined with generalized Born and surface area (MM-GBSA).

Results: Nine antibody candidates were generated post *in silico* site-directed mutagenesis followed by preliminary screening. Molecular dynamics simulation of 100 nanoseconds and MM-GBSA analysis confirmed L-K45S as a lead antibody with the highest binding affinity against the RBD compared to wild-type and mutant counterparts. Three out of the remaining mutants were also found to have distinct epitopes and binding, possessing a potential to be developed against emerging SARS-CoV-2 variants of concern.

Conclusion: The study demonstrates the use of an integrative antibody engineering protocol for enhancing affinity and neutralization potential through mutagenesis using robust open-source computational tools and predictors. This study highlights unique scoring and ranking methods for evaluating docking efficiency. It also underscores the importance of framework mutations for developing broadly neutralizing antibodies.

Key Points

- Optimization and design of *in silico* antibody affinity maturation protocol using open-source or freely accessible web servers and software.
- A unique intra-system ranking parameter was used for the selection of a representative pose post-docking via $\log(|\Delta G/Kd|)$ plots.
- Among the nine framework mutations, one mutant candidate exhibits increased binding affinity, while three other mutants exhibit distinct epitope positions on SARS-CoV-2 RBD suggesting a potential binding towards the virus's emerging variants.
- This study affirms the importance of framework mutations in the development of broadly neutralizing antibodies.

1. Introduction

Antibodies represent the most versatile group of binding molecules. The modularity of their structure provides unique leverage for engineering intervention and optimization. Reliable exploitation of antibodies from a therapeutic standpoint depends on efficient ways to develop these molecules, assess their potential for production, and approximate their efficacy for binding and neutralization functions.

An avenue for the significant contribution of computational approaches to antibody engineering is the rational design of antibody structure and function. Modeling followed by assessing stability and binding upon mutagenesis is an essential aspect served by rational design in achieving computational affinity maturation of therapeutic antibodies [1].

Computational approaches to antibody design and selection before arduous experimental production owe their robustness and reliability to the promise of faster results along with sensitive and accurate predictions, both relative and absolute.

Several structural bioinformatics utilities such as homology modeling, protein-protein docking, protein interface prediction aid in rational antibody design. However, certain hurdles concerning the need for antibody-antigen specific docking, scoring, and ranking while balancing stability-specificity trade-offs impede computational approaches for developing biologically optimized antibodies.

This study presents a methodology for performing affinity maturation of antibody variable domain using robust open-source webserver that integrate multiple computational data-driven approaches and directed evolution mutagenesis. We also introduce parameters for scoring complexes post docking that can be employed to screen and select lead antibodies. Molecular dynamics simulations have also been performed to study the antibody candidates' temporal stability, and conformational behavior, followed by MM-GBSA analysis to estimate endpoint binding free energies and establish successful results post-affinity maturation.

To demonstrate the protocol, this study performs *in silico* affinity maturation of CR3022 convalescent antibody via site-directed mutagenesis towards the S-protein receptor binding domain (RBD) of SARS-CoV-2, the causative agent of COVID-19. CR3022 is a cross-reactive neutralizing antibody initially derived from a SARS-CoV convalescent patient. Studies by Yuan et al. elucidated conserved epitopes of CR3022 between SARS-CoV and SARS-CoV-2 [2]. They found that despite 86% (24 out of 28) conservation of epitopes for CR3022 between SARS-CoV and SARS-CoV-2 RBD, the CR3022 antigen binding fragment (Fab) binds to the RBD of the former with a much higher affinity than to that of the latter. Their studies also underlined the absence of overlap between the epitopes and the ACE-2 binding site of the SARS-CoV-2 RBD. This implies neutralizing mechanism, which appears to be independent of competitive inhibition or direct blocking of the receptor binding site.

Furthermore, IgBLAST analysis of CR3022 revealed that its immunoglobulin G heavy-chain variable (IGHV) region is 3.1 % somatically mutated leading to 8 amino acid changes in germline sequence. In comparison, its IG light- κ -chain variable (IGKV) region was found to be only 1.3% somatically mutated with three amino acid changes for the germline. Six out of these eleven mutations belonged to the

framework region (FR), indicating their significance in the affinity maturation process. These findings highlight the scope of increasing the binding affinity of CR3022 through mutagenesis and the need for increasing its specificity to SARS-CoV-2 RBD, mainly the ACE-2 binding site.

Our study presents a methodology that performs hotspot mutation analysis to design antibody candidates with better binding affinities against the SARS-CoV-2 S-protein RBD. We design nine mutants of CR3022 comprising single point substitutions and observed their binding with the SARS-CoV-2 S-protein RBD, leading to the selection of a lead mutation that enables a marked increase in binding affinity against the target and identified three mutants with the potential to be designed against the emerging SARS-CoV-2 variants.

This study also elucidates the importance of framework region mutations at the interface of variable heavy and light chains for developing broadly neutralizing antibodies with increased breadth and potential against pathogens presenting rapidly evolving strains and variants of concerns.

2. Methods

2.1 Mutant Library Design using HotSpot Wizard 3.0

The crystal structure of the CR3022 antibody complexed with the SARS-CoV-2 S-protein receptor binding domain (RBD) was retrieved from RCSB Protein Data Bank (PDB ID:6W41, 3.08 Å resolution, version 1.5: 2020-07-29). The PDB file was stripped of water molecules and heteroatoms. The heavy and light chain residues of the antibody corresponding to the constant region were also removed. The antigen (RBD) and variable domain heavy and light chain of antibody CR3022 were renumbered and saved as separate PDB files using SPDBV. The PDB file containing variable domain heavy and light chain of the CR3022 antibody was uploaded as input on the HotSpot Wizard 3.0 server [3].

