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Article

Investigating AlphaFold's handling of nanobody-antigen complex prediction

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SUMMARY

AlphaFold2 revolutionized our ability to predict a protein's structure from its sequence. However, predicting antibody structures and antibody-antigen complexes remains a challenge. To investigate this issue further, we analyzed AlphaFold's accuracy in predicting nanobody-antigen complexes. Nanobodies are the smallest and simplest antibody derivatives capable of binding antigens. They represent the variable portions of a special type of antibody, called a heavy-chain antibody, which lacks light chains, reducing the structural complexity of their bound complexes. We first benchmarked AlphaFold's poor performance across 40 known nanobody-antigen complexes. Using the DockQ metric, 35 of 40 complexes ranked in the lowest accuracy category: incorrect. We then hypothesized that an inability to accurately predict nanobody contact sites may contribute to AlphaFold's poor overall nanobody-antigen modeling. By comparing contact sites of predicted nanobody-antigen complexes against those of their experimentally verified reference structures, we concluded that AlphaFold does not accurately identify nanobody contacts; 32% of the reference contacts were missed and 38% of the predicted contacts were wrong. We further hypothesized that poor intuition for nanobody structural flexibility may limit AlphaFold's ability to predict nanobody contact sites. However, by comparing nanobody conformations of AlphaFold models generated with or without antigens, we found that AlphaFold modifies nanobody structure to accommodate antigen binding, suggesting that AlphaFold may incorrectly identify nanobody contact sites despite an apparent understanding of nanobody structural flexibility. These results motivate future work in this area, such as examination of the effect of incorporating nanobody contact site information as modeling input on AlphaFold complex prediction accuracy.

INTRODUCTION

Knowing the structure of a protein is essential for understanding its function. However, experimentally determining a protein's structure is expensive, time consuming, and often technically difficult. Computational protein structure prediction, while not a complete replacement for experimental structure determination, can be instrumental in advancing our understanding of protein function and expediting drug discovery. Over the past decade, machine-learning-based tools for protein structure prediction have dramatically improved our ability to reliably model not only the structures of single proteins but also protein complexes (1).

DeepMind's unveiling of AlphaFold 1, AlphaFold 2, and AlphaFold multimer in 2018, 2020, and 2021, respectively, represented landmark breakthroughs for protein structure prediction (2). AlphaFold uses an end-to-end neural network designed and trained to predict protein structures, given only an amino acid sequence as input (3). At CASP14, the 2020 edition of the Critical Assessment of Structure Prediction conference, AlphaFold 2's performance far exceeded the competition, predicting the structures of 70 out of 100 proteins as accurately as experimental methods (4). In 2021, DeepMind released AlphaFold Multimer, an updated version trained specifically to better predict the structures of protein complexes, thus achieving a new state of the art (5).

An important area of weakness for AlphaFold lies in predicting the structures of antibody-antigen complexes (5). Antibodies are the main effectors of our adaptive immune system that recognize and bind to specific proteins of pathogenic bacteria and viruses, termed antigens. The antigen binding region of a conventional antibody is heterodimeric, consisting of the variable domains of the heavy and light chains (**Figure 1A**) (6). Camelids (camels, alpacas, and llamas) produce an additional type of antibody, the heavy-chain antibody (HCAb), which consists only of heavy chains (**Figure 1B**) (6). Nanobodies are the target-binding region of the HCAb and are, therefore, monomeric (**Figure 1C**) (6). Nanobodies have two main regions: the framework, which constitutes the majority of a nanobody's structure and is largely conserved across all nanobodies, and the complementarity determining regions (CDRs), which are short stretches of variable sequence largely responsible for target specificity and binding. CDR diversity and flexibility allow nanobodies to adopt a wide variety of paratopes, or antigen-binding regions, to target diverse epitopes, or target regions, on antigens (6).

Antibodies are useful reagents for research and represent a powerful therapeutic class with well over 100 antibody-based therapeutics approved in the US, including best-in-class cancer immunotherapies (7). Therefore, there is great interest in applying AlphaFold to assist in the analysis and development of antibody-based modalities. However, benchmarking studies have illustrated the current challenges in applying AlphaFold to antibody complex modeling. An earlier version of AlphaFold (v2.2, trained on protein structures deposited to the Protein Data Bank before May 2018) had a low success rate of 11% in predicting the structure of antibody-antigen complexes across a set of 100

