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**Grafting-Based Design of Cyclic Peptides from Nanobody CDR3 Loops for Molecular
Recognition of Protein Targets**

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1. INTRODUCTION AND BACKGROUND

1.1 Nanobodies and antigen recognition

Antibodies are among the most versatile molecular recognition tools in biology, diagnostics, and therapeutic development. Their ability to bind antigens with high affinity and specificity is mainly mediated by the paratope, a binding surface formed by the variable regions of the antibody that interacts with a complementary epitope on the antigen. In conventional antibodies, this binding site is created by six complementarity-determining regions (CDRs), three from the heavy chain and three from the light chain. These loops contribute to antigen recognition through non-covalent interactions such as hydrogen bonds, electrostatic interactions, hydrophobic contacts, and shape complementarity (Madsen et al., 2024).

Antibody–antigen recognition is inherently three-dimensional. Large-scale analyses of experimentally determined antibody–antigen complexes have shown that interface residues are not always apparent from sequence alone, because residues that are distant in the primary sequence may be brought into close proximity in three-dimensional space to form the functional binding surface (Madsen et al., 2024). This highlights the importance of structural approaches for understanding antibody recognition and guiding rational binder design.

Nanobodies, also known as VHH domains, are derived from the variable domains of camelid heavy-chain-only antibodies (HCAbs). In contrast to conventional IgG antibodies, which consist of paired heavy and light chains and have a molecular mass of approximately 150 kDa, HCAbs naturally lack light chains and recognize antigens through a single variable heavy-chain domain. The isolated VHH domain constitutes the nanobody, a compact antigen-binding unit of approximately 15 kDa (Van Holsbeeck, Martins and Ballet, 2022). As illustrated in Figure 1, progressive downsizing of conventional antibodies has generated several functional formats, including Fab fragments, single-chain variable fragments (scFvs), and single-domain antibodies, with nanobodies representing one of the smallest naturally derived antibody-binding domains.

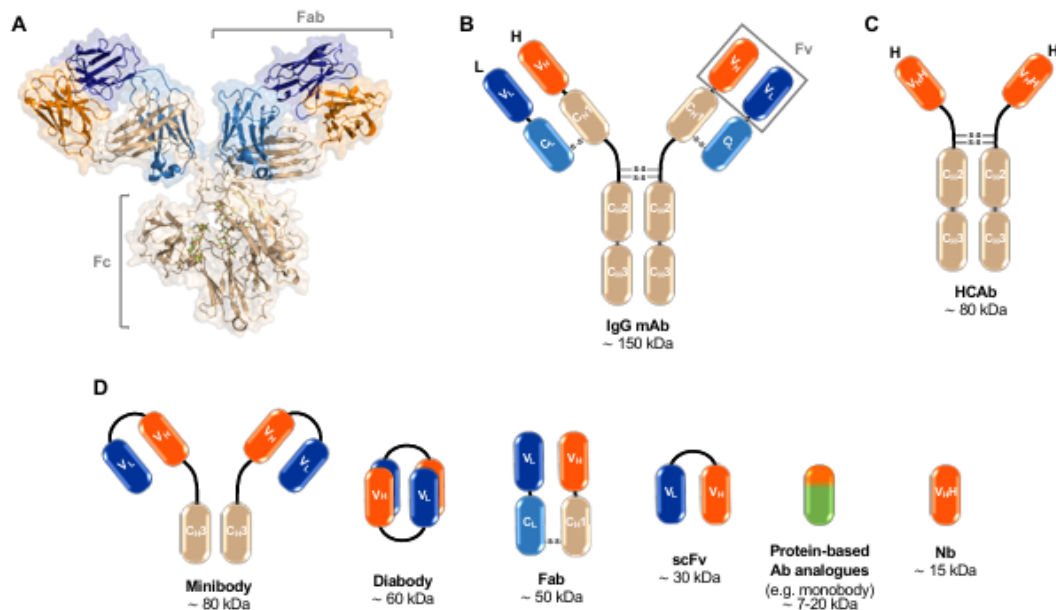


Figure 1. Structural organization and downsizing of antibody formats. (A) Three-dimensional structure of a conventional IgG antibody showing the Fab and Fc regions. (B) Schematic representation of a conventional IgG composed of paired heavy and light chains. (C) Heavy-chain-only antibody (HCAb), naturally lacking light chains. (D) Representative downsized antibody formats, including minibodies, diabodies, Fab fragments, single-chain variable fragments (scFvs), protein-based antibody analogues, and nanobodies (Nbs). Reproduced from Van Holsbeeck, Martins and Ballet (2022).

Despite their structural similarity to the VH domains of conventional antibodies, VHH domains possess specific adaptations that allow them to function independently of a paired light chain. These include hydrophilic substitutions in framework region 2, distinctive CDR conformations, and, in some cases, additional non-canonical disulfide bonds (Bahrami Dizicheh et al., 2023; Ketaren et al., 2025). Since the first description of heavy-chain-only antibodies in camelids, nanobodies have become widely used in structural biology, biotechnology, diagnostics, and therapeutic research (Hamers-Casterman et al., 1993; Muyldermans, 2013).

Their small size, high solubility, structural stability, and compatibility with recombinant expression systems facilitate their use in both research and engineered molecular formats (Ketaren et al., 2025). Their simple single-domain architecture also makes them suitable building blocks for multivalent and multispecific constructs (Bahrami Dizicheh et al., 2023).

The compact architecture of nanobodies also gives rise to distinctive antigen-binding properties. Their small dimensions and characteristic paratope organization can enable access to recessed, hidden, or cleft-like epitopes that may be less accessible to conventional antibodies. In particular, CDR3 can extend into cavities or grooves on the antigen surface and often makes a substantial contribution to molecular recognition (Yang, Li and Du, 2024). This ability to recognize structurally challenging epitopes has also been demonstrated in nanobodies targeting conserved regions of the SARS-CoV-2 spike protein (Maeda et al., 2022). Among

the structural features that distinguish nanobodies, the CDR3 loop is particularly important because it often forms a major part of the antigen-binding surface and can access cleft-like epitopes. For this reason, understanding the structural and functional role of CDR3 is essential for evaluating its potential as a template for molecular binder design.

1.2 The importance of CDR3 in nanobody binding

In nanobodies, antigen recognition is mediated by three complementarity-determining regions (CDRs), among which CDR3 often makes a major contribution to the binding interface. Structural analyses have shown that CDR3 can constitute a substantial portion of the antigen-contacting surface and may therefore partly compensate for the absence of light-chain CDRs (Ketaren et al., 2025). The contribution of CDR3 to antigen recognition depends not only on its amino-acid sequence but also on the conformation adopted by the loop within the VHH framework, which influences how it is presented to the antigen (Bahrami Dizicheh et al., 2023). VHH CDR3 loops can adopt different conformations depending on their interaction with the surrounding framework regions. Bahrami Dizicheh et al. (2023) identified two major conformational groups, referred to as kinked and extended conformations. In the kinked conformation, the CDR3 loop bends back toward framework regions 2 and 3 (FRW2 and FRW3), allowing contacts between the loop and the VHH framework. In the extended conformation, the CDR3 loop projects further away from the framework and generally forms fewer contacts with these regions. Representative kinked and extended CDR3 conformations are shown in Figure 2. Bahrami Dizicheh et al. (2023) reported that approximately 58% of the analyzed VHH structures adopted a kinked conformation, whereas approximately 29% were classified as extended.

These structural differences indicate that interactions with the VHH framework contribute to shaping the conformation of CDR3. Consequently, kinked and extended CDR3 loops differ in their spatial presentation within the VHH domain.

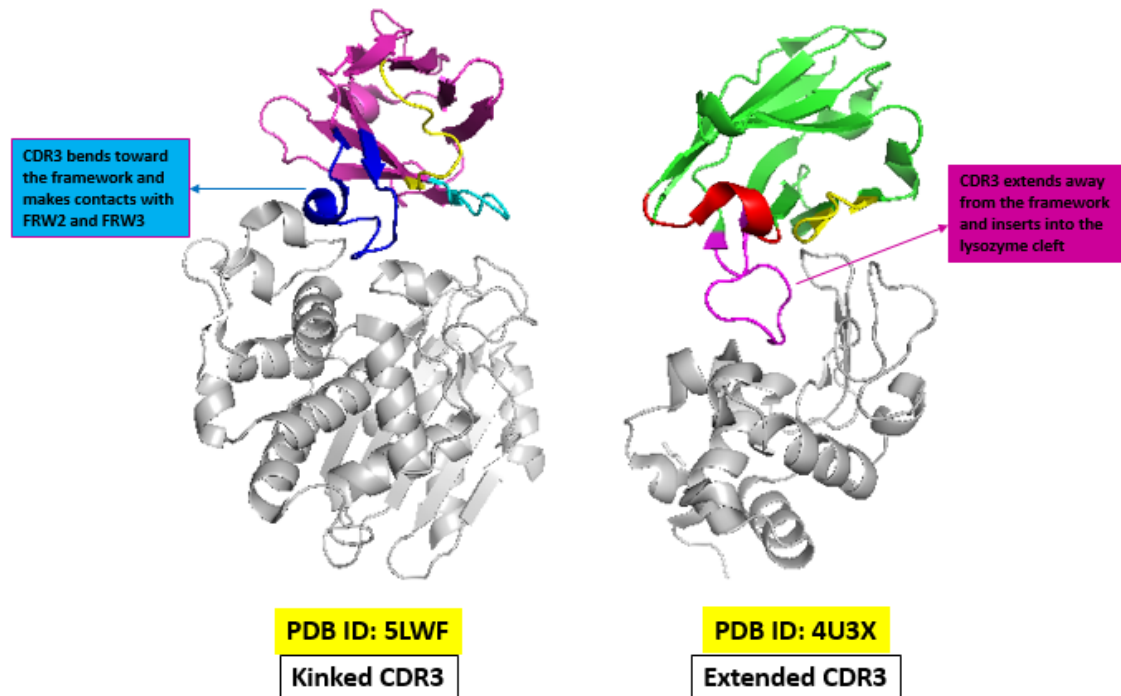


Figure 2. Representative kinked and extended CDR3 conformations in single-domain antibody-lysozyme complexes.

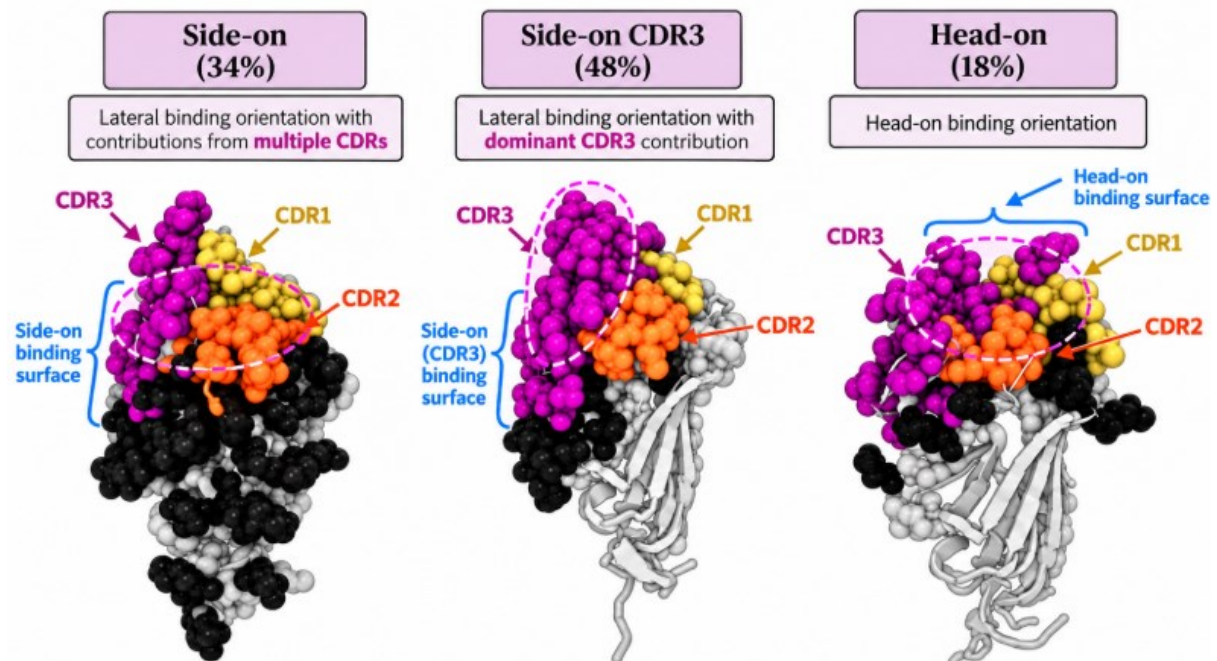


Figure 3. Nanobody paratope composition and binding orientations. Nanobody-antigen recognition can occur through different binding modes, including side-on (34 %), side-on CDR3 (48 %), and head-on (18 %) orientations. These orientations depend on the relative contribution of CDR loops and framework residues to the paratope. Adapted from Ketaren et al. (2025).