No essential residues were specified during job submission, to increase the range of hotspot prediction across the length of heavy and light chain sequences without bias. Functional hotspots were selected from the predicted outputs to focus on optimization and tailoring of functional/catalytic specificity. Residues predicted by the F-pocket tool; residing in catalytic pockets or access tunnels, with reliably high mutability scores (indicated by scores ranging 6-9) were selected to design a library of mutants.

The mutation landscape was determined by setting the percentage for loss of function tolerance at a minimum of 60%. Only stabilizing substituents at mutation positions were selected from the resultant library.

2.2 Antibody modeling and prediction of interfacial residues

Mutant antibodies were modeled using Repertoire Builder [4]. Heavy and light chain amino acid sequences of the antibody (including the desired point substitution mutation) were fed into the server. PDB files of modeled antibodies were obtained from Repertoire Builder. PDB files were processed through

the antibody paratope prediction server, Parapred, which estimates complementarity determining region (CDR) of the antibody as per the Chothia definition [5].

All the antibody files were then processed before molecular docking on the HADDOCK 2.4 server. Heavy and light chains were renamed as H and L, respectively. Unique residue numbers were assigned across the H and L chains using Swiss PDB Viewer [6]. Antibody specific epitopes were predicted using the EpiPred server [7].

2.3 Structure-guided antigen-antibody docking, refinement and pose generation using HADDOCK 2.4

The structures of 9 mutant antibodies and a wild-type antibody were processed for submission to the HADDOCK 2.4 user interface. SARS-CoV-2 RBD structure from RCSB PDB (PDB ID: 6W41) was processed as antigen for the same. There were eleven submissions altogether, comprising nine mutant systems and two controls utilizing the wild-type antibody – WT-AR and WT-PR.

2.3.1 Data input

The PDB files of antigen and modeled antibody structures were processed through manual editing before using them as input in HADDOCK 2.4 interface. The antibody structure (with 231 residues) was taken as the receptor and input as molecule 1, while the antigen (with 195 residues) was input as molecule 2 in the interface.

2.3.2 Input parameters

The HADDOCK 2.4 web interface's input parameters require the assignment of restraints in the form of active and passive residues for each of the interacting molecules termed ambiguous interaction restraints (AIR) that guide docking. The residues predicted by Parapred were fed as active residues for antibody. Buried surface residues predicted as paratopes were also included in the active residue input. The option to automatically define surface residues as passive was unchecked to restrain interface sampling space of antibody structure.

Furthermore, owing to the knowledge of a loose definition of epitopes specific to the mutant antibodies, all the three summaries of epitopes predicted by EpiPred and experimental epitopes for the wild-type antibody identified previously were loaded as input [2]. To avoid errors due to penalty on input residues found to be absent at the interface upon docking during HADDOCK scoring and clustering, these epitopes were fed as passive residues.

To observe the docking efficiency for the crystal structure, a control with wild-type antibody and antigen, and known experimental epitopes and paratopes input as active residues was additionally submitted (WT-AR). To observe efficacy of the protocol employed for addressing the uncertainty of epitopes predicted for mutant antibodies, a control comprising the wild-type antibody and antigen structures was submitted with predicted epitopes loaded as passive residues (WT-PR). The inputs were optimized for

bioinformatics prediction by increasing the sampling size for the rigid docking iteration to 10000 and the following flexible docking iteration to 400.

2.3.3. Docking parameters

Certain changes were made to the sampling, clustering, and analysis parameters under this section of the interface. The option to perform refinement with short molecular dynamics in explicit solvent (water) was included as a run parameter for sampling. The clustering method was changed to root-mean-square deviation (RMSD) from the default fraction of common contacts (FCC) with an RMSD cut-off of 7.5Å. Advanced water analysis was also included as part of the docking parameters. The remaining options were maintained at default for all job submissions.

2.4 Molecular dynamics

Molecular dynamics (MD) simulations for wild-type and mutant complexes were performed using GROMACS 2020.4 modules and CHARMM36m force field [8–10]. Using CHARMM-GUI, the complexes were solvated with TIP3P water in a rectangular box, keeping minimum distance to the edge of the box as 12 Å under periodic boundary conditions [11]. Sodium and chloride ions were added to neutralize the protein charge, followed by further additions of ions to mimic a salt solution concentration of 0.15M. Each system was energy-minimized for 5000 steps and heated (0K to 310K) with the NVT ensemble (utilizing V-rescale temperature coupling), followed by equilibration for 2 nanoseconds (ns) and production for 100 ns with the NPT ensemble (T = 310 K and P = 1 atm). All simulations were run with a time step set to 1 femtosecond (fs) and supported by V-rescale for temperature control. Trajectories were recorded after every 100 picoseconds (ps). Long-range electrostatics were evaluated through the Particle mesh Ewald (PME) method [12]. A linear constraint solver (LINCS) algorithm was employed to constrain bonds involving hydrogen atoms [13]. Simulations for each system were performed in triplicates with uniform conditions and parameters. The results were plotted using Origin Pro 2020 and were visualized using the PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC. We considered one of three trajectories as representative for further analysis.