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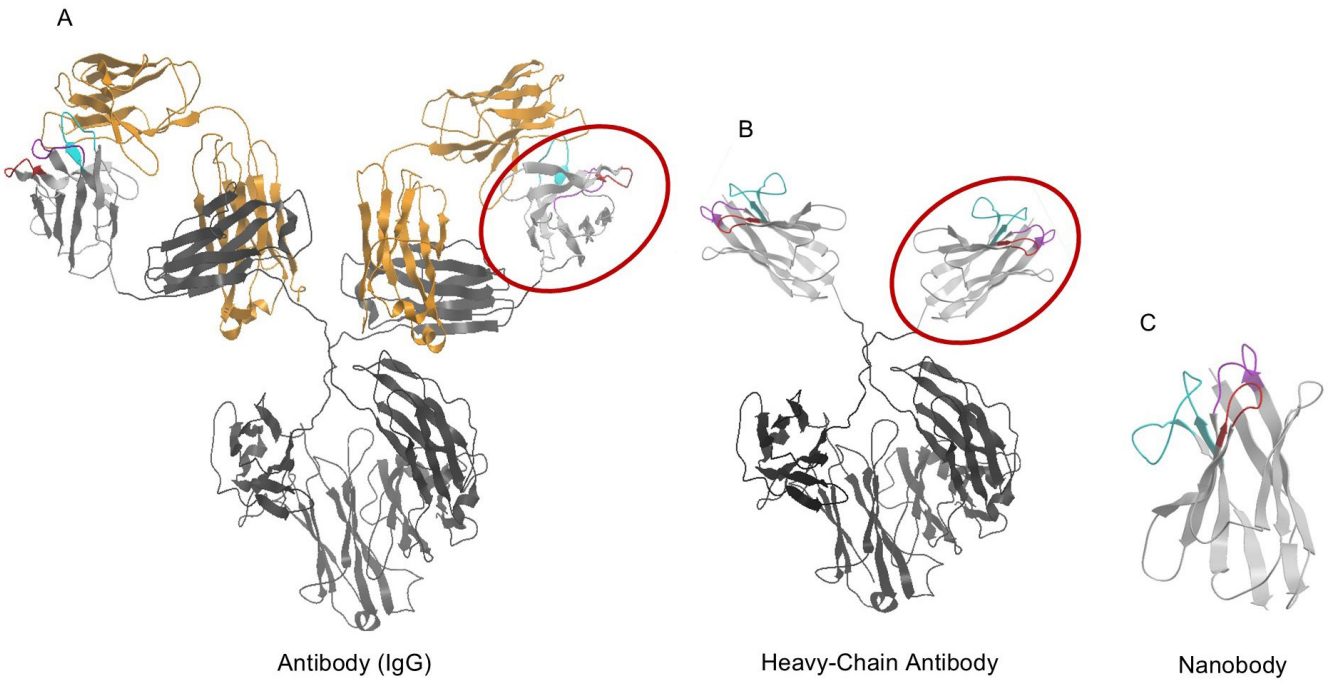

Figure 1: Canonical antibody and nanobody structure. Nanobodies represent the minimal binding fragment of a special class of antibody lacking light chains. A) Representative structure of a full-length, conventional IgG antibody (PDB ID: 1IGY). Light chains are depicted in orange, heavy chains are depicted in black, and heavy chain

structures (8). An updated AlphaFold Multimer (v2.3), trained on protein structures deposited to the Protein Data Bank before October 2021, successfully predicted 30% of 427 antibody-antigen complex structures when considering 25 predictions per complex and 26% when considering only top-ranked predictions for each complex (9). Notably, nanobody-antigen complexes in the same study were accurately predicted 27% of the time with medium or high accuracy, while antibody-antigen complex predictions were accurate only 14% of the time. The number of CDR loops, three loops for nanobodies versus six for conventional antibodies, likely contributes to this difference. While these benchmarking studies reveal modeling improvement from increasing training data and expanding the number of predictions generated for each assembly, they also highlight the persistent challenge of modeling antibody-antigen complexes overall.

A significant factor for why AlphaFold struggles to predict antibody-antigen complexes has to do with the information that AlphaFold usually relies on to intuit protein-protein interactions. Before predicting a protein-protein complex, AlphaFold assembles a multiple sequence alignment (MSA) for each protein, composed of the sequences of closely and distantly related sequences of evolutionarily related proteins (5). By identifying co-evolutionary signals between the MSAs of interacting proteins, AlphaFold can predict likely residues contributing to protein-protein interactions (4, 5). However, co-evolutionary information between nanobodies and antigens is not accessible to AlphaFold because functional nanobody evolution occurs within individuals and is not heritable (6). Given this deficit, we hypothesized that poor paratope prediction likely underlies AlphaFold's poor nanobody-antigen complex modeling.

In this study, we evaluated AlphaFold's ability to identify nanobody contact sites by comparing its predictions against experimentally-determined structures. We first generated AlphaFold models for 40 unique post-CASP14 nanobody-antigen complexes of known structure. After confirming poor model accuracies, we mapped nanobody contact sites for experimentally-determined and predicted complexes and observed poor agreement between reference and predicted paratopes. We further hypothesized that poor intuition for CDR flexibility may limit AlphaFold's ability to predict paratope structures. CDRs, unlike rigid nanobody framework regions, often exhibit some measure of structural flexibility that allows a nanobody to achieve complementarity with antigen (6). In order to examine predicted CDR flexibility, we chose a sample of 5 nanobody-antigen complexes, each with above average CDR length, and tested whether AlphaFold could predict conformational changes to CDRs when attempting to assemble nanobody-antigen complexes. We compared our findings for these predicted structures to those of an exhaustive list of reference nanobody structures in the presence and absence of antigen. To our surprise, we found good agreement between antigen-induced CDR conformational changes for predicted and reference structures, suggesting that AlphaFold may appropriately incorporate CDR flexibility into its complex predictions. Ultimately, we found that, despite taking CDR flexibility into account, AlphaFold struggles with nanobody-antigen prediction in part due to poor paratope prediction. Our study supports a strategy of supplying AlphaFold with paratope information to improve overall nanobody-antigen complex modeling in the future.