In addition to CDR3 conformation, the binding orientation of a nanobody on its antigen can also depend on the composition of the paratope. Ketaren et al. (2025) identified three major nanobody binding orientations: head-on, side-on, and CDR3-dominated side-on. These categories differ in the relative contribution of CDR3 and framework residues to antigen recognition. In the CDR3-dominated side-on orientation, antigen binding is mediated primarily by CDR3, which forms the major part of the paratope. In the head-on orientation, all three CDR loops make substantial contributions to the antigen-binding interface. In the side-on orientation, both CDR loops and framework residues contribute significantly to antigen binding, rather than the interaction being dominated by CDR3 alone. As illustrated in Figure 3, these differences in paratope composition are associated with distinct nanobody binding orientations.

Together, these observations support the view that CDR3 often acts as a central recognition element in nanobodies. Its contribution to paratope composition, epitope selection, and antigen-binding geometry makes it a promising candidate for extraction and redesign into smaller molecular formats. At the same time, its dependence on framework support and the overall architecture of the nanobody–antigen interface introduces an important challenge for peptide-based design.

1.3 Peptide design as a strategy for molecular recognition

Peptides represent attractive molecular recognition scaffolds because they are substantially smaller and structurally simpler than antibodies and nanobodies. Their reduced size can facilitate chemical synthesis, sequence modification, and the incorporation of non-natural amino acids or other chemical functionalities. In addition, peptide-based binders may provide access to molecular surfaces that are less accessible to larger protein scaffolds and can offer a relatively simple platform for the development of target-specific recognition molecules (Van Holsbeeck et al., 2022).

Despite these advantages, isolated peptides also present important limitations. Removal of a recognition sequence from its parent protein scaffold can increase conformational flexibility and reduce the probability that the peptide adopts the bioactive conformation required for efficient target recognition. Peptides may also show reduced structural stability and, depending on their sequence and conformation, lower binding affinity compared with the parent antibody or nanobody. For this reason, peptide design often requires strategies that restrict conformational freedom and stabilize geometries compatible with target binding.

One approach to address this limitation is peptide cyclization. By constraining the peptide backbone, cyclization can reduce the number of accessible conformations and increase the

probability of maintaining a structure compatible with molecular recognition (Tyler et al., 2023). In the context of nanobody-derived peptide design, this strategy is particularly relevant because antigen-binding CDR loops can be extracted from the parent nanobody and subsequently constrained in an attempt to preserve structural features associated with antigen recognition.

1.4 Nanobody CDR3 loops as templates for peptide design

The use of CDR loops as templates for peptide design is based on the possibility that a short antibody-derived sequence may retain part of the antigen-recognition properties of the parent antibody. This strategy is particularly attractive when a single loop makes a substantial contribution to the binding interface. In nanobodies, CDR3 represents a promising template because it often contains a large proportion of the antigen-contacting residues and can play a dominant role in molecular recognition.

Translating an antibody CDR3 loop into an independent peptide, however, is not straightforward. Within the parent nanobody, the loop is structurally supported by the surrounding framework and by interactions with other regions of the VHH domain. Once removed from this scaffold, the same sequence may become substantially more flexible and may no longer preferentially adopt the binding-competent conformation observed in the native complex. Loss of this conformational support is therefore a major challenge in the development of CDR-derived peptides as independent molecular binders (Burkovitz et al., 2013).

Cyclization offers one strategy to reduce this conformational freedom. Compared with linear peptides, cyclic peptides generally occupy a more restricted conformational space, and disulfide-mediated cyclization can be used to stabilize loop-like structures. Such conformational constraint may increase the probability of retaining geometries compatible with molecular recognition, although cyclization alone does not guarantee preservation of the native antibody-bound conformation (Tyler, Durek and Craik, 2023). As illustrated in Figure 4, oxidation of terminal cysteine thiol groups can lead to the formation of an intramolecular disulfide bond, thereby constraining the peptide backbone.

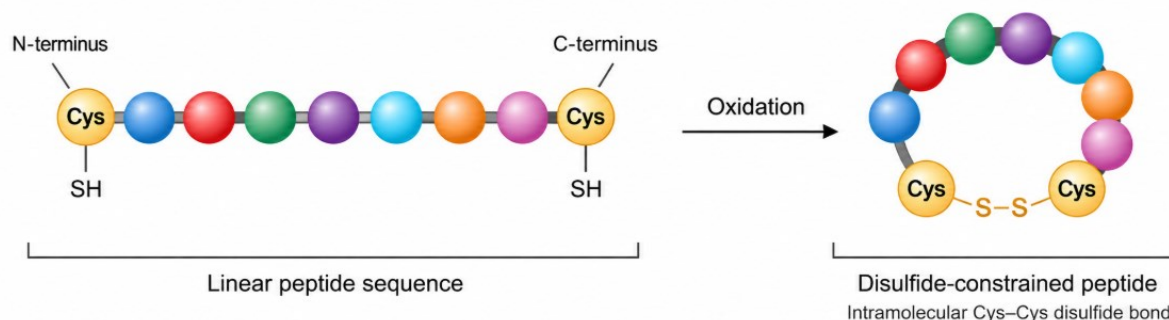


Figure 4 . Schematic representation of cysteine-mediated peptide cyclization through disulfide bond formation. Oxidation of terminal cysteine thiol groups can produce an intramolecular disulfide bond, resulting in a conformationally constrained cyclic peptide.

Based on this principle, selected nanobody CDR3 sequences in this study were redesigned by introducing terminal cysteine residues to enable potential intramolecular CYS–CYS disulfide formation. The resulting CDR3-derived peptide designs were then evaluated to determine whether they could retain structural features of the parent CDR3 loop, including its antigen-binding orientation and local interface geometry.

1.5 Computational modeling, structural comparison, and molecular dynamics

Computational modeling provides a useful framework for investigating biomolecular structure and molecular recognition when experimental characterization of every candidate is not feasible. Structural models can be used to generate and test hypotheses, compare predicted and experimentally determined conformations, and examine how molecular geometry may influence recognition (Schlick, 2010; Hinchliffe, 2008). This is particularly relevant to antibody- and nanobody-derived binders, where molecular recognition depends strongly on the three-dimensional organization of residues at the binding interface.

Recent advances in deep-learning-based structure prediction have substantially improved the modeling of proteins and molecular complexes. AlphaFold demonstrated highly accurate protein structure prediction (Jumper et al., 2021), while AlphaFold 3 extended this framework to the prediction of biomolecular complexes containing multiple molecular components (Abramson et al., 2024). Nevertheless, prediction of antibody–antigen and peptide–antigen interfaces remains challenging, particularly when recognition involves flexible regions such as CDR3-derived peptides. Predicted peptide–antigen complexes should therefore be regarded as structural hypotheses rather than direct evidence of molecular binding.

In this study, predicted peptide–antigen complexes were compared with the corresponding experimentally determined nanobody–antigen structures to assess the structural plausibility of the CDR3-derived peptide designs. The antigen was used as the common structural reference

for superposition, allowing the predicted peptide to be evaluated relative to the position of the native CDR3 loop. This approach makes it possible to determine whether the predicted peptide occupies a similar region of the antigen surface, adopts a comparable binding orientation, and preserves structural features of the native CDR3–antigen interface.

Root-mean-square deviation (RMSD) provides a quantitative measure of structural similarity between corresponding atoms after structural superposition. In this work, C α RMSD was used to compare the native CDR3 backbone with the corresponding residues of the predicted peptide after antigen-based alignment. Lower RMSD values indicate closer structural agreement, whereas higher values indicate greater deviation. However, RMSD alone does not fully describe preservation of a binding mode, because similar overall positioning may coexist with differences in local orientation or residue-level interactions. RMSD was therefore interpreted together with qualitative structural features, including peptide orientation, local antigen contacts, and the geometry of the terminal cysteine residues.

Structure prediction and structural comparison provide static representations of peptide–antigen complexes. Molecular dynamics (MD) simulations complement these analyses by examining their time-dependent structural behavior and intermolecular interactions. In MD simulations, atomic motions are propagated according to classical equations of motion using a molecular mechanics force field, allowing conformational behavior, flexibility, and the persistence of intermolecular interactions to be investigated over time (Schlick, 2010; Hinchliffe, 2008). In this study, MD simulations were used to evaluate structural rearrangements of the selected peptide–lysozyme complexes and to monitor peptide RMSD, center-of-mass distance, and intermolecular contacts over time. This dynamic analysis was used to complement the static AlphaFold 3 structural predictions.

1.6 Objectives

The aim of this thesis is to investigate whether antigen-binding CDR3 loops derived from experimentally determined single-domain antibody–antigen structures can serve as templates for the design of constrained peptides capable of retaining structural and interaction features of the native antigen-recognition mode. To address this aim, single-domain antibody–antigen complexes are selected from the Protein Data Bank (PDB) and structurally analyzed to identify CDR3 loops directly involved in antigen binding. The CDR3–antigen interfaces are characterized using criteria including interface area, number of interacting residues, hydrogen bonds, salt bridges, and residue-level contact patterns. Candidate CDR3 regions are then comparatively assessed using structural and interaction-based descriptors relevant to antigen recognition.

Selected CDR3 sequences are redesigned by introducing terminal cysteine residues to enable intramolecular disulfide-mediated cyclization. AlphaFold 3 is then used to generate structural models of the designed peptide–antigen complexes. These models are compared with the corresponding experimentally determined nanobody–antigen structures by superimposing the antigen chains and examining the position of the designed peptide relative to the native CDR3 loop. RMSD, terminal CYS–CYS geometry, binding orientation, and local interface contacts are evaluated together to determine the extent to which the designed peptides reproduce structural features of the parent nanobody–antigen interface. Figure 5 illustrates the general workflow adopted in this thesis, from the selection of CDR3 loops in experimental nanobody–lysozyme complexes to constrained peptide design, AlphaFold 3 modeling, structural comparison, molecular dynamics simulations, trajectory analysis, and DFIRE scoring.

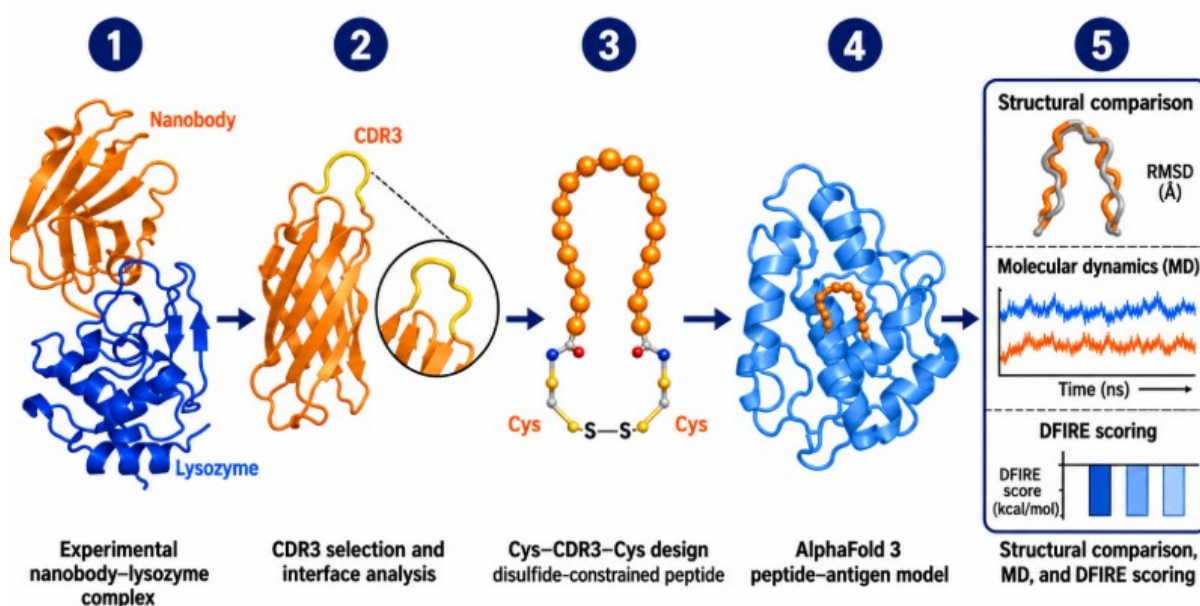


Figure 5. Overall workflow of CDR3-derived peptide design and computational evaluation.

Molecular dynamics simulations were subsequently used to investigate the structural behavior of the selected peptide–lysozyme complexes over 200 ns. Trajectory-based analyses were used to monitor peptide RMSD, center-of-mass distance, and the number of intermolecular contacts throughout the simulations.

DFIRE scoring was then applied to conformations extracted from the MD trajectories to provide an additional scoring-based comparison of the interaction energy of the three peptide–lysozyme complexes. The DFIRE results were interpreted together with the structural and dynamic descriptors.

Overall, this work integrates structural interface analysis, CDR3-derived constrained peptide design, AlphaFold 3 modeling, structural comparison, molecular dynamics simulations, trajectory analysis, and DFIRE scoring to evaluate the potential of CDR3 regions as templates for constrained peptide binders.

2. MATERIALS AND METHODS

2.1 Identification and screening of single-domain antibody–antigen complexes

Experimentally determined single-domain antibody–protein complexes were identified from the Structural Antibody Database (SAbDab). Lysozyme was selected as the main proof-of-concept antigen because of the relatively large number of experimentally determined single-domain antibody–lysozyme structures available for comparative analysis. Additional single-domain antibody complexes with other protein antigens, including BlaP β -lactamase, were also examined to compare alternative CDR3 binding geometries.