The testbed configuration utilized involved Dell power edge 2 socket server R7525 with AMD EPYC 7742 64 - (Core processor 2.6GHz (Base)). The operating system configuration was RHEL 8.2 (4.18.0-193.el8x86_64) with memory and BIOS of DDR4 256G (16Gb X 16) 3200MT/s and 1.17 respectively.

2.5 MM-GBSA

Selected representative complexes of one of the trajectories were obtained for calculating the binding energy of each system using the MM-GBSA method [14]. The HawkDock server created by G Weng and colleagues was used for this purpose [15]. The PDB file of the selected complex for each system was uploaded on the server along with specifications of chains identifying the receptor (antibody) and ligand (antigen).

3. Results

3.1 Hotspot mutation analysis

The mutant library designed using Hotspot Wizard 3.0 is presented in Table 1. M40 and G44, and K45, Q48, V89 and V91 were found to be functional hotspots present in the heavy (H) and light (L) chains of the variable domain, respectively. The functional hotspots were not observed to overlap with the stability and correlated hotspots also identified by the server.

All the identified mutation hotspots lie in the structurally necessary FR of the antibody that lines the more variable CDRs. H-M40 and H-G44 lie in the H-FR2, L-K45 and L-Q48 lie in L-FR2 and, L-V89 and L-V91 reside in L-FR3 (Table 1).

Upon annotation of the crystal (PDB ID: 6W41) derived CR3022 antibody structure using PDBePISA; H-M40, L-K45, L-V89 and L-V91 were found to be solvent accessible residues, while H-G44 and L-Q48 were found to be present as partially buried residues at the interface between H and L chains with L-Q48 residue particularly forming an H-bond at the interface (Supplementary File S1) [16].

After generation of a mutant library based on the mutation landscape specified above (section 2.1) and screening of thermodynamically stabilizing and destabilizing mutants in Rosetta module of Hotspot Wizard 3.0, substituent residues; A, H, M, S, W were selected for L-K45; F, W, Y were selected for L-Q48 and I for L-V91 positions, respectively. No stabilizing substituent was identified for the hotspots residing in the H chain within the mutation landscape.

3.2 Antibody modeling and prediction of interacting residues

The Repertoire Builder generates atomic-resolution, three-dimensional models of antibodies from their amino acid sequence by applying a unique multiple sequence alignment extension technique [4]. It is available as an accessible web server and performs better in terms of ease, speed and accuracy of processing and generation compared with its contemporaries such as LYRA, ABodyBuilder, and PigsPro. It also includes scoring of generated CDR loops followed by refinement with Rosetta Antibody. It is an integrated modeling solution specific to antibodies.

Parapred is a freely accessible webserver utilizing a probabilistic sequence-based machine learning algorithm for paratope prediction. Using deep learning architecture, the algorithm leverages features from local residue neighborhoods and across the entire sequence to identify stretches of hypervariable regions in an antibody sequence input. It specifies CDRs as per the Chothia definition for variable heavy and light chains. It requires only the antibody sequence as input, making it independent of antigen information. It has been used to improve the accuracy and speed of rigid docking algorithms and binding pose sampling [5]. The results from Parapred are highlighted in Figure 2 and Supplementary File S2, Appendix 1.

Information on antibody-specific epitopes of the antigen was derived from data generated by Yuan and colleagues and EpiPred [17,18]. EpiPred combines a specific antibody-antigen score with antibody-

antigen structures to predict antigen residues involved at the binding interface (epitopes). It utilizes structures of antigen and the antibody as input. It has shown to improve the number of near-native poses upon rigid docking. EpiPred generates three summaries of predicted epitopes. All three summaries along with experimentally determined epitopes of CR3022 were used as inputs for docking to increase sampling of binding poses for the mutant antibodies (Supplementary File S2, Appendix 1).

The choice of these algorithms was employed to affirm optimal integration of both sequence and structure features. This aided greatly in generating information for improving the docking protocol's accuracy and efficiency for finding near-native binding conformations of the designed antibodies.

3.3.1 Structure-guided antigen-antibody docking, refinement and pose generation using HADDOCK 2.4

HADDOCK integrates global and local docking with refinement to generate binding poses. Prior to docking, HADDOCK repairs the input antibody and antigen structures and automatically assigns protonation states to histidine residues using MolProbity. The binding poses generated after global and local docking require refinement to assume near-native conformations.

Short molecular dynamics in explicit solvent (water) are performed for this purpose. It comprises building a solvent shell around the complex, restraining all atoms at the interface to their original position except the side-chain atoms, followed by 1250 MD steps at 300K with position restrained heavy atoms not involved in intermolecular contacts within 5 Å. Following this, the system is cooled down (1000 MD steps at 300, 200 and 100 K) with position restraints on the backbone atoms of the protein complex, excluding the interface atoms. This achieves relaxation of the complex and the comprising structures along with energy refinement for nearing native conformations.

The protocol mentioned in section 2.3 is tailored for antibody-antigen docking. It involves RMSD-based clustering following rigid docking, local docking, and MD refinement to group distinct resultant representative conformations of docked complexes for each system. HADDOCK 2.4 forms clusters by assigning HADDOCK scores to each cluster.