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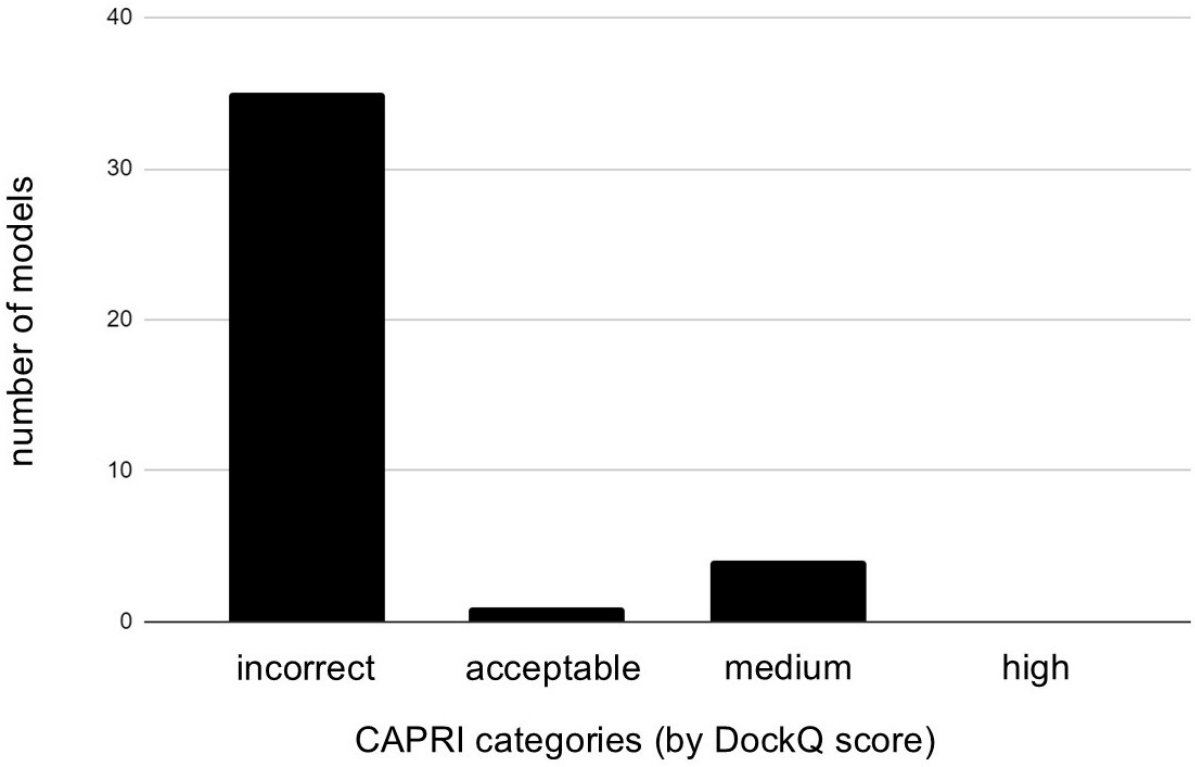

Figure 2: AlphaFold exhibits poor modeling accuracy for nanobody-antigen complexes. Bar graph showing modeling accuracy categories for 40 nanobody-antigen models generated by AlphaFold using ColabFold. Accuracy was measured by DockQ score comparing the predicted nanobody-antigen model to the known reference nanobody-antigen complex, and categories follow CAPRI guidelines.

RESULTS

Measuring Prediction Accuracy

In order to benchmark AlphaFold multimer version 2.3 (now to be referred to as "AlphaFold") performance over nanobody-antigen complexes, we compiled a dataset of all 40 non-redundant nanobody-antigen complexes deposited to the Protein Data Bank (PDB) after AlphaFold's training cutoff. We then generated predicted models (without the use of templates) for these 40 nanobody-antigen complexes with AlphaFold via ColabFold, a user-friendly and streamlined implementation of AlphaFold available to the public using Google Colab (10). Predictions for each of the 40 nanobody-antigen complexes were rated using DockQ score, an established metric for measuring model quality of predicted protein complexes (11). Each prediction was binned in one of the four following established Critical Assessment of Predicted Interactions (CAPRI) categories: incorrect, acceptable, medium, and high (**Figure 2**) (12). Of the models tested, 87% (35 total) were categorized as incorrect. Only 10% achieved a medium level of accuracy, and no models achieved high accuracy, corroborating previous work that showed AlphaFold struggles to accurately predict nanobody-antigen complexes (5,8,9).

Comparing Nanobody Contact Sites across predicted and Reference Real Structures

We analyzed reference (experimentally determined) and predicted complexes for our dataset of 40 nanobodies and mapped antigen-contacting sites across each nanobody (**Figure 3A**). A residue was considered in contact with antigen if it was within 5 angstroms of at least one atom in the antigen structure (13). Across all reference structures, there was an average of 21 contacts per nanobody, compared to an average of 23.025 nanobody contacts across all predicted structures. Although this appears consistent, prediction sensitivity, which is the percentage of reference contacts that were identified in predicted complexes, was only 68%. Prediction precision, which is the percentage of predicted contacts that were correct (based on reference contacts), was only 62% (**Figure 3B**). While modeled contacts are generally concentrated within CDR regions, our contact map shows that AlphaFold has a high rate of false positive contact sites and overall poor accuracy for intuiting subtler aspects of nanobody paratopes (**Figure 3A**).