The collected structures were screened according to structural criteria related to CDR3 conformation, binding location, structural completeness, and suitability for subsequent peptide design. Structures were considered suitable when the CDR3 region was structurally resolved, directly participated in the antibody–antigen interface, and adopted a protruding or extended geometry compatible with engagement of a defined antigen cleft or pocket.

CDR-H3 conformation was assessed with reference to the structural classification described by Bahrami Dizicheh et al. (2023). In that study, extended CDR-H3 loops were described as protruding away from the antibody framework, whereas kinked CDR-H3 loops folded back toward framework regions. For structures included in the dataset of Bahrami Dizicheh et al. (2023), the CDR-H3 conformation was verified using the PDB-specific classifications reported in Supplementary Data 1. The study also showed that kinked CDR-H3 conformations frequently interact with framework regions, particularly FWR2 and FWR3, whereas extended conformations generally show less framework association. The authors further reported that PDB identifiers of the analyzed structures are provided in Supplementary Data 1.

For lysozyme-binding complexes, particular attention was given to CDR3 regions engaging the catalytic cleft. Extended or protruding CDR3 conformations were preferred because they were considered more suitable for extraction as independent peptide sequences. In contrast, kinked or strongly framework-associated CDR3 conformations were considered less suitable for the intended peptide-design strategy.

Structures showing predominantly surface-oriented binding, only partial cleft engagement, or interaction with receptor-associated pockets rather than the selected antigen cleft were

excluded from further prioritization. Closely related structures, sequence variants, mutants, alternative crystal forms, and other structurally redundant complexes were also considered during candidate selection to avoid over-representation of highly similar CDR3 binding modes.

Structures satisfying these screening criteria were subsequently subjected to detailed CDR3–antigen interface analysis.

2.2 Identification of CDR3 regions

For each retained single-domain antibody–antigen complex, the antibody and antigen chains were identified, and the CDR3 region located at the binding interface was examined in the corresponding three-dimensional structure.

The analysis focused specifically on CDR3 residues involved in the antibody–antigen interface. CDR3 interface-contacting residues were defined as residues identified by PDBePISA (Krissinel & Henrick, 2007) as contributing to the interface between the single-domain antibody and the antigen. Residue-level interface data from PDBePISA were used to determine which CDR3 residues formed intermolecular contacts with the antigen. Where residue numbering differed among PDB structures, the corresponding residues were identified according to their position within the CDR3 loop and the residue-level interface information provided by PDBePISA.

The experimentally observed positions of these contact residues were used as structural references for the subsequent evaluation of CDR3-derived peptide designs.

2.3 Visualisation

Molecular structures were visualized using PyMOL version 3.1.8 (Schrödinger, LLC, 2026). PyMOL was used to inspect the single-domain antibody–antigen complexes, visualize the CDR3 regions and their spatial relationship to the antigen surface, examine antigen-contacting residues, and assess the overall orientation of the CDR3 loop within the binding interface.

PyMOL was also used for structural superposition and visual comparison between the experimentally determined nanobody–antigen complexes and the corresponding predicted peptide–antigen models.

2.4 Structural interface analysis

The structural interface between the CDR3 region and the corresponding antigen was analysed using visual inspection together with interaction data obtained from PDBePISA (Krissinel & Henrick, 2007).

For each single-domain antibody–antigen complex, PDBePISA was used to characterise the interface between the antibody and antigen chains. The analysis included interface area, interacting residues, hydrogen bonds, salt bridges, and other reported intermolecular contacts. CDR3 residues participating directly in the antigen interface were identified from the residue-level interface data.

The relative contribution of CDR3 to the antibody–antigen interface was calculated using the residue-level buried surface area (BSA) reported by PDBePISA. For each complex, the summed BSA of the CDR3 interface residues was divided by the total BSA of all antibody residues participating in the interface and multiplied by 100.

PDBePISA also reports the solvation free-energy gain upon interface formation (ΔiG , kcal/mol). This value is calculated as the difference between the total solvation energies of the isolated structures and the corresponding interfacing structures. It therefore represents the calculated change in solvation energy associated with interface formation and was used here as an interface-level energetic descriptor rather than as an experimentally measured binding free energy. The reported ΔiG value does not include the energetic contribution of satisfied hydrogen bonds and salt bridges across the interface (Krissinel & Henrick, 2007).

The spatial distribution of the CDR3 interface residues relative to the antigen surface was additionally examined in the corresponding three-dimensional structures. The combined analysis therefore considered the number of antigen-contacting CDR3 residues, interface area, relative CDR3 contribution to the interface, hydrogen bonds, salt bridges, residue-level interaction patterns, and the local orientation of the CDR3 region relative to the antigen surface.

The resulting interface parameters were recorded for the eight retained complexes and used for comparative assessment and subsequent candidate prioritization.

2.5 Comparative assessment and selection of candidate CDR3 regions

Following structural interface analysis, the identified CDR3 regions were compared using structural and interaction-based descriptors relevant to antigen recognition.

The descriptors considered included the number of antigen-contacting CDR3 residues, residue-level interaction patterns, hydrogen bonds, salt bridges, interface area, the spatial orientation of the CDR3 region relative to the antigen surface, relative CDR3 contribution to the antibody–antigen interface, and Sequence redundancy. The main descriptors used for the comparative

assessment are summarized in Table 1. Candidate prioritization was based on the combined comparison of these descriptors rather than on any single parameter. For example, interface area was considered together with the number and type of intermolecular interactions, the relative contribution of CDR3 to the interface, and the spatial organization of the antigen-contacting CDR3 residues.

The comparative analysis was subsequently used to identify representative CDR3 templates for peptide design. The selected CDR3 regions were then modified by introducing terminal cysteine residues to enable potential disulfide-mediated conformational constraint.

Table 1. Structural and interaction-based descriptors used for comparative assessment of candidate CDR3 regions.

Criterion	Description	Role in comparative assessment
Number of CDR3 contact residues	Number of CDR3 residues identified by PDBePISA as participating in the antibody–antigen interface and forming intermolecular contacts with the antigen.	Higher values indicate that a larger portion of CDR3 participates in the interface.
Hydrogen bonds	Hydrogen bonds were identified according to the standard PDBePISA geometric criteria, using an approximate donor–acceptor distance cutoff of 3.89 Å. (Kumar et al., 2017; Das et al., 2021).	Describes the contribution of specific polar interactions to the CDR3–antigen interface.
Salt bridges	Number of salt bridges identified by PDBePISA between oppositely charged CDR3 and antigen residues. A distance cutoff of approximately 4.0 Å is used for salt-bridge identification. (Kumar et al., 2017; Das et al., 2021).	Indicates the presence of specific electrostatic interactions across the interface.
Interface area (Å ²)	Interface area was calculated by PDBePISA from the decrease in solvent-accessible surface area upon complex formation, corresponding to the difference between the total accessible surface areas of the isolated and interacting structures divided by two.	Larger values indicate a more extensive physical interface between the interacting components.
Binding orientation	Spatial arrangement of the CDR3 region relative to the antigen surface, examined using the three-dimensional complex structure.	Used as a qualitative descriptor of how CDR3 approaches and engages the antigen surface.
Relative CDR3 interface contribution (%)	Similarity between antigen-contacting CDR3 sequences among structurally related candidates.	Used to avoid carrying forward highly similar peptide templates.
Sequence redundancy	Similarity between antigen-contacting CDR3 sequences among structurally related candidates.	Used to avoid carrying forward highly similar peptide templates.

2.6 Design of terminal-cysteine-containing CDR3-derived peptides

Following candidate prioritization, the selected CDR3 sequences were used as templates for the design of terminal-cysteine-containing peptides.

For each selected candidate, the amino acid sequence corresponding to the selected CDR3 region was extracted from the parent nanobody structure. Terminal cysteine residues were introduced where required to generate a cysteine pair capable of potential intramolecular disulfide formation. For 1MEL and 2I26, the native CDR3 sequence already contained a C-terminal cysteine; therefore, only an additional N-terminal cysteine was introduced. In contrast, the selected 4U3X sequence lacked terminal cysteine residues, and cysteines were added at both the N- and C-termini. The native CDR3-derived sequence was otherwise retained unchanged.

The resulting peptide sequences were used together with their corresponding lysozyme sequences as inputs for AlphaFold 3 complex prediction. AlphaFold 3 model confidence was evaluated using the predicted TM-score (pTM), interface predicted TM-score (ipTM), and predicted local distance difference test (pLDDT). Predicted aligned error (PAE) estimates the expected positional error between pairs of residues in the predicted structure. Lower PAE values indicate greater confidence in the relative positioning of the corresponding regions, whereas higher values indicate greater uncertainty. The pTM score was used as an indicator of confidence in the overall predicted structure, whereas ipTM was used to assess confidence in the relative arrangement of the interacting peptide and antigen chains. pLDDT was used as a residue-level measure of local structural confidence, with higher values indicating greater confidence in the predicted local geometry. For the peptide chain, the reported mean peptide atom pLDDT was calculated as the arithmetic mean of the atom-level pLDDT values assigned to peptide chain A.

The terminal cysteine pairs were introduced to permit potential disulfide-mediated conformational constraint; however, covalent disulfide formation was not assumed solely from the predicted structures.

The predicted peptide–antigen models were subsequently evaluated according to terminal CYS–CYS geometry, peptide position and orientation relative to the corresponding experimental CDR3 region, structural similarity after antigen-based superposition, $C\alpha$ RMSD, and preservation of local peptide–antigen contacts.

2.7 RMSD-based structural comparison

Structural similarity was quantified using C α root-mean-square deviation (RMSD), which measures the average positional deviation between corresponding C α atoms of two structures. In this study, RMSD was calculated after structural superposition using the antigen as the common reference. The experimentally determined CDR3 region was then compared with the corresponding residues of the predicted peptide without further fitting of the peptide itself. The C α RMSD was calculated according to Equation 1.

$$RMSD = \sqrt{\frac{1}{N} \sum_{i=1}^N \sum_{j=1}^3 (X_{ij}^{native} - X_{ij}^{model})^2} \quad (1)$$

In the RMSD equation, x_{ij}^{native} represents the Cartesian coordinate j of C α atom i in the experimentally determined structure, whereas x_{ij}^{model} represents the corresponding coordinate in the predicted structure. The index i refers to the corresponding C α atoms included in the comparison, j represents the three Cartesian coordinates (x , y , and z), and N is the total number of compared C α atoms.

2.8 Molecular dynamics simulations and trajectory analysis

Molecular dynamics (MD) is a computational simulation method used to investigate the time-dependent behavior of molecular systems. In MD simulations, the motion of atoms is described according to classical mechanics, allowing the conformational and dynamical behavior of biomolecular systems to be investigated over time. The interactions between atoms are described by a molecular mechanics force field, which accounts for bonded and non-bonded contributions to the molecular potential energy. (Schlick, 2010; Hinchliffe, 2008).

Molecular dynamics (MD) simulations were performed by Prof. Soler's group using GROMACS 2022.3 to investigate the structural behavior and interaction characteristics of the selected peptide–protein complexes (Abraham et al., 2015). The best AlphaFold model for each peptide–lysozyme complex was used as the initial structure for the MD simulations. Each complex first underwent in vacuo energy minimization, followed by solvation in a cubic TIP3P water box and neutralization with Na⁺/Cl[−] ions. A subsequent energy minimization was then performed, and the systems were parametrized using the AMBER99SB-ILDN force field (Lindorff-Larsen et al., 2010).

The minimized solvated systems were subsequently subjected to two successive equilibration stages: 1 ns of NVT followed by 1 ns of NPT equilibration at 310 K. During equilibration, the geometry of the peptide–protein complex was restrained to maintain its structural integrity while allowing the surrounding solvent and ions to equilibrate. In the NVT ensemble, the number of particles, volume, and temperature are maintained constant, whereas the NPT ensemble maintains a constant number of particles, pressure, and temperature while allowing the system volume to fluctuate (Schlick, 2010; Hinchliffe, 2008).

Following equilibration, unrestrained production MD simulations were performed for 200 ns under NPT conditions at 310 K and 1 bar. Periodic boundary conditions were applied, and a Verlet integrator with a 2 fs time step was used. Bond constraints were handled using the LINCS algorithm (Hess et al., 1997). Temperature and pressure were regulated using a modified Berendsen thermostat and a Parrinello–Rahman barostat, respectively. A 1 nm cutoff was applied to van der Waals and electrostatic interactions.

The resulting production trajectories were analyzed in this work after removal of solvent and counterions. RMSD analysis was performed using GROMACS (gmx rms) by first aligning the lysozyme backbone and subsequently calculating the RMSD of the peptide with respect to its initial structure. The number of intermolecular atomic contacts and the center-of-mass (COM) distance between the lysozyme and peptide were calculated using gmx mindist and gmx distance, respectively. An intermolecular distance cutoff of 0.4 nm was used to define a contact between any peptide atom and any lysozyme atom.

2.9 DFIRE scoring

The DFIRE scoring function was applied to the three peptide–protein complexes to assess their interaction throughout the molecular dynamics simulations. In protein–protein docking, scoring functions are primarily used after the sampling step to evaluate and rank candidate binding conformations and to distinguish near-native structures from non-native models (Shirali et al., 2025). DFIRE is a knowledge-based statistical potential developed for protein structure selection and protein–protein recognition (Liu et al., 2004). In addition to evaluating static docking structures, scoring functions can also be applied to conformations sampled along MD trajectories. Previous work has shown that evaluating multiple MD conformations and averaging the resulting scores after structural equilibration can provide meaningful estimates of the relative binding affinity of protein–protein complexes, particularly when the initial modeled structures undergo conformational rearrangements during the simulation (Soler et al., 2018). Therefore, in the present study, DFIRE was applied to structural frames extracted from the MD trajectories of the three complexes to support their comparative interaction analysis.