Van der Waals, electrostatic, desolvation, interaction energy components, and buried surface area are considered while computing the HADDOCK scores for each cluster. However, this score doesn't serve to select a single representative docked complex from the four representatives generated per cluster. The cluster analysis requires closer inspection concerning binding pose and interface.

3.3.2 Clustering analysis and ranking

A unique method was employed to rank complexes among the docking results for each system to prioritize maximal interface between antibody and antigen among the docking results.

For each system, the top four clusters were chosen. The best four representatives of each cluster were selected, i.e., 16 representative complexes for each system were selected for input in protein binding energy (PRODIGY) prediction. PRODIGY is a predictor of binding energy and affinity of protein-protein

complexes from their 3D structure. It is based on structural determinants, namely the number of intermolecular contacts at the interface. This parameter, combined with information on non-interacting surfaces's properties, is used for predicting binding free energy (ΔG) and affinity (K_d). It has demonstrated a Pearson's Correlation coefficient of 0.73 between the predicted and measured binding affinity on the benchmark (P-value <0.0001) as reported by Xue et al. [19].

ΔG and K_d were predicted at 37°C for each of the 16 representative structures corresponding to every system through PRODIGY. We utilized PRODIGY for relative estimations. Both parameters (ΔG and K_d) were combined into a single entity $\log(|\Delta G|/K_d)$ for easier comparison across the 16 structures (Figure 3). This entity combines specificity, affinity and stability displayed by each representative binding pose within a system and can be reliably used for ranking top poses within a system, i.e., for intra-system comparison. The pose with the highest value of $\log(|\Delta G|/K_d)$ from each system was selected as a representative conformation (Figure 3).

3.4 Molecular dynamics simulations and MM-GBSA

To analyze docked RBD-antibody complexes' temporal behaviour and conformations, molecular dynamics simulations were carried out for 100 ns using GROMACS 2020.4 software package. The temperature and enthalpy measures were monitored throughout the production to ensure the maintenance of uniform conditions. MD trajectories for each simulation were analysed using RMSD and root-mean-square fluctuation (RMSF) plots of backbone carbon-alpha ($C\alpha$) atoms about the initial conformation of the production run. These frames were analyzed using the GROMOS clustering method based on RMSD distributions across neighbors (with a cut-off set to 2 nm) [20]. For each system, it contained snapshots of centroids of all the clusters. Centroids representing the top 5 clusters in each system were taken for further analysis. They were ranked based on their interface using the method utilizing PRODIGY described in section 3.3. Out of three set of runs, second set of runs were chosen as representative for further analysis (highlighted as red in Figure 6). The top-ranked conformation from among the five models for every system in second set of runs was selected for MM-GBSA analysis.

The HawkDock server performs MM-GBSA calculations based on the GB^{OBC1} model and the ff02 force field for protein-protein binding free energies [15,21,22]. The result presents five components that compose the total binding energy between the receptor and ligand, namely (i) Van-der Waals energy, (ii) Electrostatic energy, (iii) Generalised Bonn desolvation energy, and (iv) Surface Area (Figure 4). It is worth noting that, the electrostatic energy component is a significant contributor to the total energy for the wild-type complex. Simultaneously, the lead demonstrates a comparatively higher contribution of the Van der Waals energy and a slight increase in the SA contribution to the total energy. This increased dependence of binding upon Van der Waals energy greatly implicates the designed antibody's increased flexibility. Analyzing the binding affinities of all wild-type and mutant antibody candidates set the seal on K45S to be the lead antibody (Figure 5).

The RMSD across the trajectory for WT-PR, V-6W41 and the lead mutant L-K45S indicates convergence of the simulations in the last 20 ns (Figure 6). The RMSF plots for per residue fluctuations in antibody across the lead mutant L-K45S and control WT-PR indicate the presence of more conformationally flexible residues in the mutant (Figure 7). The distinctly higher peak in the mutant RMSF plot (compared to WT-PR) belongs to the second FR of the light chain. This observation implicates the contribution of the single substitution mutation to the flexibility of the designed antibody. The RMSD histogram distributions for the lead mutant (L-K45S) and the two controls (WT-PR and crystal V-6W41) depict a single prevalent conformation representing their respective trajectories (Figure 8).

For affirming effective binding and neutralizing potential of the lead mutant, its end point free energy was also compared with that of SARS-CoV RBD-CR3022 complex (PDB ID: 7JN5). Upon comparison, the binding energy of the mutant (L-K45S) to SARS-CoV-2 was found to be better than that of 7JN5 (Table 2). This emphasises the success of the mutation in supplementing the cross-reactive CR3022 antibody (originally anti-SARS-CoV) in achieving improved binding energy, affinity and possible neutralization potential against the evolved SARS-CoV-2. It is also imperative to note that compared to the binding energy between SARS-CoV-2 S protein RBD and its human target receptor ACE-2 (PDB ID: 6MOJ), the binding energy between the SARS-CoV-2 S protein RBD and the lead mutant antibody (L-K45S) has increased two-fold.

The key residues contributing significantly to the total interaction energy upon binding with SARS-CoV-2 RBD for CR3022 include H-Y27, H-T31, H-Y52, H-Q57, H-G101, H-I102, H-T104, H-D107, L-I39, H-W61 and for K45S include H-M2, H-S100, H-D107, H-I102, and L-N37 (Figure 9). All the key residues reside within the predicted paratopes. Furthermore, it is interesting to note that while K45S has fewer key residues contributing to energy concerning the wild type CR3022, they contribute significant proportions of the energy landscape calculated by MM-GBSA.