We alo examined amino acid frequencies present in the paratopes of reference and predicted structures. We counted the total number of contacts made by each amino acid type for the reference and predicted structures and compared those totals to the total available contacts for each amino acid type across those structures (**Figure 3C**). Amino acids were considered available to form contacts if they fell in positions that had at least one observable contact across our dataset of 40 reference and 40 predicted structures (80 structures total). Only glycine (G) and arginine (R) exhibited statistically significant differences in their inclusion in paratopes between reference and predicted structures (paired two-tailed *t*-test,

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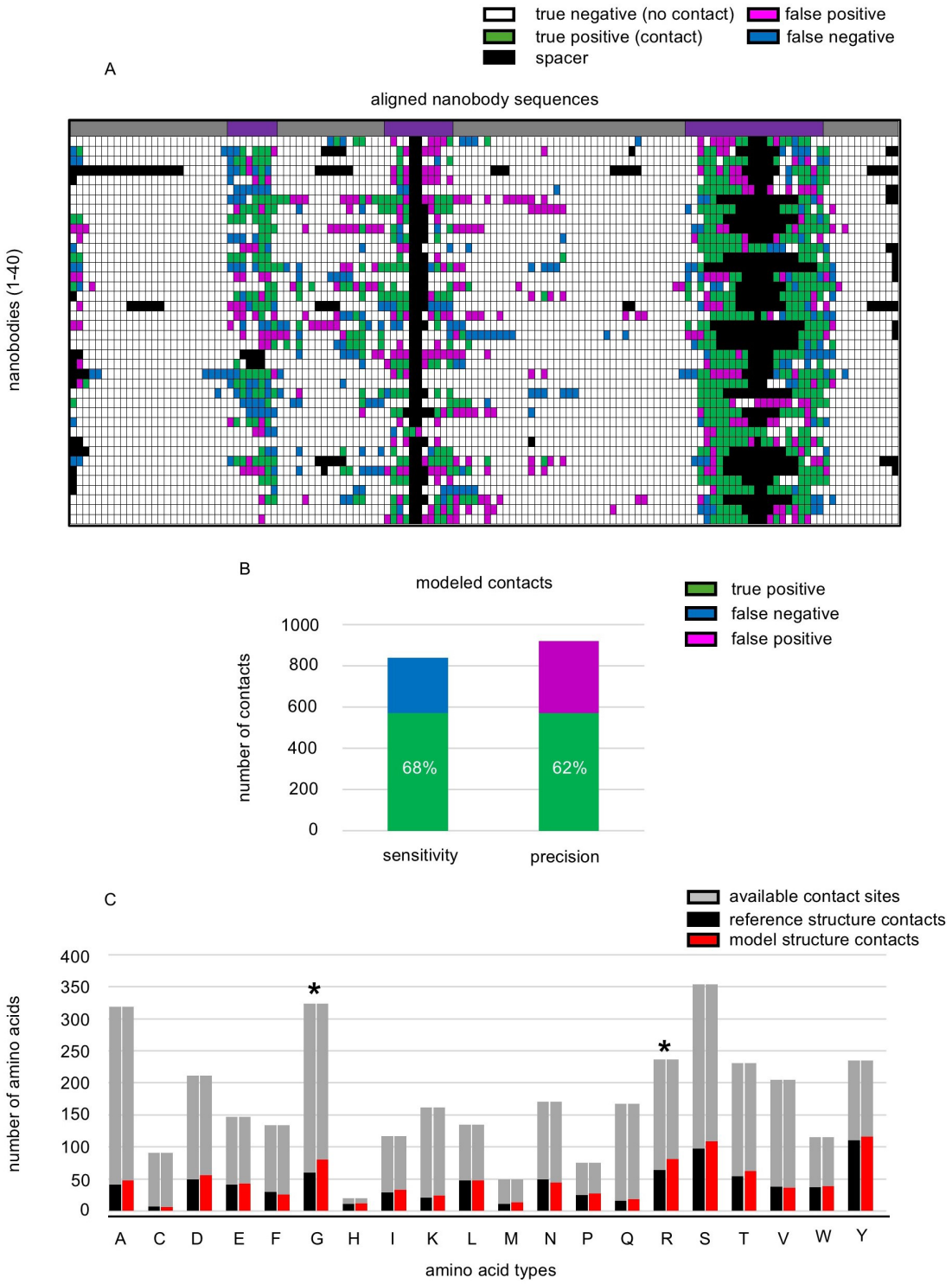

Figure 3: AlphaFold displays poor paratope prediction. Profiles of contact sites across real and predicted nanobody structures. A) For 40 nanobody-antigen complexes, AlphaFold models were compared to reference, experimentally-derived, structures. The contact map illustrates positions where both reference and predicted nanobody structures agree on contacts (green for positive contact, white for no contact), and where predicted structures make false positive (magenta) or false negative (blue) contacts compared to reference structures. Top bar is color coded for framework (grey) and CDR (purple) regions. Black boxes were used for sequence alignment purposes. B) Stacked bar graphs illustrate the precision and sensitivity of AlphaFold's predicted contacts with respect to reference contacts across all predicted structures (data from contact map in A). C) For each amino acid, stacked bar graphs illustrate the proportion of the total number of available residues (grey) forming antigen contacts in reference (black) and predicted (red) structures. Asterisk denotes statistical significance at $p<0.05$ by paired *t*-test for contacts between reference and predicted structures.

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$p<0.05$).

Investigating Loop Flexibility with AlphaFold

After confirming AlphaFold's poor performance in paratope prediction, we wanted to investigate the extent to which AlphaFold leverages CDR flexibility when attempting to dock nanobodies onto antigens. Nanobodies each have 3 CDRs, termed CDR1–3, and CDR3 is the most highly variable loop, with a broad range of lengths and structures (6). We hypothesized that poor intuition of CDR flexibility or of intermolecular influences on CDR structure may limit AlphaFold's ability to appropriately model paratopes. For five nanobodies of above-average CDR3 length in our dataset (PDB: 7E53, PDB: 7NXX, PDB: 7QBE, PDB: 8B7W, and PDB: 8BH5), we generated two AlphaFold-predicted structures: one for the nanobody alone (without antigen), and one with the nanobody bound to antigen. The nanobody portion of the bound structure was superimposed onto the structure of the nanobody alone. Then, the root mean square distance (RMSD) between the two structures was calculated across each of the three CDRs. RMSD is an averaged distance metric that compares two superimposed structures of equivalent sequence across one or more corresponding atoms. Higher RMSD values indicate a greater conformational difference between structures, and lower RMSD values indicate greater conformational similarity (14).