3. RESULTS AND DISCUSSION

3.1 Screening and selection of lysozyme-binding complexes

According to the screening criteria described in Section 2.1, 20 of the 28 initially examined single-domain antibody–protein complexes were excluded, while eight representative complexes were retained for subsequent comparative analysis.

Among the excluded structures, five complexes (3P0G, 4LDE, 4LDO, 4LDL, and 7BTS) were not retained because their CDR3 regions interacted with receptor-associated binding pockets rather than the selected antigen cleft. Four structures (4I0C, 1OP9, 3EBA, and 4M3K)

displayed predominantly surface-oriented or non-cleft-inserting binding modes. Three structures (5LWF, 4N1H, and 1SQ2) were excluded because of CDR3 geometries considered unsuitable for the intended peptide-design strategy; 5LWF and 4N1H were classified as kinked in the supplementary structural dataset of Bahrami Dizicheh et al. (2023), whereas 1SQ2 showed only partial engagement of the antigen cleft.

A further eight structures (1T6V, 1JTP, 1JTT, 1JTO, 6JB2, 6JB5, 6JB8, and 1XFP) represented closely related, mutant, alternative-form, or structurally redundant cleft-binding complexes and were therefore not retained as independent candidates.

The final selected set comprised 1MEL, 1ZMY, 2I25, 2I26, 4PGJ, 4U3X, 9FGX, and 9FGY. These eight complexes were subsequently subjected to detailed CDR3–antigen interface analysis.

3.2 Overview of selected single-domain antibody and related lysozyme-binding structures

The eight retained PDB entries represented structurally distinct single-domain antibody and related lysozyme-binding systems, allowing CDR3 interface characteristics to be compared within a common antigen system. The selected structures comprised five scaffold or construct categories: camelid VHH domains, a CDR-grafted camelid VHH framework, shark IgNAR/VNAR variable domains, engineered human VH domains, and engineered nanobody-derived Di-Gluebody constructs. An overview of the eight selected lysozyme-binding structures and their corresponding domain or construct types is shown in Figure 6.

The camelid VHH group included 1MEL, a camelid single-domain VH in complex with hen egg-white lysozyme (Desmyter et al., 1996), and 1ZMY, a cAbBCII-10 VHH framework carrying the CDR loops of the anti-lysozyme VHH cAb-Lys3 (Saerens et al., 2005). The latter therefore represents a structurally relevant example of CDR-loop grafting within a VHH framework.

The shark-derived structures 2I25 and 2I26 represent IgNAR/VNAR variable domains in complex with hen egg-white lysozyme and correspond to somatically matured and ancestral forms, respectively (Stanfield et al., 2007). These structures provide a non-camelid single-domain comparison for CDR3-mediated antigen recognition.

The engineered human VH structures 4PGJ and 4U3X were included as examples of human single-domain antibodies recognizing hen egg-white lysozyme (Rouet et al., 2015). Both structures were reported in the same study examining stable human VH domains capable of cleft recognition.

Finally, 9FGX and 9FGY represent related cryo-EM structures of a lysozyme homodimer assembled using an engineered anti-lysozyme Di-Gem/Gluebody construct (Yi et al., 2026). 9FGX represents the overall reconstruction, whereas 9FGY corresponds to a locally refined

structure of the same system. These entries were therefore treated as related structural representations rather than independent scaffold types.

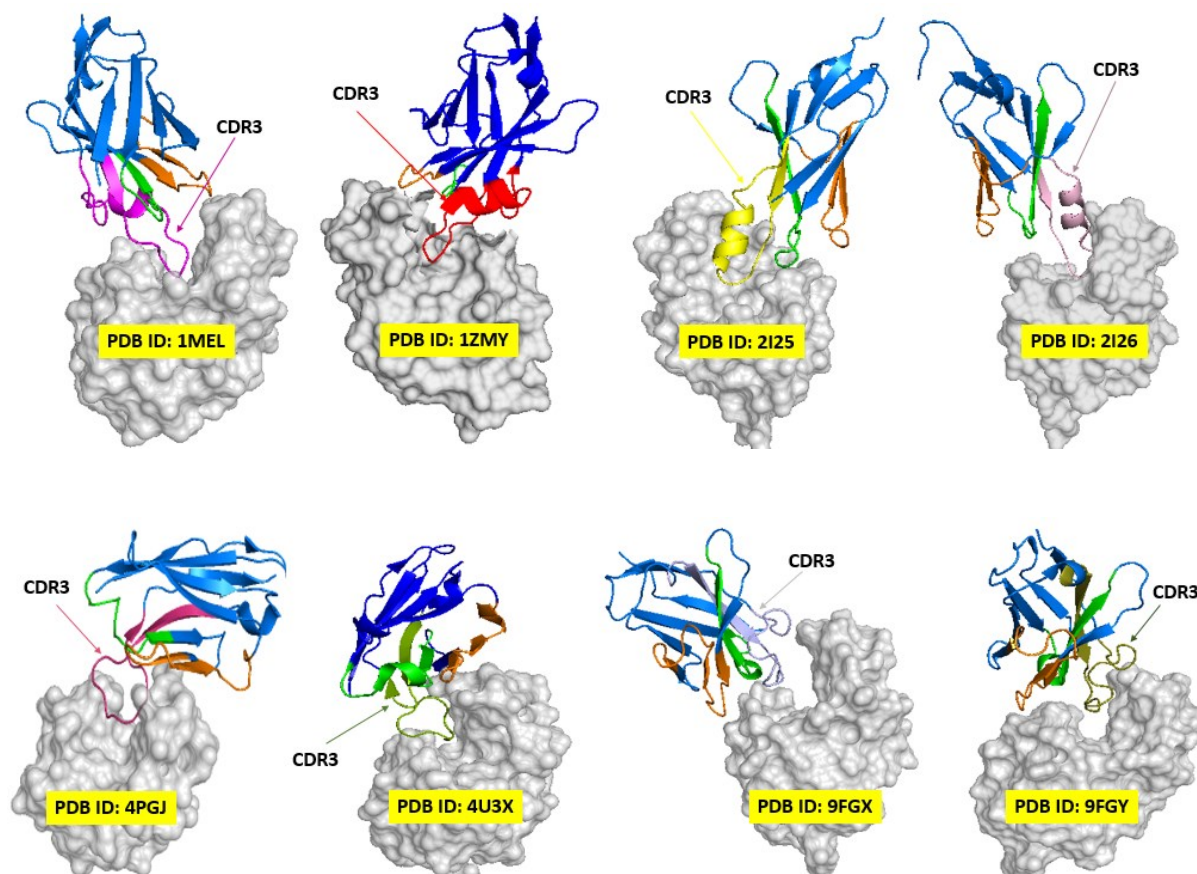


Figure 6. Overview of the eight selected lysozyme-binding structures used for comparative CDR3-interface analysis, showing the corresponding PDB IDs and domain types.

3.2 Comparison of CDR3–antigen interface properties

The CDR3 regions of the eight selected complexes were compared according to their contribution to the antibody–antigen interface. The analysis included the number and identity of CDR3 residues participating in antigen contact, hydrogen bonds, salt bridges, CDR3-associated buried interface area, the relative percentage contribution of CDR3 to the antibody-side interface area, and the PDBePISA-reported solvation free-energy gain upon interface formation (ΔiG). These parameters were used to compare the structural contribution of CDR3 across the selected complexes and are summarized in Table 2.

Table 2. CDR3–antigen interface properties of the eight selected nanobody–antigen complexes.

PDB ID	Experimental (nM)	K _D	CDR3 atoms in interface (CDR3 atomic contribution to VHH binding)	CDR3 residues in interface	CDR3 residues / sequence	CDR3 H-bonds / salt bridges	CDR3-lysozyme Interface area (Å ²)	ΔG on CDR3-lysozyme complex formation (kcal/mol)
4U3X	19 ± 5 nM ^a		57 (78.5%)	11	99-109 Y T S P P Q N H G F D	19 / 4	614.90	−2.10
4PGJ	19 ± 5 nM ^a		54 (74.3%)	11	99-109 Y T S P P Q N H G F D	15 / 3	580.20	−2.00
2I26	9.4 ± 1.2 nM ^b		50 (82.4%)	9	88-96 R Y G S Y D A E C	13 / 6	589.40	−1.40
2I25	9.4 ± 1.2 nM ^b		44 (75.0%)	6	87-92 S R Y G S Y	12 / 4	563.50	−1.30
1MEL	5 ± 4 nM ^c		50 (66.5%)	11	99-109 D S T I Y A S Y Y E C	11 / 0	599.00	−4.80
1ZMY	7.6 nM ^d		43 (70.36%)	8	101-108 T I Y A S Y Y E	6 / 0	557.60	−5.30
9FGX	0.353 nM ^e		30 (55.5%)	7	100-107 R K A V P V R	0 / 2	410.70	−0.90
9FGY	0.316 nM ^e		28 (61.0%)	7	100-107 R K A V P V R	0 / 1	411.00	−1.80

^a(Rouet et al., 2015), ^b(Stanfield et al., 2007), ^c(Birchenough et al., 2021), ^d(Saerens et al., 2005), ^e(Yi et al., 2026)

Among the analyzed structures, 4U3X showed the most extensive CDR3 involvement in the antigen interface, with the highest number of CDR3 contact atoms and residues, together with a high number of hydrogen bonds and salt bridges and the largest CDR3–antigen interface area. The closely related structure 4PGJ showed a highly similar antigen-contacting CDR3 sequence and a comparable interaction profile, although with slightly lower contact values. These observations indicate that the CDR3 contact regions of these complexes make a substantial structural contribution to antigen recognition.

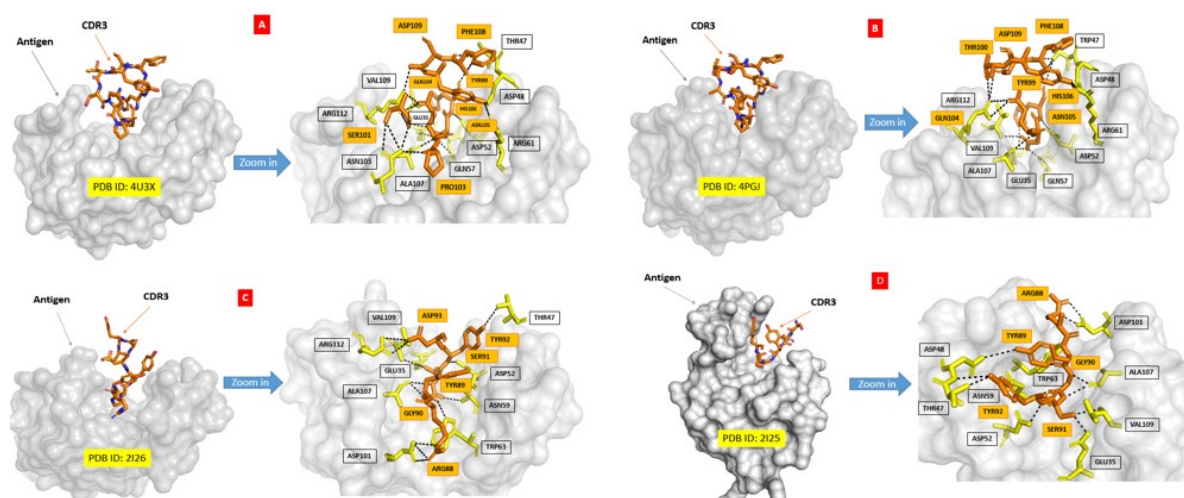
The 2I26 and 2I25 complexes also showed substantial CDR3 involvement, particularly through polar and electrostatic interactions. Although their interface areas were smaller than those observed for 4U3X and 1MEL, both structures formed multiple hydrogen bonds and salt bridges, indicating a well-defined interaction network between the CDR3 contact region and the antigen.

In contrast, the Di-Gluebody structures 9FGX and 9FGY showed the smallest CDR3 contact footprints and the lowest CDR3–antigen interface areas among the analyzed structures. They also displayed few or no hydrogen bonds and only limited salt-bridge formation involving the analyzed CDR3 residues. These observations suggest a more limited local contribution of this CDR3 contact region to the antigen interface compared with the other complexes.

The camelid VHH structures 1MEL and 1ZMY showed comparatively favorable PDBePISA-reported solvation free-energy gains upon interface formation (ΔiG). In particular, 1MEL also displayed extensive CDR3 involvement, with 11 contact residues, a relatively large CDR3-associated interface area, and a CDR3 contribution of 66.5% to the antibody-side interface. Neither 1MEL nor 1ZMY showed CDR3-associated salt bridges in the analyzed interface, suggesting that their interaction profiles may instead involve other non-covalent contributions, including hydrogen bonding and van der Waals contacts. The ΔiG values reported by PDBePISA represent calculated solvation free-energy changes associated with interface formation and were therefore used as interface-level energetic descriptors rather than as direct measurements of binding affinity.

Based on the overall interface comparison, 4U3X, 4PGJ, 2I26, and 1MEL showed comparatively extensive CDR3 involvement and favorable interface characteristics at this stage of the analysis. These structures combined relatively high CDR3 participation with multiple intermolecular contacts, including hydrogen bonds and, in some cases, salt bridges. They were therefore prioritized for subsequent comparative ranking and final candidate selection.