The trends of binding affinities computed are in agreement with previously reported studies on the interaction between SARS-CoV-2 RBD and CR3022 [23,24]. The computed binding affinity between SARS-CoV RBD and CR3022 (PDB ID: 7JN5) is higher than that found between SARS-CoV-2 RBD and CR3022. It is imperative to note that the binding affinity between the lead mutant and SARS-CoV-2 RBD is higher than both affirming its status as an affinity matured lead antibody candidate. It is also interesting to note that three other mutant candidates expressed binding affinities greater than that found between ACE2 and SARS-CoV-2 RBD.

3.5 Antigen-antibody interaction analysis

The interfacing residues were analyzed for complexes of SARS-CoV-2 S-protein RBD with lead mutant (L-K45S) and mutants expressing binding energies higher than that of SARS-CoV-2 S-protein RBD and hACE2 complex against the control of wild type CR3022.

3.5.1 CR3022 and K45S mutant

As is evident from the alignment in Figure 2, the epitopes for CR3022 on the SARS-CoV-2 S-protein RBD are distinct from its residues that bind to the human ACE-2 receptor implying a non-competitive neutralization mechanism involving allosteric structural blocking, driven by hydrophobic interactions as also discussed by M. Yuan and colleagues [2]. Interestingly, the lead mutant L-K45S gains additional epitopes on the SARS-CoV-2 S-protein RBD (A411, P412, G413, Q414, F464) (Figure 2). Resembling wild-type, the lead mutant also does not boast of any epitopes overlapping with the human ACE-2 binding residues implying a similar neutralization mechanism and binding based on allosteric blocking through conformational change that does not include competitive inhibition. Moreover, PRODIGY results indicate that the influence of hydrophobic interactions with SARS-CoV-2 reduces upon introduction of the L-K45S mutation in the wild type CR3022.

3.5.2 K45W, K45M and Q48W Mutants

It is important to note how some of the epitopes across all three (L-K45M, L-K45W, and L-Q48W) overlap with SARS-CoV-2 residues binding to human ACE-2. This suggests the possibility of a partial blocking mechanism for neutralization. Moreover, these mutants bind to epitopes on the RBD that coincide with mutant positions in emerging variants of concern, namely, B.1.1.7, P.1, B.1.351, B.1.427, and B.1.429 [25].

The K417 mutation position found in variants P.1 and B.1.351 overlaps with epitopes of mutants L-K45W, L-K45M, and L-Q48W. The L452R mutant position present in variants B.1.427 and B.1.429 also lines the epitopes of all the three aforementioned mutants. Similarly, E484 position in variants B.1.1.7, P.1 and B.1.351 lines the epitopes of all the three concerned mutants. The S494 mutant position unique to the B.1.1.7 variant lies between 2 epitopes of L-K45M and coincides with the epitope of the L-Q48W mutant. The N501 mutant position present in B.1.1.7, P.1, and B.1.351 coincides with the epitope position of L-Q48W and resides in the close vicinity of L-K45M and L-K45W epitopes.

Despite the direct overlap of the epitopes with residues binding to the human ACE-2 receptor and higher binding affinity than SARS-CoV-2 S protein RBD and hACE-2, these three mutants display average interaction energies for the wild type CR3022 and the lead L-K45S. Wu et al. demonstrate that the P384 residue is responsible for determining the affinity between the cross-reactive antibody and SARS-CoV-2 RBD [26]. In SARS-CoV, this position consists of alanine instead of proline and is the reason behind successful neutralization by CR3022, unlike the case with binding of the same antibody with SARS-CoV-2 RBD. Interestingly, this position does not coincide or fall near any of the epitopes of the L-K45M, L-K45W, L-Q48W mutants and can form a plausible explanation for their reduced interaction energy in the context of SARS-CoV-2. Given the observation regarding their epitopes, these three mutants show promise to be developed as neutralizing antibodies against emerging variants of concerns.

The interface and the binding poses of all the discussed mutants compared to wild-type upon interaction with SARS-CoV-2 S-protein RBD are shown in Figure 10.

4. Discussion And Conclusion

4.1 Mutations

Of particular intrigue are the resultant mutations, all of which reside in the framework region of the light chain of antibody variable domain. Contrary to the expectation that hotspots would be better suited to be found at the hypervariable complementarity determining regions, these mutations lie in close structural proximity of the CDRs. The results of this study affirm the importance of framework mutations in the variable domain of an antibody, specifically in effecting change in orientation and conformation through increased structural flexibility (as indicated by the RMSF plots of the lead with respect to the wild type). Moreover, given the context of a neutralizing antibody like CR3022 which cross-reacts with a rapidly evolving antigen, our results agree with studies demonstrating that the manipulation of the FR and antibody flexibility through mutations is crucial for the development of broadly neutralizing antibodies [27,28]. This study underscores the role of single point substitution mutations in the FR, in enabling an increase in breadth and the potential of a neutralizing antibody as indicated by the varied epitopes gained by mutants L-K45M, L-K45W, L-Q48W and the increased binding interaction of the lead mutant L-K45S, respectively. This observation holds significant importance for the engineering and development of broadly neutralizing and effective vaccine and antibody candidates against aggressively mutating pathogens such as SARS-CoV-2 and HIV. This also proves the relevance of this study in the context of the current COVID-19 pandemic that is facing continually emerging variants of concern.