As a control set, we found 12 nanobodies that had both antigen-bound and unbound structures deposited to the PDB (unbound structures for nanobodies in our dataset were unavailable). After calculating changes in loop conformations between bound and unbound structures for these 12 controls using the RMSD, we were able to compare loop flexibilities observed between our AlphaFold predicted structures and this control set. Interestingly, we found good agreement between the observed flexibilities across each CDR group for control and predicted structures (**Figure 4A-4B**). On average, CDR3 exhibited the largest conformational changes upon binding for predicted and control structures (2.95 angstroms for predictions and 2.84 angstroms for controls), followed by CDR1 (1.59 angstroms for predictions and 1.48 angstroms for controls), and CDR2 (1.24 angstroms for predictions and 1.32 angstroms for controls). The main observed difference between our prediction and control sets was the increased standard deviation observed across CDR3 conformational changes upon binding (1.37 angstroms for predictions and 2.99 angstroms for controls).

Inspecting the predicted structures of 7E53 and 7NXX illustrates how differences in measured conformational changes between bound and unbound structures may be influenced by differences in CDR binding contribution (**Figure 5**). For 7E53, CDR3 contributes more to antigen binding than CDR1, which is reflected in RMSD differences between bound and unbound structures (1.2 and 1.6 angstroms for CDRs 1 and 3, respectively) (**Figure 5A**). Alternatively, for 7NXX, CDR1 has a broader interaction interface with antigen than CDR3, which likely contributes to the large conformational difference observed between bound and unbound structures across CDR1 (2.7 and 1.4 angstroms for CDRs 1 and 3, respectively) (**Figure 5B**).

To examine framework rigidity, we looked at all the CDRs as one domain, and the framework region as the other domain, and calculated total RMSD across each following superimposition of predicted nanobody structures with and without the antigen (**Figure 4C**). The framework regions were most similar for 7E53, with an RMSD of around 0.5 angstroms and the most different for 8B7W, with an RMSD of 1.1 angstroms. In both cases, however, the RMSDs are quite low, implying structural rigidity within the framework region. While AlphaFold produced predicted complexes of poor quality, it appears to leverage CDR flexibility and enforces framework rigidity in predicting structures in the presence of antigens.

Finally, while visually examining the structures of some of our predicted complexes, we observed a crucial flaw in AlphaFold's nanobody-antigen docking: steric clashes. Normally, when proteins interact, they are in close proximity with one another but can never overlap in space. AlphaFold, on the other hand, tends to produce numerous steric clashes, where regions of both proteins pass through one another (**Figure 6**). While AlphaFold is able to modify CDR structure in the presence of antigen, it appears to ignore, in certain cases, general steric constraints.

DISCUSSION

Our finding that AlphaFold's prediction of nanobody-antigen complexes is generally inaccurate corroborates previous benchmarking studies investigating AlphaFold's handling of antibody-antigen complexes more broadly (8,9). However, we were most interested in comparing the paratopes of predicted and reference structures, as AlphaFold lacks insight into coevolutionary signals between nanobodies and antigens that normally encode information about what residues are likely involved in protein-protein interactions. While this deficit should impact predictions of both paratopes and epitopes, our analysis focused on paratopes because nanobodies (unlike antigens) are a standardized protein class, and anything we learned might ultimately be applied to the general problem of poor nanobody-antigen modeling accuracy.

We found that in AlphaFold's predictions of nanobody-antigen complexes, 32% of reference contacts were missed and 38% of their predicted contacts were incorrect, on average**.** While AlphaFold correctly predicted that the majority of contacts are concentrated in CDR positions, this is a general feature apparent across the hundreds of antibody and nanobody structures that AlphaFold was trained on (5,9). For example, virtually all nanobodies have strong binding contribution coming from CDR3, and this region has the highest predicted binding contribution for both predicted and reference structures (15). However, clusters of false positive and false negative binding sites for predicted structures, combined with modest overall precision and sensitivity, testify to poor overall paratope prediction.

In terms of AlphaFold's tendencies to include specific amino acids in nanobody paratopes, glycine and arginine exhibit a statistically significant number of false positive inclusions in predicted interfaces. The glycine false positives are less problematic since glycine lacks a functional group and is therefore not an "active" binder. Glycine interactions in our database represent interactions between the target and peptide backbone, a likely byproduct of poor paratope positioning. Arginine, however, has a large, charged functional group that is more commonly involved in the formation of protein-protein interactions (16). Therefore, AlphaFold may be over-relying on arginine for contact formation due to its lack of a more nuanced grasp of nanobody-antigen interfaces.