For six of the eight structures included in Table 2, the CDR3–antigen interfaces were additionally examined in PyMOL to visualize the spatial position and orientation of the antigen-contacting CDR3 region relative to the antigen surface and to identify the specific CDR3 and antigen residues contributing to the interactions summarized above (Figure 7).



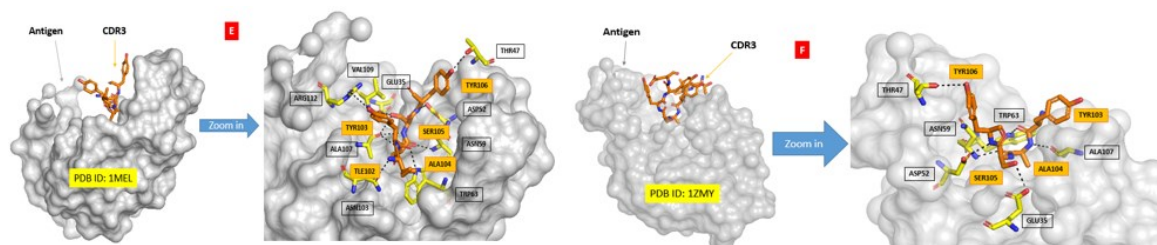


Figure 7. CDR3–antigen interface of six representative single-domain antibody–lysozyme complexes. For each panel, the left view shows the overall position of the CDR3 loop (orange sticks) on the surface of lysozyme (grey surface); the right view shows a close-up of the boxed interface, with CDR3 residues (orange labels) and antigen contact residues (white/yellow labels) shown as sticks and hydrogen bonds/salt bridges indicated as black dashed lines. (a) 4U3X; (b) 4PGJ; (c) 2I26; (d) 2I25; (e) 1MEL; (f) 1ZMY. The two Di-Gluebody constructs, 9FGX and 9FGY, are not shown, consistent with their comparatively small CDR3 contact footprint and interface area (Table 3).

Across the six panels, the extent of the orange CDR3 stick representation broadly reflects the contact-residue patterns reported in Table 2. In 4U3X (a) and 4PGJ (b), the CDR3 contact region extends deeply into the antigen-binding cleft and is associated with a dense network of polar interactions, consistent with the comparatively high numbers of hydrogen bonds and salt bridges reported for these complexes (19/4 and 15/3, respectively). In 2I26 (c) and 2I25 (d), the CDR3 contact regions form a more compact but still well-defined interaction zone, consistent with their intermediate CDR3–antigen interface areas.

In the camelid VHH complexes 1MEL (e) and 1ZMY (f), the CDR3 contact regions engage a comparatively narrower portion of the antigen surface. The interaction pattern visualized in these structures is less dominated by hydrogen-bond and salt-bridge networks than in 4U3X and 4PGJ. Other non-covalent contributions, including van der Waals interactions, may therefore contribute substantially to stabilization of these interfaces.

3.3 Selection of representative candidates for peptide design

Three CDR3-derived candidates from 4U3X, 2I26, and 1MEL were selected for subsequent peptide design and AlphaFold 3 structural evaluation. Final candidate selection was guided by the comparative interface descriptors summarized in Table 3, while also considering sequence redundancy among candidates with extensive CDR3 interface involvement.

Although 4PGJ showed extensive CDR3 involvement in the antigen interface, its antigen-contacting CDR3 sequence was highly similar to that of 4U3X. To avoid carrying forward two closely related peptide templates, 4U3X was retained as the representative of this sequence-similar group based on the combined interface descriptors.

2I26 was selected as a second candidate because it showed high CDR3 involvement in the antigen-binding interface together with a distinct CDR3 sequence. 1MEL was additionally retained as a sequence-distinct candidate, thereby increasing the structural and sequence diversity of the set selected for subsequent analysis.

Thus, the final selection of 4U3X, 2I26, and 1MEL was based on the combined interface analysis together with consideration of sequence redundancy among the candidate CDR3 regions.

3.4 AlphaFold 3-predicted CDR3-derived peptide–antigen models

Five AlphaFold 3 models were generated for each of the three selected CDR3-derived peptide–lysozyme complexes containing terminal cysteine residues introduced to enable potential disulfide-mediated conformational constraint. Model confidence was evaluated using the interface predicted TM-score (ipTM), predicted TM-score (pTM), minimum inter-chain predicted aligned error (PAE), and mean peptide atom pLDDT values. The corresponding confidence metrics are summarized in Table 3.

Table 3. AlphaFold confidence metrics for the five models generated per CDR3-derived cyclic peptide candidate.

Candidate	Model	pTM	ipTM	Mean peptide atom pLDDT	Minimum inter-chain PAE (Å), peptide→lysozyme / lysozyme→peptide	Ranking score
1MEL	0	0.92	0.85	90.14	1.02 / 0.93	0.90
1MEL	1	0.92	0.85	89.59	1.03 / 0.91	0.90
1MEL	2	0.92	0.85	88.39	0.99 / 0.91	0.90
1MEL	3	0.92	0.84	89.72	1.04 / 0.93	0.90
1MEL	4	0.92	0.84	89.19	1.02 / 0.92	0.90
2I26	0	0.92	0.86	89.42	1.24 / 1.07	0.91
2I26	1	0.92	0.85	89.56	1.25 / 1.11	0.91
2I26	2	0.92	0.85	89.48	1.32 / 1.16	0.91
2I26	3	0.92	0.85	89.54	1.28 / 1.13	0.90
2I26	4	0.91	0.85	88.60	1.31 / 1.15	0.90

4U3X	0	0.90	0.72	76.84	3.33 / 2.87	0.81
4U3X	1	0.89	0.70	75.46	3.60 / 3.10	0.79
4U3X	2	0.89	0.70	74.09	3.56 / 2.92	0.79
4U3X	3	0.89	0.68	74.21	3.91 / 3.31	0.77
4U3X	4	0.89	0.68	73.43	3.99 / 3.31	0.77

The 1MEL- and 2I26-derived peptides both showed consistently high ipTM values (0.84–0.86) and low minimum inter-chain PAE values (approximately 0.9–1.3 Å) across all five models, with no steric clashes. These results were consistent with higher confidence in the predicted peptide–lysozyme interfaces. In contrast, the 4U3X-derived peptide showed lower ipTM values (0.68–0.72) and higher minimum inter-chain PAE values (approximately 2.9–4.0 Å) across all models. Although the original 4U3X crystal structure showed extensive CDR3–antigen interface involvement (Table 3), the corresponding isolated peptide models were predicted with lower confidence in the relative peptide–lysozyme arrangement than the 1MEL- and 2I26-derived peptides.

The AlphaFold 3 predictions showed that all three CDR3-derived peptide designs adopted defined loop-like conformations at the lysozyme surface. The pLDDT representations showed generally high confidence for the lysozyme structures, while confidence in the peptide regions varied among the three candidates.

Differences in the geometry of the terminal cysteine pairs were also observed among the three predicted peptide models, as shown in Figures 8–10, and are evaluated separately in Section 3.6. Overall, the AlphaFold 3 models supported the structural plausibility of the three CDR3-derived peptide designs and provided structures for subsequent comparison with the corresponding experimental CDR3 regions. The selected AlphaFold 3 models were subsequently used as the starting structures for the molecular dynamics simulations described in Section 3.8.

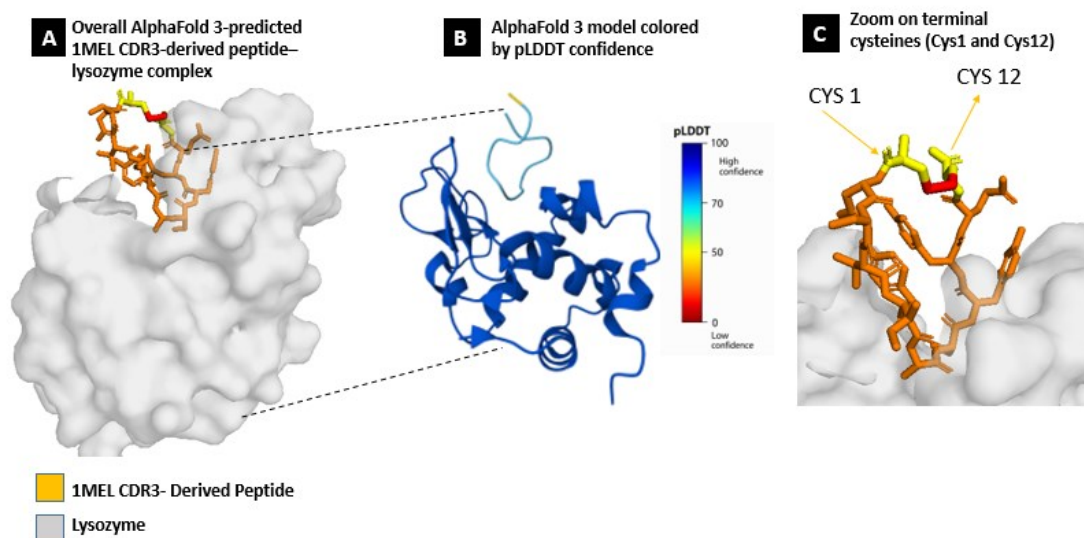


Figure 8. AlphaFold 3 prediction of the 1MEL-derived CDR3 peptide-lysozyme complex. (A) Overall predicted peptide-lysozyme complex. (B) pLDDT-based confidence representation of the AlphaFold 3 model. (C) Close-up of the introduced terminal Cys1 and Cys12 residues.

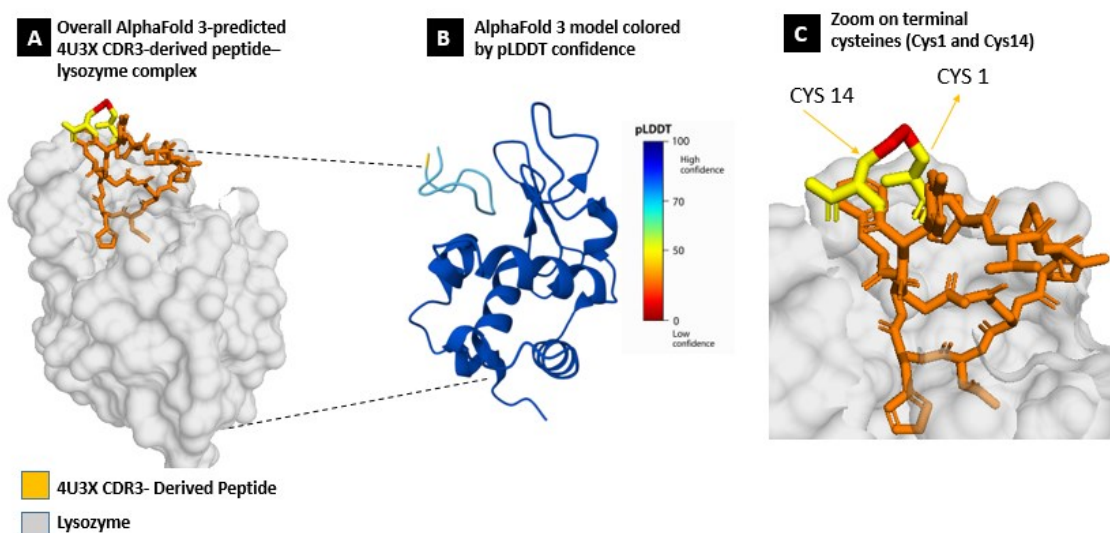


Figure 9. AlphaFold 3 prediction of the 4U3X-derived CDR3 peptide-lysozyme complex. (A) Overall predicted peptide-lysozyme complex. (B) pLDDT-based confidence representation of the AlphaFold 3 model. (C) Close-up of the introduced terminal Cys1 and Cys14 residues.

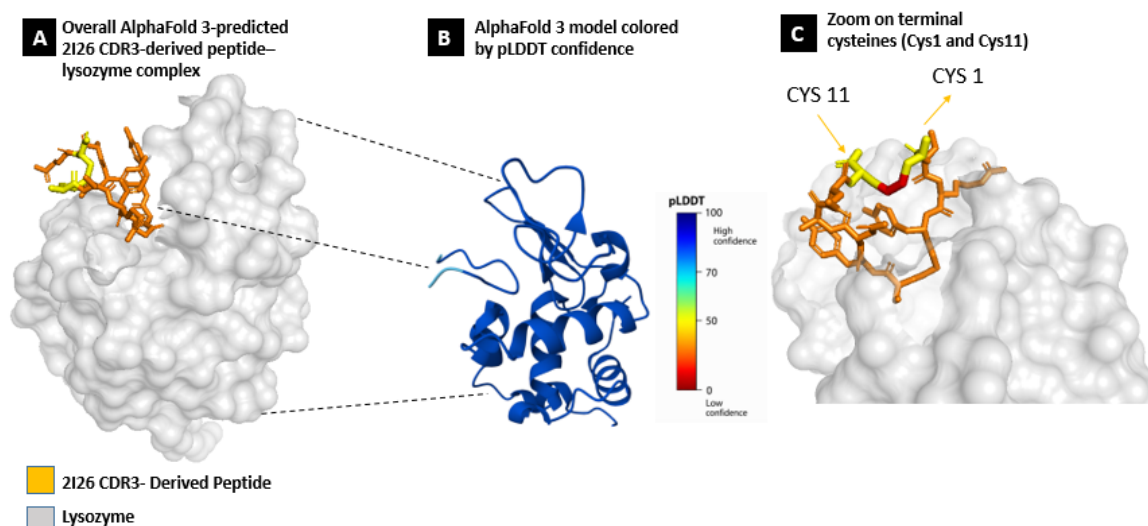


Figure 10. AlphaFold 3 prediction of the 2I26-derived CDR3 peptide–lysozyme complex. (A) Overall predicted peptide–lysozyme complex. (B) pLDDT-based confidence representation of the AlphaFold 3 model. (C) Close-up of the introduced terminal Cys1 and Cys11 residues.