Another interesting point of discussion is that the designed mutants (L-K45S, L-K45M, L-K45W, L-Q48W) lie at the interface of variable domain heavy and light chains. As noted in section 3.1, the mutant position L-K45 is a solvent accessible residue while L-Q48 is responsible for forming polar contacts with the heavy chain. The performance of these mutants affirms the importance of mutations at the heavy and light chain interface in improving binding affinity specificity as also shown by Cisneros and colleagues [29]. The increase in stability can also be attributed to these interface mutations noted by Warszawski et al. [30].

Mutations that increase binding affinity are often found to be destabilizing [30,31]. The stability-specificity trade-off mediated by framework mutations is another caveat that this study aimed to keep in line with. The selection of stabilizing mutations before their selection as well-performing enhancers of binding affinity ensured that the antibody's stability was not compromised, thus demonstrating an efficient way to computationally design stable, affinity matured developable antibodies.

4.2 Integrated docking and ranking

Our protocol involves tailoring of options and parameters of HADDOCK to customise its protein-protein docking algorithm for antibody-antigen docking. Compared to standalone rigid and local docking algorithms such as those followed by ClusPro and SnugDock, docking through HADDOCK can be used in an information guided manner to perform with improved speed and accuracy [32,33].

Giving priority to structural specificity, which is mediated particularly well by inspection of interfacial contacts, we used the PRODIGY algorithm to estimate relative binding free energy and affinity values for

comparing poses generated within a system i.e., a method of intra-system comparison. PRODIGY predicts binding energy values based on the number and type of interfacial contacts, thus integrating our concerns. The ΔG and K_d values generated by PRODIGY were integrated into a single entity- $\log(|\Delta G|/K_d)$.

ΔG value is critical in understanding how firmly the antigen binds its respective antibody. However, it does not talk about the tendency of dissociation of antigen-antibody interaction at a particular temperature. Hence, to overcome this uncertainty, it was essential to bring K_d into binding affinity picture. K_d provides a quantitative measure of dissociation of a complex binding at a given temperature. Hence, we incorporated K_d to calculate binding affinities and devised a novel way of visualizing and comparing the data through $\log(|\Delta G/K_d|)$ bar graphs. Higher columns on $\log(|\Delta G/K_d|)$ plots correspond to the better binding. Thus, the $\log(|\Delta G/K_d|)$ plot provides a clearer and quantitative understanding of antigen-antibody binding. This ranking method ensures a balanced estimate of stability, specificity and affinity and can be used to compare binding poses of the same protein-protein complex post docking.

The main advantage of the workflow followed for antibody engineering in this study is using automated freely accessible webserver that integrates and leverages information from sequence and structural features with speed and efficiency.

Conclusively, this study provides a succinct workflow for computational antibody engineering, design, and selection using tools and algorithms that can be used modularly. The methodology also provides a way to screen lead antibody candidates based on desired properties with efficient speed and accuracy. The algorithms used can be combined with algorithms that assess developability and immunogenicity to further the scope of this methodology to develop and produce therapeutic antibodies.

Declarations

Author contributions

MA, SS and AP conceptualized and designed the study. MA and SS performed mutagenesis, modeling and docking. SP, SS, MA and AK performed molecular dynamics simulations. SS, AP and MA prepared the tables and figures. MA, AP and SS wrote the manuscript. SS and AP compiled the manuscript. MA, SS, GD and YK analysed the data. GD and YK guided, reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare no financial interests or conflicts of interest related to this research.

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Tables

Table 1. Hotspot Wizard 3.0 results. Predicted stability (Kcal/mol) of designed functional hotspot mutations. All residues selected for designing the library had a reliable mutability score between 6 and 9, and probability of function tolerance above 60% Destabilizing mutation are colored red whereas stabilizing mutations are colored dark green.

Chain	Position	Residue	Ala	Arg	Asn	Asp	Cys	Gln	Glu	Gly	His	Ile	Lys	Leu	Met	Phe	Pro	Ser	Thr	Val	Trp	Tyr
H	40	Met	0.8	0.4	1.4	2.3	3	0	0.2	1.7	0.8	10	0.7	0.6	-	2.9	18	1.6	6.1	9.7	-	3.5
H	44	Gly	1.7	-	2.8	3	2.5	4	25	-	3.2	-	6.7	15	8	5.1	9.5	3.2	8	16	-	-
L	45	Lys	-3.2	0.5	2	4.7	1.4	-1.4	0	-	-5.3	11	-	3.8	-1.5	1	-	-1.7	3.4	11	-0.6	3.1
L	89	Val	1.7	2.2	2	2.8	-	0.9	2	-	-	-	-	2	1.5	4.4	-	-	-	-	-	-
L	91	Val	2.5	2.4	3.1	3.6	4.6	1.3	2.1	-	2	-0.3	2.1	1.7	1.2	1	-	3.1	1.5	-	-	1.1

Table 2. Binding energy (ΔG) computed using MM-GBSA (HawkDock webserver).