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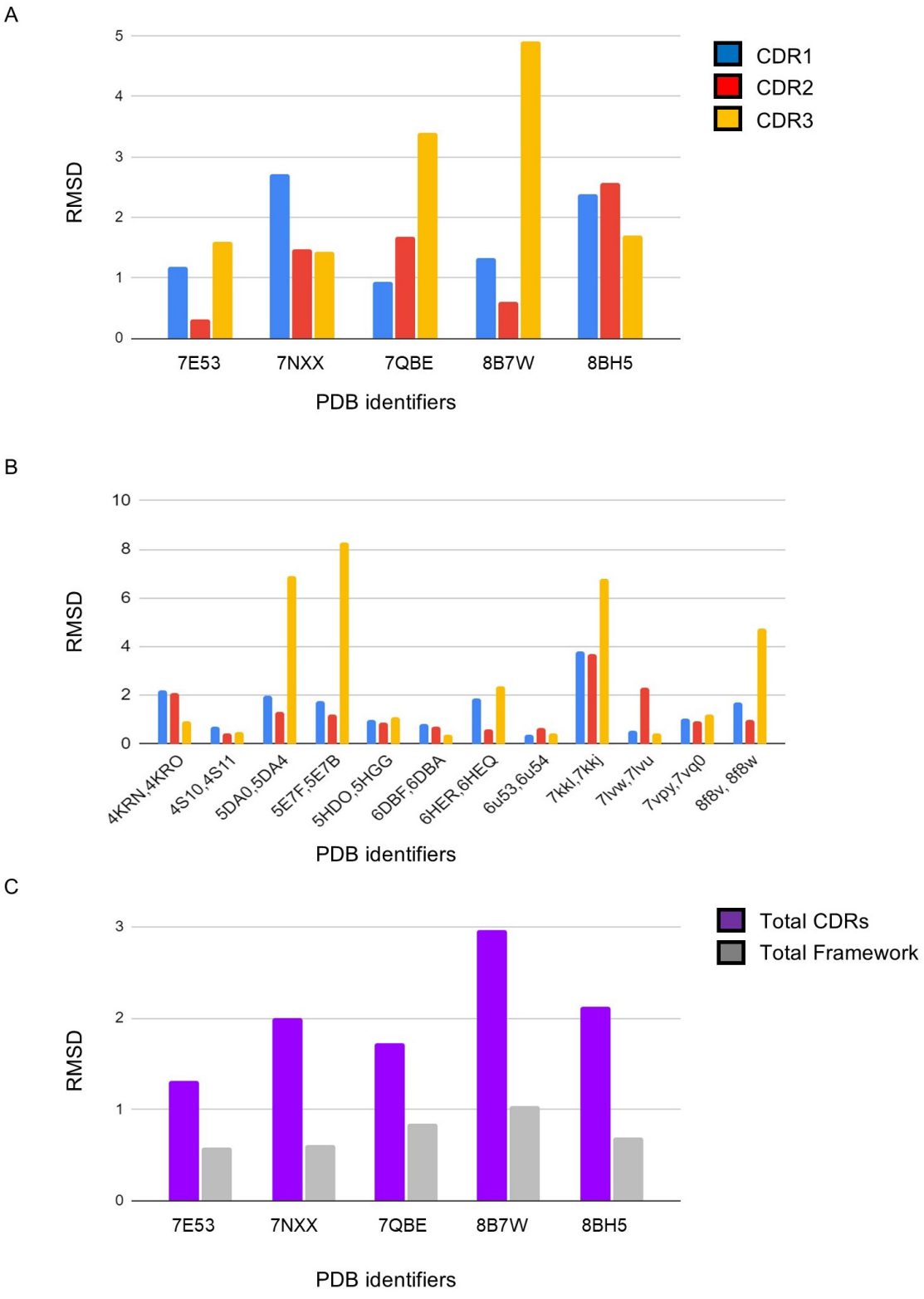

Figure 4: AlphaFold leverages loop flexibility when modeling nanobody-antigen complexes. Bar graphs showing RMSD calculations between nanobody structures in the presence and absence of antigen. A) For five nanobodies, AlphaFold predictions were generated in the presence and absence of antigen. Graph shows the RMSD by CDR between these paired predictions. B) RMSD by CDR for a control set of experimentally-determined nanobody structures. Structures for each nanobody in the presence and absence of antigen were compared. C) For predicted bound and unbound nanobody structures described in A, RMSDs were calculated across total CDRs and total framework regions.