3.6 Evaluation of terminal CYS–CYS geometry

The terminal cysteine geometries differed among the three predicted peptide models, as summarized in Table 4. The 1MEL-derived peptide showed an $S\gamma$ – $S\gamma$ distance of 1.8 Å between Cys1 and Cys12, whereas the 2I26-derived peptide showed a distance of 1.7 Å between Cys1 and Cys11. In contrast, the 4U3X-derived peptide showed an $S\gamma$ – $S\gamma$ distance of 2.04 Å between Cys1 and Cys14. The latter value is close to the typical S–S bond length of approximately 2.04 Å observed in protein disulfide bonds (Wiedemann et al., 2020), whereas the shorter distances observed for 1MEL and 2I26 indicate very close sulfur proximity and should be interpreted cautiously.

Table 4. Cys–Cys $S\gamma$ – $S\gamma$ distances measured in the terminal-cysteine-containing CDR3-derived peptide models.

Candidate	Cyclization residues	$S\gamma$ – $S\gamma$ distance (Å)	Interpretation
1MEL	Cys1–Cys12	1.8	Very close sulfur proximity; shorter than a typical disulfide bond length
2I26	Cys1–Cys11	1.7	Very close sulfur proximity; shorter than a typical disulfide bond length
4U3X	Cys1–Cys14	2.04	Geometrically compatible with a typical disulfide bond length

The three models showed close proximity between the terminal cysteine sulfur atoms, but the geometries differed substantially among candidates. The 4U3X-derived peptide showed an S γ –S γ distance of 2.04 Å, which is consistent with the typical geometric range of a disulfide bond. In contrast, the 1MEL- and 2I26-derived peptides showed shorter S γ –S γ distances of 1.8 and 1.7 Å, respectively. These values indicate close sulfur proximity but should not be interpreted as evidence of a formed covalent disulfide bond. Overall, the terminal-cysteine designs produced geometries that support further evaluation of potential disulfide-mediated conformational constraint, with 4U3X showing the most typical S–S geometry among the three candidates.

3.7 RMSD analysis between the predicted peptides and the experimental CDR3 conformations

To quantify the structural similarity between the AlphaFold 3-predicted CDR3-derived peptides and the corresponding experimentally observed CDR3 conformations, C α RMSD values were calculated after structural superposition using lysozyme as the common reference. Following antigen alignment, the predicted peptide was not further fitted to the native CDR3 region, allowing the RMSD values to reflect both conformational and positional differences of the peptide relative to the corresponding experimental CDR3 conformation. An example of the structural superposition is shown in Figure 11, while the RMSD values obtained for all five AlphaFold 3 models of each candidate are summarized in Table 5.

As shown in Figure 10, the largest structural deviations were mainly observed for peptide residues located farther from the lysozyme-binding pocket, whereas residues positioned within or close to the antigen-contacting region showed greater agreement with the corresponding experimental CDR3 conformation. This indicates that the binding region was comparatively well preserved, while larger deviations occurred primarily in the more distal regions of the peptide.

For 1MEL, the five AlphaFold 3 models showed C α RMSD values ranging from 1.128 to 1.497 Å, with Model 0 showing the closest agreement with the experimental CDR3 conformation (1.128 Å). The relatively narrow range among the five models indicates that the predicted 1MEL-derived peptide adopted broadly similar positions and conformations across the AlphaFold 3 predictions.

The 2I26-derived peptide showed the closest overall structural agreement with the experimental CDR3 among the three candidates. RMSD values ranged from 0.667 to 1.191 Å, with Model 0 providing the lowest value (0.667 Å). These results indicate that the predicted 2I26-derived peptide reproduced the experimentally observed CDR3 position and backbone geometry more closely than the corresponding 1MEL and 4U3X designs.

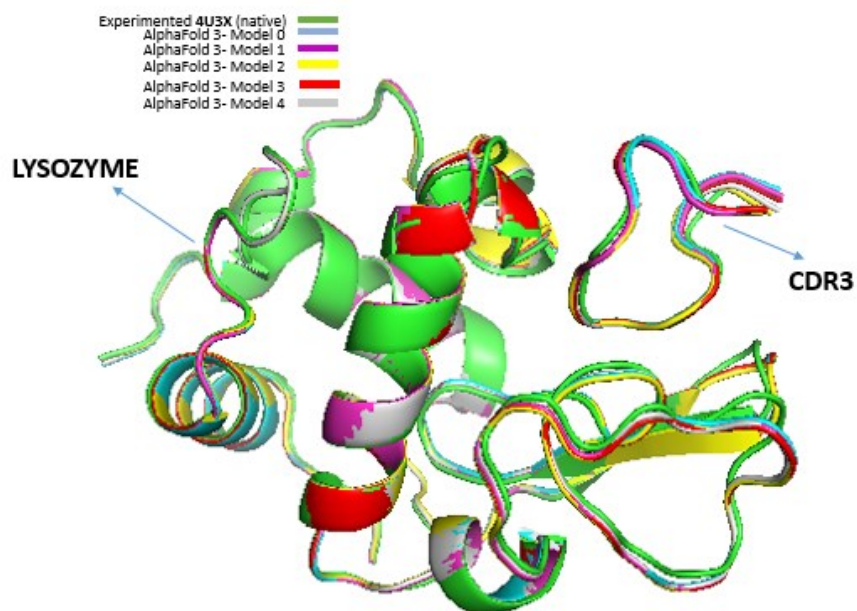
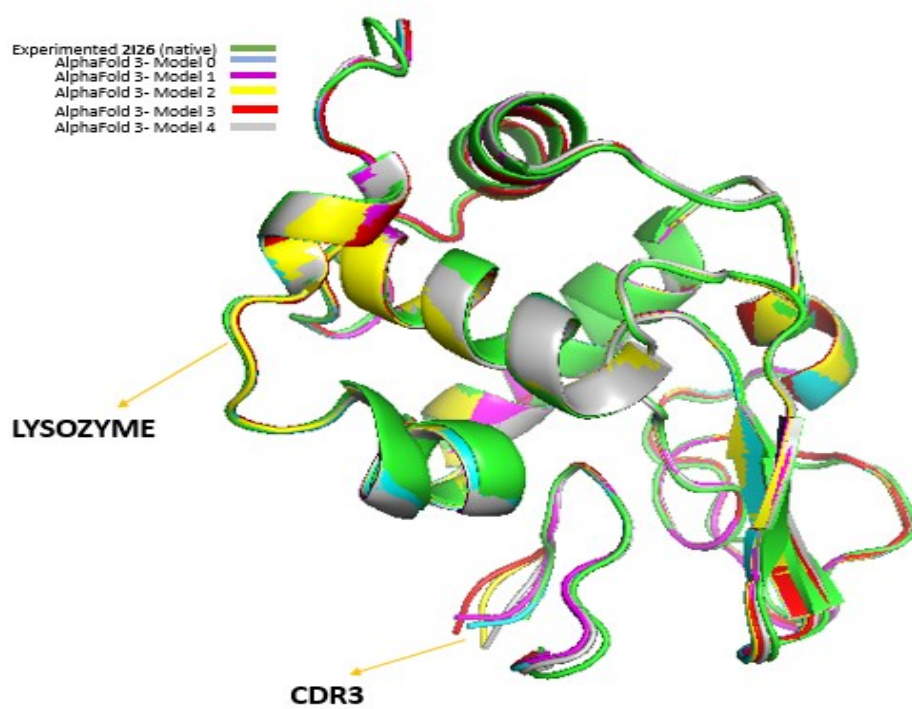
For 4U3X, greater variability was observed among the five predicted models. RMSD values ranged from 1.190 to 1.694 Å, with Model 4 showing the closest agreement with the

experimental CDR3 conformation (1.190 Å). Models 0–3 displayed higher deviations, ranging from 1.531 to 1.694 Å, indicating greater model-to-model variability in the predicted position and conformation of the isolated CDR3-derived peptide.

Overall, the AlphaFold 3-predicted peptides reproduced binding conformations that were highly similar to those adopted by the corresponding CDR3 regions in the experimental structures. The relatively small deviations observed in the antigen-contacting regions suggest that the terminal-cysteine-based cyclization strategy preserved the native-like binding geometry to a substantial extent, while larger structural differences were mainly located farther from the binding pocket. The close agreement between the predicted and experimentally determined conformations may also indicate that the AlphaFold 3 predictions are influenced by structural information related to previously characterized complexes, resulting in a possible memory or overfitting effect toward the experimental binding conformations.

Table 5. C α RMSD values for the five AlphaFold 3 models of each CDR3-derived peptide after lysozyme-based structural superposition.

Candidate	Model 0	Model 1	Model 2	Model 3	Model 4	Best Model
1MEL	1.128 Å	1.135 Å	1.366 Å	1.223 Å	1.497 Å	Model 0, 1.128 Å
2I26	0.667 Å	0.731 Å	1.027 Å	1.191 Å	0.792 Å	Model 0, 0.667 Å
4U3X	1.610 Å	1.694 Å	1.618 Å	1.531 Å	1.190 Å	Model 4, 1.190 Å



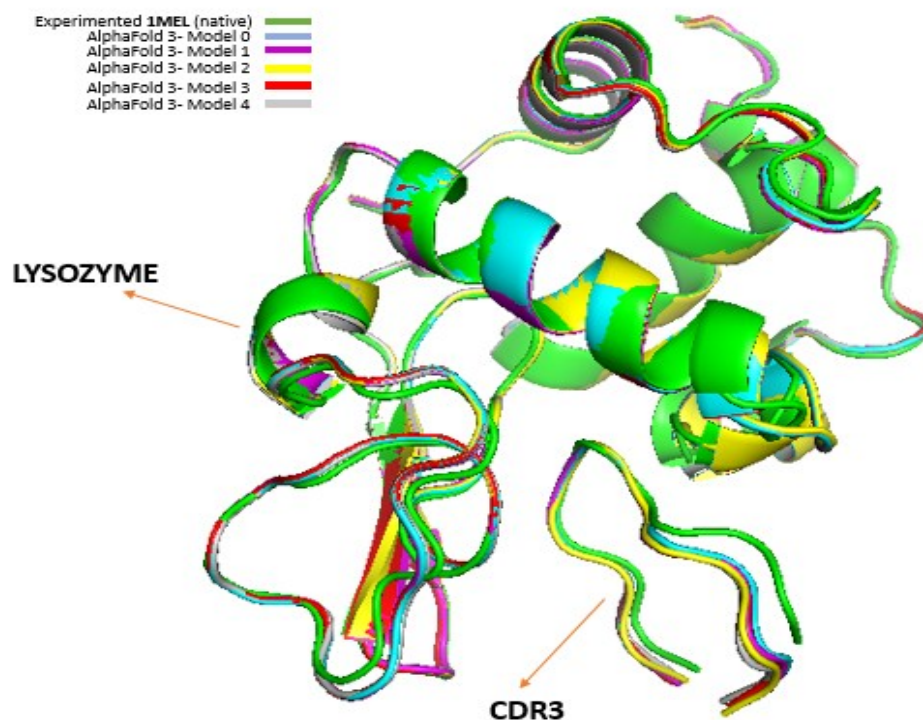


Figure 11. Structural superposition of experimental and AlphaFold 3-predicted peptide–lysozyme complexes for 1MEL, 2I26, and 4U3X.

3.8 Molecular dynamics analysis of the selected peptide–lysozyme complexes

3.8.1 RMSD analysis

Comparison of the RMSD profiles revealed distinct patterns of structural deviation among the three peptide–protein complexes (Figure 12). The 1MEL complex exhibited the highest RMSD values during most of the simulation, with the RMSD predominantly fluctuating between approximately 0.30 and 0.45 nm after the initial stage. The 2I26 complex showed intermediate RMSD values, with the RMSD mainly fluctuating between approximately 0.30 and 0.38 nm during the later stages of the simulation. In contrast, 4U3X displayed the lowest RMSD values, predominantly fluctuating between approximately 0.24 and 0.29 nm after the first 40 ns. Overall, the three complexes exhibited an increment of RMSD values during the first stages of the MD trajectory, indicating that the starting peptide-lysozyme binding conformations undergo different structural rearrangements. The highest RMSD values observed with 1MEL shows a major structural deviation of the complex conformation with respect to the other peptide binders.