Complex	ΔG (Kcal/mol)
V_6W41	-81.73
L-K45A	-55.12
L-K45H	-35.12
L-K45M	-68.82
L-K45S	-101.43
L-K45W	-70.17
L-Q48F	-30.55
L-Q48W	-73.52
L-Q48Y	-28.94
L-V91I	-46.13
WT-AR	-85.7
WT-PR	-84.73
V-7JN5 (SARS-CoV RBD CR3022)	-91.77
6M0J (SARS-CoV-2 RBD ACE2)	-58.73

Table 3. Binding affinities and dissociation constant of docked antigen-antibody complexes. Binding affinities (ΔG) are measured in Kcal/mol. Dissociation constants (Kd) are measured in M at 37°C.

scFv	Mutation/WT	Pre-MDS		Post-MDS	
		ΔG (kcal/mol)	Kd (M) at 37.0°C	ΔG (kcal/mol)	Kd (M) at 37.0°C
6W41	WT	-15.2	1.80E-11	-10.9	2.20E-08
SAM1	K45A	-13.2	5.10E-10	-11.2	1.20E-08
SAM2	K45Q	-13.7	2.00E-10	-11.2	1.20E-08
SAM3	K45S	-15.8	7.10E-12	-9.2	3.30E-07
SAM4	K45W	-14.4	6.50E-11	-10.4	4.40E-08
SAM5	Q48F	-13.1	5.90E-10	-9.1	3.60E-07
SAM6	Q48W	-12.7	1.20E-09	-8.8	5.90E-07
SAM7	Q48Y	-12.9	7.90E-10	-8.8	5.90E-07

Figures

Figure 1

Overview of the methodology employed for in-silico antibody affinity maturation.

A

SARS-CoV-2 319 RVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRRKRISNCVADYSVLYNSASFSTFK
SARS-CoV-2 319 RVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRRKRISNCVADYSVLYNSASFSTFK
SARS-CoV-2 319 RVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRRKRISNCVADYSVLYNSASFSTFK
SARS-CoV-2 319 RVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRRKRISNCVADYSVLYNSASFSTFK
SARS-CoV-2 319 RVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRRKRISNCVADYSVLYNSASFSTFK
SARS-CoV-2 319 RVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRRKRISNCVADYSVLYNSASFSTFK

SARS-CoV-2 379 CYGVSPTKLNLDLCFTNVYADSFVIRGDEVQRQIAPGQTGKIADYNYLPDDFTGCVIAWNS
SARS-CoV-2 379 CYGVSPTKLNLDLCFTNVYADSFVIRGDEVQRQIAPGQTGKIADYNYKLPDDFTGCVIAWNS
SARS-CoV-2 379 CYGVSPTKLNLDLCFTNVYADSFVIRGDEVQRQIAPGQTGKIADYNYKLPDDFTGCVIAWNS
SARS-CoV-2 379 CYGVSPTKLNLDLCFTNVYADSFVIRGDEVQRQIAPGQTGKIADYNYKLPDDFTGCVIAWNS
SARS-CoV-2 379 CYGVSPTKLNLDLCFTNVYADSFVIRGDEVQRQIAPGQTGKIADYNYKLPDDFTGCVIAWNS
SARS-CoV-2 379 CYGVSPTKLNLDLCFTNVYADSFVIRGDEVQRQIAPGQTGKIADYNYKLPDDFTGCVIAWNS

SARS-CoV-2 439 NNLDISKVGGNINYLRLFRKSNLKPFFERDISTEIQAGSTPCNGVEGFNCYFPLQSYGFQ
SARS-CoV-2 439 NNLDISKVGGNINYLRLFRKSNLKPFFERDISTEIQAGSTPCNGVEGFNCYFPLQSYGFQ
SARS-CoV-2 439 NNLDISKVGGNINYLRLFRKSNLKPFFERDISTEIQAGSTPCNGVEGFNCYFPLQSYGFQ
SARS-CoV-2 439 NNLDISKVGGNINYLRLFRKSNLKPFFERDISTEIQAGSTPCNGVEGFNCYFPLQSYGFQ
SARS-CoV-2 439 NNLDISKVGGNINYLRLFRKSNLKPFFERDISTEIQAGSTPCNGVEGFNCYFPLQSYGFQ
SARS-CoV-2 439 NNLDISKVGGNINYLRLFRKSNLKPFFERDISTEIQAGSTPCNGVEGFNCYFPLQSYGFQ

SARS-CoV-2 499 PTNGVGYPYRVVLSFELLHAPATVCGPKKSTNLVKNKCVNFSG 543
SARS-CoV-2 499 PTNGVGYPYRVVLSFELLHAPATVCGPKKSTNLVKNKCVNFSG 543
SARS-CoV-2 499 PTNGVGYPYRVVLSFELLHAPATVCGPKKSTNLVKNKCVNFSG 543
SARS-CoV-2 499 PTNGVGYPYRVVLSFELLHAPATVCGPKKSTNLVKNKCVNFSG 543
SARS-CoV-2 499 PTNGVGYPYRVVLSFELLHAPATVCGPKKSTNLVKNKCVNFSG 543
SARS-CoV-2 499 PTNGVGYPYRVVLSFELLHAPATVCGPKKSTNLVKNKCVNFSG 543

B

H 1 QMLVQSGTEVKKPGESLKISCKGSGYGFITYWIGWVRQMPGKLEWMIIPGDSERY
H 1 QMLVQSGTEVKKPGESLKISCKGSGYGFITYWIGWVRQMPGKLEWMIIPGDSERY
H 1 QMLVQSGTEVKKPGESLKISCKGSGYGFITYWIGWVRQMPGKLEWMIIPGDSERY
H 1 QMLVQSGTEVKKPGESLKISCKGSGYGFITYWIGWVRQMPGKLEWMIIPGDSERY
H 1 QMLVQSGTEVKKPGESLKISCKGSGYGFITYWIGWVRQMPGKLEWMIIPGDSERY