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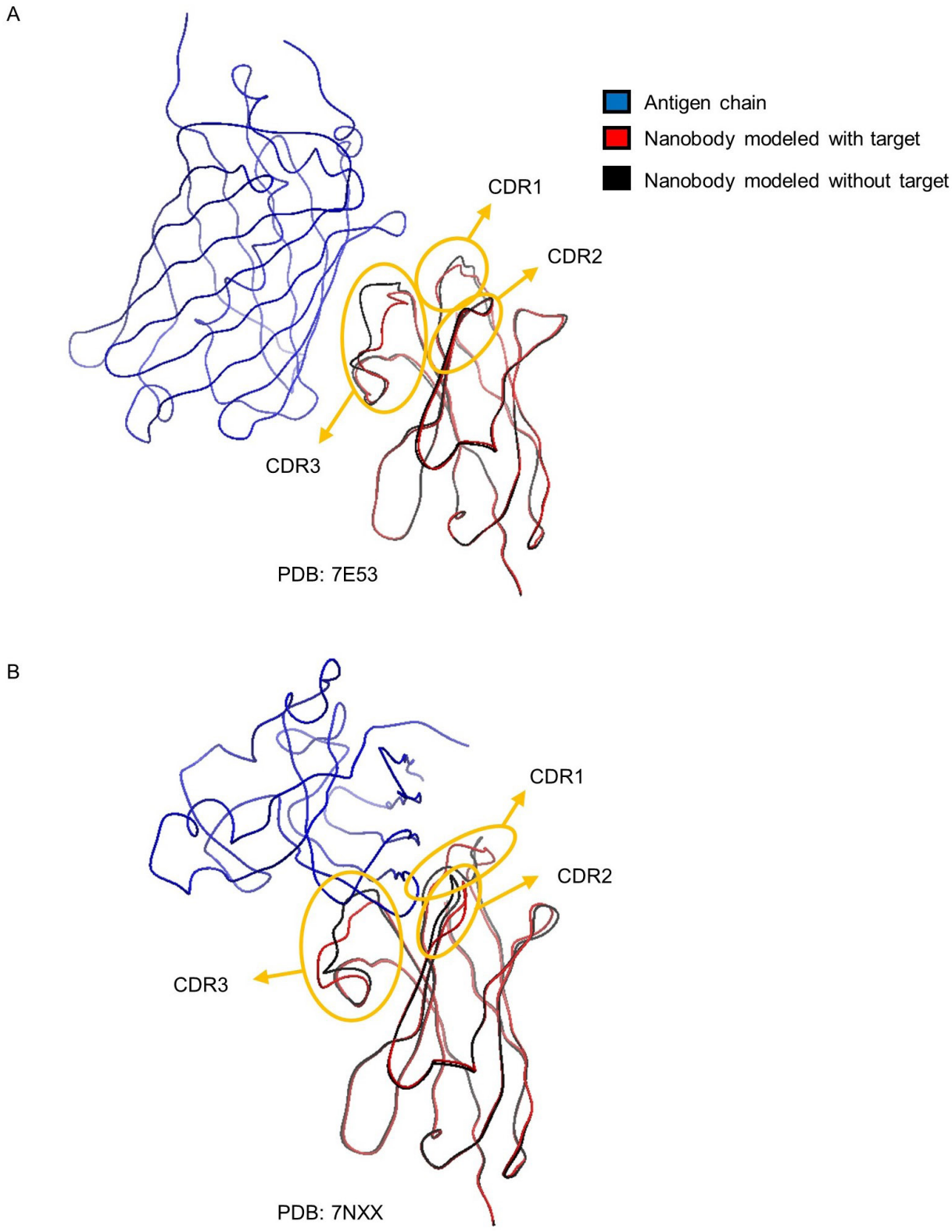

Figure 5: AlphaFold displays CDR flexibility with respect to target structure and complementarity. AlphaFold models showing change in CDR regions of nanobody depending on the presence or absence of antigen. A) Nanobody 7E53 is predicted with visual differences between CDRs in the presence and absence of target. B) Nanobody 7nxx is predicted with visual differences between CDRs in the presence and absence of target. The yellow circles and arrows highlight each of the three CDRs in the nanobody and their involvement in binding with the target antigen.

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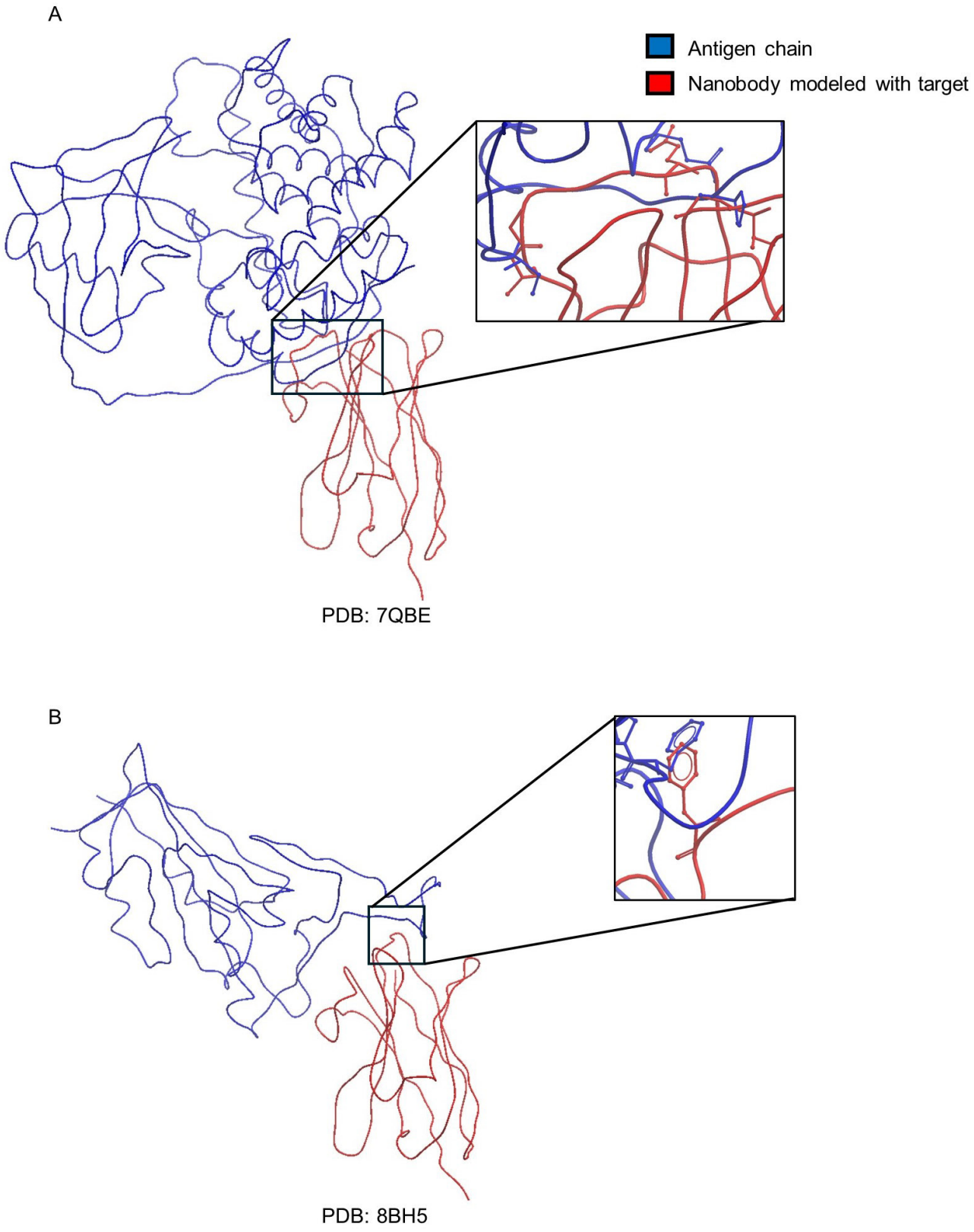