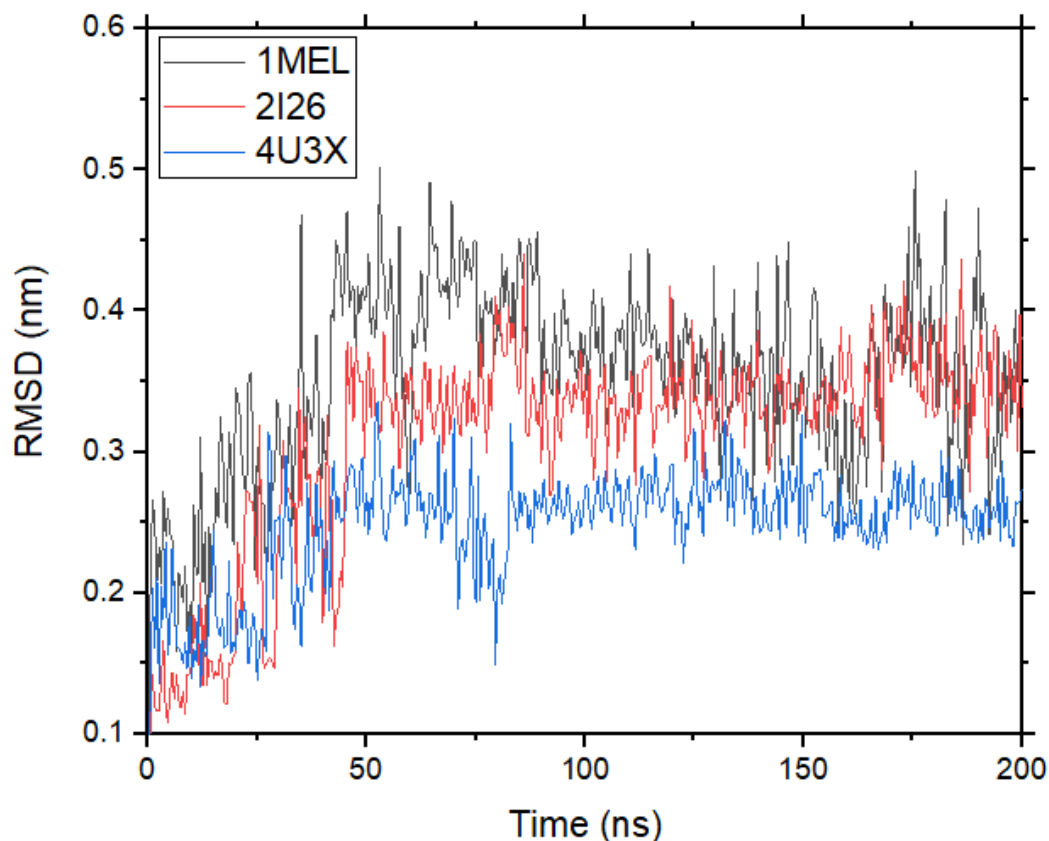


Figure 12. Peptide RMSD profiles of the 1MEL-, 2I26-, and 4U3X-derived peptide-lysozyme complexes during the 200 ns molecular dynamics simulations.

To further visualize the structural changes indicated by the RMSD analysis, the final MD structures were superimposed on the corresponding AlphaFold 3 models using the lysozyme backbone as the common reference.

The structural superposition of the initial AlphaFold 3 models and the final MD structures is shown in Figure 13. The overall architecture of the three peptide-lysozyme complexes was largely preserved after 200 ns of simulation. The main structural differences were localized to flexible regions, while most of the lysozyme backbone remained closely superimposed. Among the three complexes, 1MEL showed more evident deviations between the initial and final structures, whereas 2I26 and 4U3X displayed a higher degree of overall structural overlap. These observations are consistent with the RMSD profiles, which showed larger structural deviations for 1MEL and comparatively smaller deviations for the other two complexes.

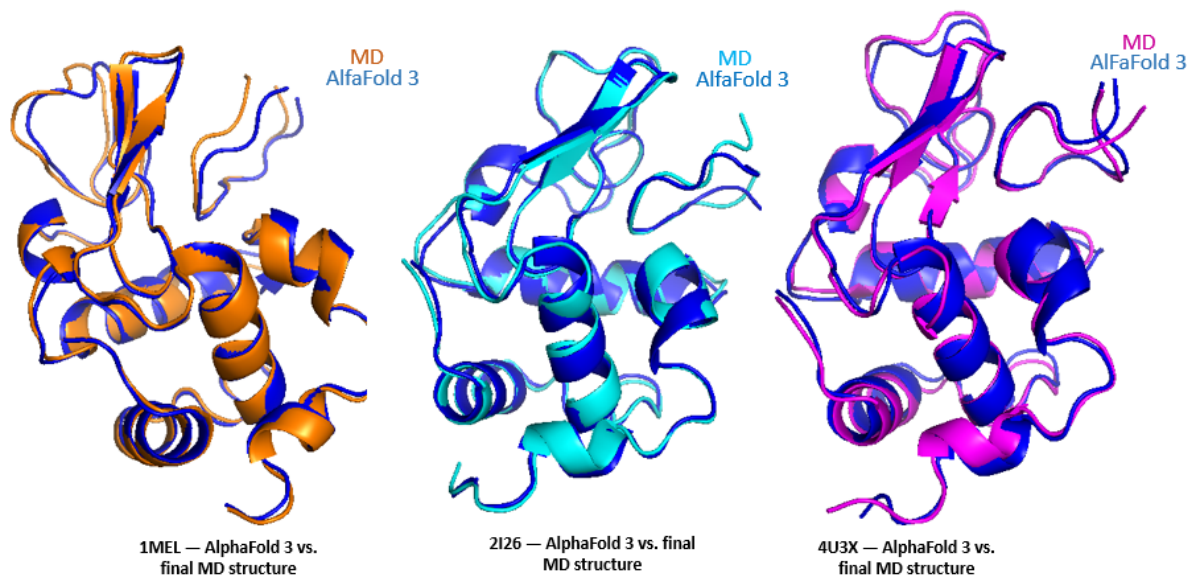


Figure 13. Structural comparison of the AlphaFold 3-predicted and final MD structures of the 1MEL, 2I26, and 4U3X-derived peptide–lysozyme complexes after 200 ns of simulation.

3.8.2 Center-of-mass distance analysis

Comparison of the center-of-mass (COM) distances between each peptide and lysozyme along the MD trajectories revealed distinct patterns among the three complexes. The evolution of the peptide–lysozyme COM distance throughout the 200 ns simulations is shown in Figure 14.

The 1MEL complex showed COM distances mainly within the range of approximately 1.50–1.65 nm throughout the simulation, with relatively limited fluctuations over time. In 2I26, the COM distance was initially lower, fluctuating mainly around 1.40–1.50 nm, and gradually increased during the simulation, reaching predominantly approximately 1.47–1.59 nm during the latter part of the trajectory. The 4U3X complex generally exhibited higher COM distance values, predominantly fluctuating between approximately 1.53 and 1.65 nm, with transient peaks reaching approximately 1.73 nm. Overall, the COM-distance profiles indicate that the relative separation between each peptide and lysozyme remained within a limited range throughout the 200 ns trajectories, although small shifts were observed. The 2I26 complex showed the clearest gradual increase in COM distance over time, whereas 1MEL and 4U3X displayed comparatively smaller changes in their average separation.

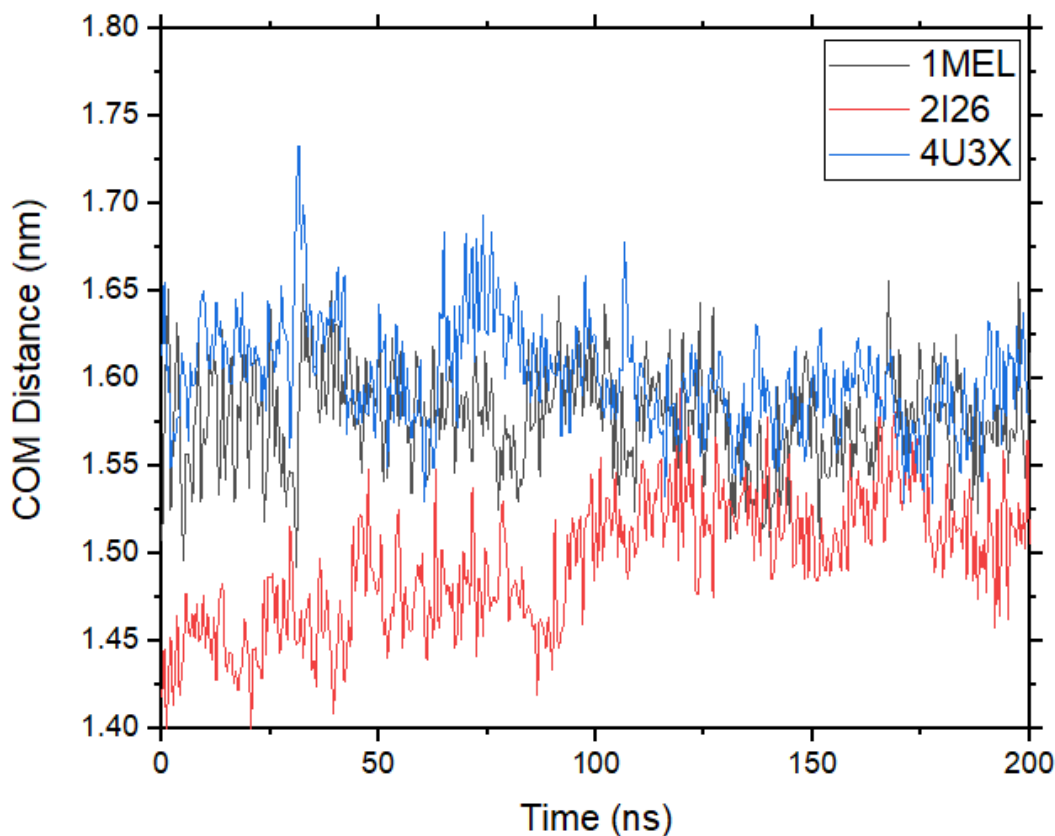


Figure 14. Evolution of the center-of-mass (COM) distance between the CDR3-derived peptide and lysozyme for the 1MEL-2I26, and 4U3X derived peptide-lysozyme complexes during the 200 ns molecular dynamics simulations.

3.8.3 Intermolecular contact analysis

Comparison of the number of intermolecular contacts revealed distinct contact profiles among the three complexes (Figure 15). 1MEL generally exhibited the lowest number of contacts, predominantly fluctuating between approximately 750 and 900 contacts. The 2I26 complex showed intermediate contact numbers, with values mainly ranging from approximately 850 to 1000 contacts and gradual increases to around 1000 contacts toward the end of the trajectory. In contrast, 4U3X generally displayed the highest number of contacts, with values predominantly fluctuating between approximately 900 and 1100 and transient peaks reaching approximately 1150–1200 contacts. Overall, 4U3X exhibited the highest number of intermolecular contacts, whereas 1MEL showed the lowest contact counts, with 2I26 displaying intermediate values.

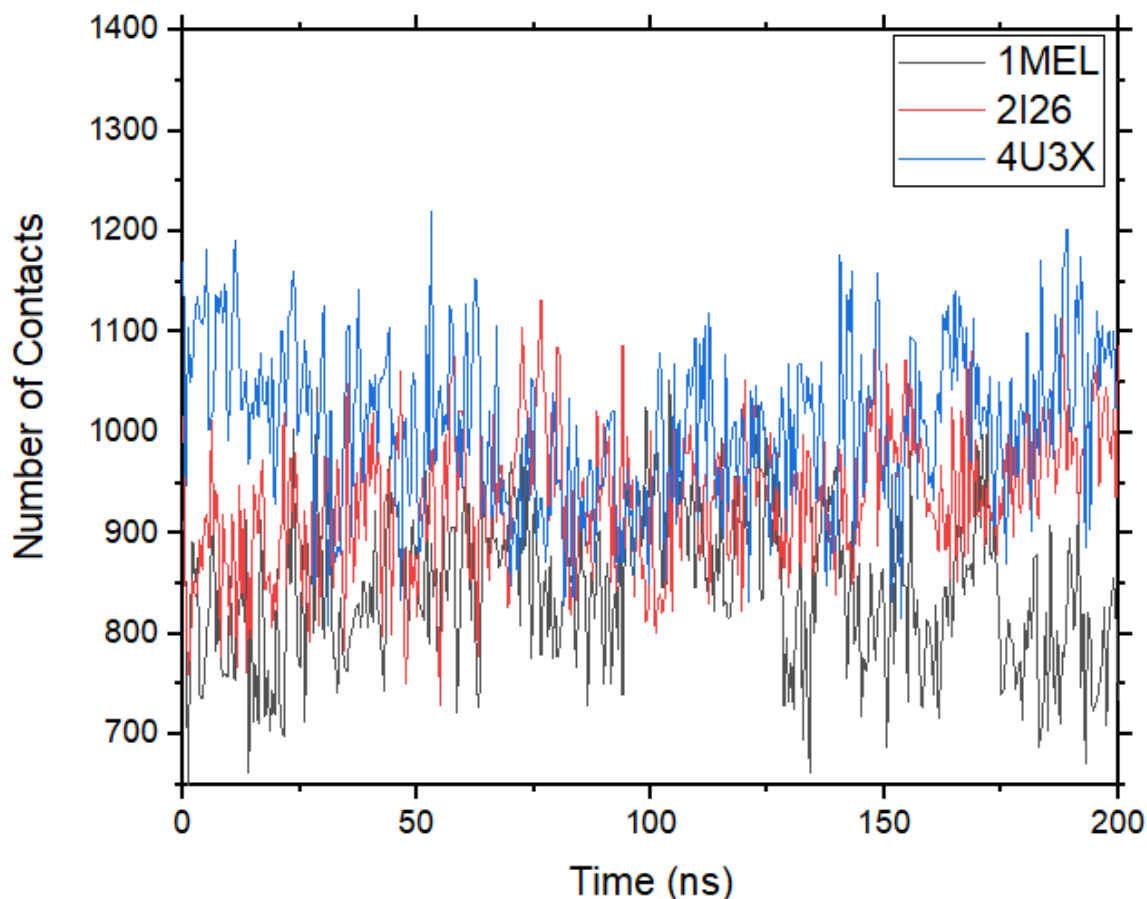


Figure 15. Number of intermolecular atomic contacts between each CDR3-derived peptide and lysozyme during the 200 ns molecular dynamics simulations for the 1MEL, 2I26, and 4U3X derived peptide–lysozyme complexes.