H 1 SPSFQGQVTISADKSINTAYLQWSSLKASDTAIYYCAGSGISTPMDVWGQGTTVTVS 113
H 1 SPSFQGQVTISADKSINTAYLQWSSLKASDTAIYYCAGSGISTPMDVWGQGTTVTVS 113
H 1 SPSFQGQVTISADKSINTAYLQWSSLKASDTAIYYCAGSGISTPMDVWGQGTTVTVS 113
H 1 SPSFQGQVTISADKSINTAYLQWSSLKASDTAIYYCAGSGISTPMDVWGQGTTVTVS 113
H 1 SPSFQGQVTISADKSINTAYLQWSSLKASDTAIYYCAGSGISTPMDVWGQGTTVTVS 113

C

L-K45M 1 DIQLTQSPDSLAVSLGERATINCKSSQSVLYSSINKNYLAWYQQMPGQPPKLLIYWASTR
L-K45W 1 DIQLTQSPDSLAVSLGERATINCKSSQSVLYSSINKNYLAWYQQMPGQPPKLLIYWASTR
L-K45S 1 DIQLTQSPDSLAVSLGERATINCKSSQSVLYSSINKNYLAWYQQSPGQPPKLLIYWASTR
L-Q48W 1 DIQLTQSPDSLAVSLGERATINCKSSQSVLYSSINKNYLAWYQQKPGWPPKLLIYWASTR
WT_L 1 DIQLTQSPDSLAVSLGERATINCKSSQSVLYSSINKNYLAWYQQKPGQPPKLLIYWASTR

L-K45M ESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQYYSTPTFTFGQGTKVEIK 108
L-K45W ESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQYYSTPTFTFGQGTKVEIK 108
L-K45S ESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQYYSTPTFTFGQGTKVEIK 108
L-Q48W ESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQYYSTPTFTFGQGTKVEIK 108
WT_L ESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQYYSTPTFTFGQGTKVEIK 108

■ WT CR3022 ■ L-K45S ■ L-K45W ■ L-Q48W ■ L-K45M ■ hACE-2 Epitopes ■ Parapred predictions ■ Variant mutant positions

Figure 2

Antibody-antigen interface residues. (A) Variation of epitopes across wild-type and designed mutant antibodies with respect to ACE2 binding residues of the SARS-CoV-2 RBD. (B) Variation of H-chain paratopes interacting with SARS-CoV-2 RBD across wild-type and designed mutant antibodies. (C) Variation of L-chain paratopes interacting with SARS-CoV-2 RBD across wild-type and designed mutant antibodies.

Figure 3

Symbolic bar graphs of $\log(|\Delta G/K_d|)$ -based ranking for selection of representative binding pose post-docking for (A) WT-PR (wild-type control) and (B) K45S (lead mutant) antibodies. Four top-ranked poses of the top four clusters post-docking for all systems (only WT-PR and K45S are shown here) were scored to select the best representative binding pose of each system.

Figure 4

Contribution of Van der Waals energy, electrostatic energy, generalized born model, surface area, and the corresponding total energy for complexes, V-6W41, V-SARS-CoV, WT-PR, and K45S. V-6W41: complex of the variable domain of WT antibody, CR3022, with SARS-CoV-2 RBD. V-SARS-CoV: complex of the variable domain of WT antibody, CR3022, with SARS-CoV (PDB ID: 7JN5).

Figure 5

Free energy decomposition (ΔG) values from MM-GBSA (left to right): V-6W41, WT-AR, WT-PR, K45A, K45H, K45M, K45S, K45W, Q48F, Q48W, Q48Y, and V91I.

Figure 6

RMSD plots corresponding to all alpha-carbons (Ca) in an antigen-antibody complex with reference to the first frame of the 100 ns production run. Each graph depicts three trajectories corresponding to each of the triplicate production runs for a complex. Black: run 1; red: Run 2; Blue: run 3. (A) V-6W41, (B) WT-AR, (C) WT-PR, (D) K45A, (E) K45H, (F) K45M, (G) K45S, (H) K45W, (I) Q48F, (J) Q48W, (K) Q48Y, (L) V91I.

Figure 7

Superimposed RMSF plots of WT-PR (control) and K45S (lead mutant candidate) complexes.

Figure 8

RMSD distribution histograms of (A) V-6W41, (B) WT-PR, and (C) K45S.

Figure 9
Per residue contribution to the total energy of (A) WT-PR and (B) K45S complexes.

Figure 10

Visualization of interfacial residues and binding poses. Interfacial residues of interaction between (A) SARS-CoV-2 RBD and CR3022 (wild-type), (B) SARS-CoV-2 RBD, and K45S (lead mutant). (C-G) Surface visualization of (C) wild-type control, (D) K45S, (E) K45M, (F) K45W, and (G) Q48W. (H-L) Cartoon visualization of (H) wild-type control, (I) K45S, (J) K45M, (K) K45W, and (L) Q48W. For each figure, the antigen is colored cyan; antibody heavy chains are colored raspberry with CDRs highlighted in light green; antibody light chains are colored pink with CDRs highlighted in dark pink.

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