Figure 6: Steric clashes in AlphaFold model predictions. AlphaFold models showing steric clashes—instances where chains on the nanobody and target are less than 1 angstrom apart. A) Chains in nanobody 7QBE and its antigen target are entangled B) Phenylalanine in nanobody 8BH5 and its antigen target are entangled.

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While we hypothesized that AlphaFold's poor paratope prediction may be due to a poor understanding of nanobody structural flexibility, we found that AlphaFold is able to incorporate CDR flexibility in its predictions. While the RMSDs between predicted bound and unbound structures were variable for each CDR across AlphaFold's predictions for different nanobodies, this may be due to varying levels of binding contribution across different CDRs for different complexes. In contrast, the RMSDs across framework regions between predicted bound and unbound structures all remained between 0.5 and 1.1 angstrom, which are low enough for the structures to be considered almost identical, showing that AlphaFold handled the framework as a rigid structure. Additionally, we see that conformational changes across predicted CDRs are comparable to the conformational changes across the CDRs of a control set of nanobodies for which we have structures in the presence and absence of antigen**.** However, we cannot fully conclude that AlphaFold is able to appropriately modify CDR conformation to achieve target complementarity. To show this, we would need to show RMSD calculations between the CDRs of a nanobody complex predicted by AlphaFold and the CDRs of that same nanobody complex solved experimentally. Additionally, the low sample size of our available control set, as well as the high variability in CDR3 conformational changes observed for the control set, limit our ability to evaluate representative levels of CDR flexibility.

We conclude that predicting the structure of nanobody-antigen complexes continues to be challenging for AlphaFold. Despite AlphaFold's ability to leverage structural flexibility when predicting complexes, paratope prediction, among other factors, is a limitation for overall accuracy. Structural modeling with AlphaFold will likely continue to improve with time. Increased training data and increased model prediction numbers have been shown to have a positive impact on prediction performance for antibody-antigen complexes (9). Moreover, AlphaFold 3, which was recently released on May 8, 2024, has reported significant improvements compared to its predecessor for antibody-antigen complex prediction, though thorough benchmarking studies will need to corroborate the initial report (17). Our study suggests that enabling AlphaFold to identify nanobody contact sites accurately, perhaps by feeding it high confidence contact site information predicted for a given nanobody in complex, may be one way to further improve prediction accuracy.

MATERIALS AND METHODS

Model Generation with ColabFold

40 nanobody-antigen complex structures were compiled using SAbDab-nano with a similarity cutoff of 85% and a Protein Data Bank (PDB) release date cutoff of 09/30/2021 (18). Amino acid sequences for nanobodies and antigens were downloaded directly from the PDB (19). Sequences were then used to generate prediction models using ColabFold (10) version 1.5.2, which invoked the most recent update version of AlphaFold multimer (v2.3). Models were generated with default settings (20 recycles) without the use of templates.

Model Quality Assessment

Models predicted using ColabFold were compared to their corresponding reference structures from the PDB and assessed for accuracy using the DockQ metric. Code to run the DockQ calculations was invoked from the author's GitHub (11). CAPRI categories of incorrect, acceptable, medium, and high were applied to calculated DockQ scores.

Conformation Comparisons

To evaluate nanobody conformational flexibility between AlphaFold models generated in the presence or absence of antigen (bound or unbound), 5 nanobodies with known structures (deposited in PDB after 09/30/2021) and CDR3 lengths above the average length for nanobodies in our dataset were chosen arbitrarily for modeling and evaluation. RMSD measurements between top-ranked nanobody models generated in the presence and absence of the antigen were calculated using a custom script (20).

Briefly, nanobody chains from bound and unbound structures were superimposed using the Superimposer function from the Bio.PDB package of the Biopython library (21). Following superimposition, a region of comparison was defined (either a CDR or framework region) at the residue level, and RMSD was calculated across the region for all backbone atoms (C, N, O, Cα) as well as Cβ atoms.

Profiling Contact Sites

Nanobody residues directly involved in the binding interface with their antigens were profiled and analyzed for 40 nanobody-antigen complexes obtained from the PDB as well as for 40 corresponding AlphaFold prediction models for those sequences. Residues were considered directly involved in the binding interface if they contained any atoms with 5 angstroms of an atom in the antigen chain.

To analyze contact sites by amino acid type for both reference and predicted structures, the total number of available amino acids of each type across all structures was counted, and the total number of contacts made by each type of amino acid for reference and predicted structures was counted. Amino acids were considered available if they fell in positions that had at least one observable contact across our dataset of 40 reference and 40 predicted structures. Differences between the number of contacts for each amino acid type between pairs of reference and predicted structures were considered statistically significant if they exhibited a *p*-value less than 0.05 by a paired, two-tailed *t*-test.

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