3.9 DFIRE scoring

The DFIRE analysis yielded scores of 19.09, 17.75, and 17.62 for 1MEL, 2I26, and 4U3X, respectively. Higher DFIRE scores indicate higher predicted binding affinity; therefore, the predicted affinity followed the order 1MEL > 2I26 > 4U3X. This trend is compared with the reported experimental binding affinities of the corresponding parent single-domain antibody-derived binders. The experimental K_D values were approximately 5 ± 4 nM for 1MEL (Birchenough et al., 2021), 9.4 ± 1.2 nM for 2I26 (Stanfield et al., 2007), and 19 ± 5 nM for 4U3X (Rouet et al., 2015). Since lower K_D values correspond to higher binding affinities, the experimental data showed the same affinity order as the DFIRE predictions. Thus, the DFIRE results were qualitatively consistent with the experimentally reported binding-affinity trend for the three complexes, confirming first the prominent role of each nanobody CDR3 in the binding and the success in conserving the binding characteristics of the CDR3 after engineering them into cyclic peptides.

The DFIRE score profiles showed different time-dependent behavior among the three peptide–lysozyme complexes (Figure 16). For 1MEL, the DFIRE score increased during the first part of the simulation, reaching higher values approximately between 50 and 80 ns, followed by a partial decrease and continued fluctuations around a relatively high level for the remainder of the trajectory. In contrast, 2I26 and 4U3X showed larger fluctuations during the first half of the simulation but reached comparatively more stable score ranges after approximately 100 ns. The two profiles largely overlapped during this later period, whereas 1MEL remained consistently higher. Overall, the average DFIRE scores were 19.09, 17.75, and 17.62 for 1MEL, 2I26, and 4U3X, respectively, corresponding to the predicted affinity order 1MEL > 2I26 > 4U3X.

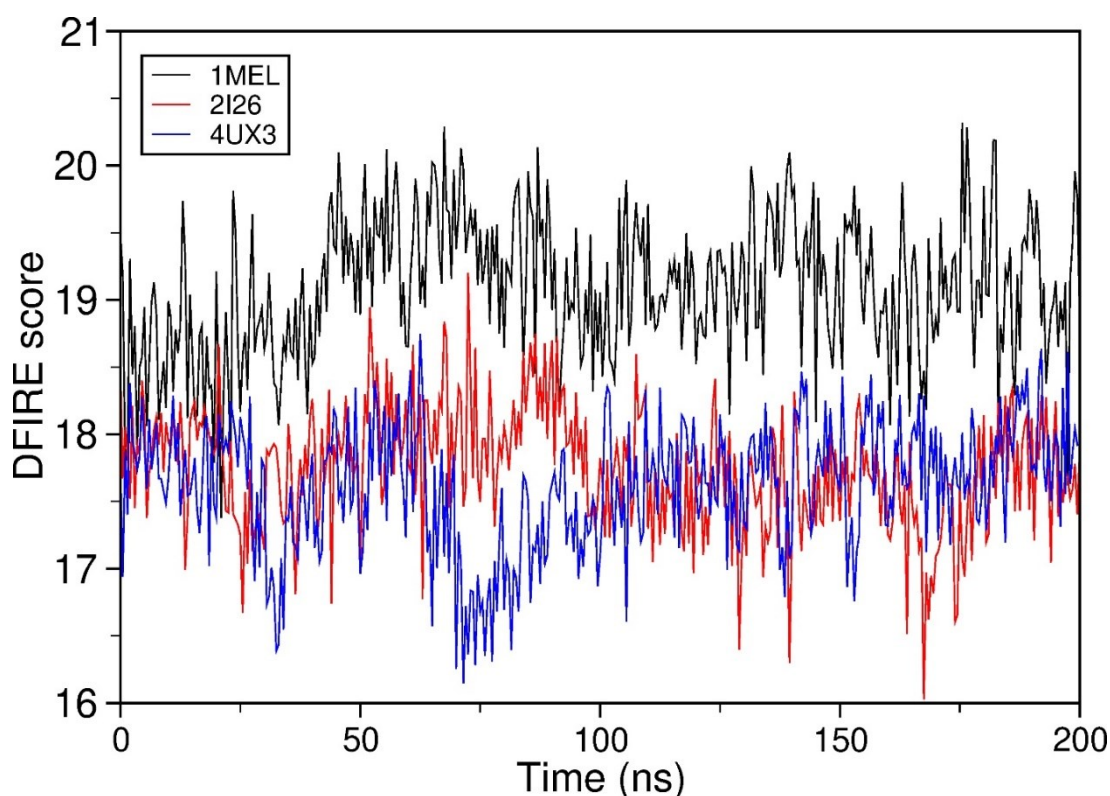


Figure 16. Evolution of DFIRE scores for the 1MEL, 2I26, and 4U3X derived peptide–lysozyme complexes during the 200 ns molecular dynamics simulations.

3.10 Overall comparison of the three complexes

No single parameter provides a complete assessment of the overall behavior or interaction properties of a peptide–protein complex, as each molecular dynamics analysis describes a different aspect of the system. RMSD reflects structural deviations during the simulation with respect to the initial binding conformation, COM distance describes changes in the relative positioning of the interacting components, and the number of intermolecular contacts provides information about the extent and persistence of the interaction interface. DFIRE provides an

additional scoring-based estimate of binding affinity, with higher scores indicating higher predicted binding affinity. Therefore, the three complexes were evaluated by considering these parameters together rather than relying on a single analysis.

An important observation from the structural analyses was that all three peptide–protein complexes underwent structural rearrangements during the MD simulations relative to their original AlphaFold 3-predicted binding conformations. Thus, none of the initial binding poses was fully preserved throughout the simulations, although all peptides remained in the lysozyme binding pocket along the whole MD trajectory. Among the three systems, 4U3X showed comparatively lower RMSD values during most of the simulation and maintained a relatively high number of intermolecular contacts, indicating comparatively stable structural behavior according to these descriptors. However, the DFIRE analysis showed a different trend, ranking 1MEL highest in predicted binding affinity, followed by 2I26 and 4U3X. Therefore, the structural descriptors and DFIRE scoring provided complementary but not fully concordant information for the three complexes. In summary, all three engineered cyclic peptides show in general a stable binding to the lysozyme catalytic pocket after some initial conformational arrangements, while small structural and energetic differences are observed among them. This result enhances the potential of the grafting-based design method of cyclic peptides from nanobodies studied in this work. Nevertheless, further computational and experimental studies should be necessary in the future to confirm this positive outcome.

4. CONCLUSION

4.1. Summary of main findings

This study investigated whether antigen-contacting CDR3 regions from single-domain antibody–lysozyme complexes could serve as structural templates for the design of constrained CDR3-derived peptides. Following structural screening and comparative interface analysis, three representative candidates, 1MEL, 2I26, and 4U3X, were selected for peptide design, AlphaFold 3 modeling, structural comparison, and molecular dynamics analysis.

Terminal cysteine residues were introduced to permit potential disulfide-mediated conformational constraint. AlphaFold 3 predictions showed that the resulting peptide designs retained substantial structural similarity to the corresponding experimentally observed CDR3 conformations. Among the three candidates, 2I26 showed the closest agreement with the experimental CDR3 structure, with a best C α RMSD of 0.667 Å, compared with 1.128 Å for 1MEL and 1.190 Å for 4U3X.

Analysis of terminal cysteine geometry showed that the 4U3X-derived peptide had an S γ –S γ distance of approximately 2.04 Å, which was geometrically compatible with a typical disulfide bond. In contrast, 1MEL and 2I26 showed shorter distances of 1.8 and 1.7 Å, respectively.

These values indicated close sulfur proximity but did not by themselves demonstrate the formation of a covalent disulfide bond.

Molecular dynamics simulations further showed that all three peptide–lysozyme complexes underwent structural rearrangements relative to their initial AlphaFold 3 conformations. Nevertheless, the peptides remained associated with the lysozyme binding pocket throughout the 200 ns simulations. The three systems displayed different dynamic characteristics: 4U3X showed comparatively lower RMSD values and the highest number of intermolecular contacts, whereas 1MEL exhibited larger structural deviations.

DFIRE analysis provided an additional energetic comparison. The average scores were 19.09 for 1MEL, 17.75 for 2I26, and 17.62 for 4U3X, corresponding to the predicted affinity order 1MEL > 2I26 > 4U3X according to the scoring convention used in this study.

Overall, the three CDR3-derived peptide designs investigated in this study retained important structural features of the corresponding parent antigen-recognition modes in the computational models, while subsequent molecular dynamics simulations reveal that these peptide–antigen complexes remain dynamic and undergo conformational adaptation. The different structural and scoring descriptors were not fully concordant, indicating that no single parameter is sufficient to characterize the behavior of these designed peptides.

4.2 Comparative interpretation of the three candidates

The three selected peptide designs showed complementary characteristics rather than a single candidate consistently performing better across all analyses. The 2I26-derived peptide showed the closest structural reproduction of the experimentally observed CDR3 conformation in the AlphaFold 3 comparison. In contrast, 4U3X showed the most geometrically compatible terminal cysteine arrangement and, during MD simulations, exhibited comparatively lower RMSD values and the highest number of intermolecular contacts. 1MEL showed larger structural deviations during MD but achieved the highest average DFIRE score among the three complexes.

These observations demonstrate that structural similarity to the experimental CDR3, dynamic behavior, interface contacts, cysteine geometry, and DFIRE scoring describe different aspects of peptide–antigen recognition. Therefore, the three candidates should be considered complementary designs for subsequent investigation rather than being reduced to a single universally superior candidate.

4.3 Limitations of the study

Several limitations should be considered when interpreting the results of this study. First, the structural screening was restricted to a relatively small set of single-domain antibody–lysozyme complexes. Although the use of a common antigen facilitated direct comparison among

different CDR3 regions, the conclusions cannot yet be generalized to other nanobody–antigen systems.

Second, the designed peptides were derived from CDR3 regions that normally function within the structural context of a complete antibody domain. Removal from the parent framework may alter conformational preferences, local interactions, and binding behavior. Nevertheless, this risk has been minimized by selecting CDR3 regions previously classified as extended (and therefore they have minimal interaction with the VHH framework), as well as by performing a computational analysis of the binding behavior of the peptide-protein complexes along MD trajectories.

Third, the AlphaFold 3 models and molecular dynamics simulations provide computational evidence but do not constitute experimental confirmation of peptide binding. In particular, structural similarity, low RMSD values, persistent contacts, or favorable DFIRE scores should not be interpreted as direct measurements of experimental binding affinity.

Finally, the experimental affinity values used for comparison correspond to the parent binders rather than to the newly designed peptides. Consequently, the apparent agreement between the DFIRE ranking and the reported experimental affinity trend should be regarded as supportive rather than as direct experimental validation of the peptide designs.

4.4 Future perspectives

The next step would be to test the designed CDR3-derived peptides experimentally. After synthesis, their structural stability should be assessed to determine whether the conformations predicted *in silico* can also be maintained under experimental conditions. Their ability to bind lysozyme could then be measured quantitatively and compared with the computational predictions obtained in this study.

The computational analysis could also be extended. Longer or replicate molecular dynamics simulations would help assess whether the observed peptide–lysozyme interactions are reproducible over time. In addition, approaches such as MM/PBSA or MM/GBSA could provide a further estimate of the relative binding energetics of the different peptide–lysozyme complexes.

It would also be useful to apply the same design strategy to other single-domain antibody–antigen complexes. This would help determine whether the approach is specific to the lysozyme system or whether it can be transferred to other targets. A direct experimental comparison between the parent nanobody, the isolated linear CDR3 sequence, and the corresponding disulfide-constrained peptide would be especially informative for understanding how constraining the CDR3 sequence affects its structure and antigen recognition.



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Declaration on the use of Artificial Intelligence tools

During the preparation of this thesis, Artificial Intelligence (AI)-based tools were used as supportive tools for language revision and scientific illustration development. ChatGPT (OpenAI) was used to improve grammar, sentence clarity, and readability of selected parts of the manuscript. In addition, ChatGPT was used to assist in the preparation of two illustrative figures included in this thesis.

The use of AI-generated or AI-assisted content was limited to graphical support and language refinement. The scientific analysis, methodological decisions, structural evaluations, data interpretation, and conclusions presented in this thesis were independently performed by the author. All AI-assisted outputs were critically reviewed and verified by the author for accuracy, scientific consistency, and appropriate representation of the underlying concepts.

Grammarly was also used as a language-support tool for grammar checking and improving English writing clarity.

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