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Review

Affinity maturation of antibody fragments: A review encompassing the development from random approaches to computational rational optimization

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ABSTRACT

Routinely screened antibody fragments usually require further *in vitro* maturation to achieve the desired biophysical properties. Blind *in vitro* strategies can produce improved ligands by introducing random mutations into the original sequences and selecting the resulting clones under more and more stringent conditions. Rational approaches exploit an alternative perspective that aims first at identifying the specific residues potentially involved in the control of biophysical mechanisms, such as affinity or stability, and then to evaluate what mutations could improve those characteristics. The understanding of the antigen-antibody interactions is instrumental to develop this process the reliability of which, consequently, strongly depends on the quality and completeness of the structural information. Recently, methods based on deep learning approaches critically improved the speed and accuracy of model building and are promising tools for accelerating the docking step. Here, we review the features of the available bioinformatic instruments and analyze the reports illustrating the result obtained with their application to optimize antibody fragments, and nanobodies in particular. Finally, the emerging trends and open questions are summarized.

1. Introduction

More than 30 years have passed since the United States Food and Drug Administration (US FDA) approved the first monoclonal antibody in 1986 [1]. In this period, antibody engineering has become progressively simpler due to the introduction of more precise and versatile techniques of gene manipulation. This technical progress allowed the development of a large number of biotherapeutics based on monoclonal antibodies [2] that have been effectively humanized and produced in multivalent and multifunctional formats [3,4]. Multivalent constructs can exploit avidity to compensate for low affinity, that for therapeutic drug development should be lower than 1 nM. Multispecific constructs provide even more selective binders by combining ligands specific for independent epitopes [5]. In the perspective of constructs combining more ligands, recombinant antibody fragments offer clear advantages over conventional IgGs (mAbs) because their short sequences can be engineered with basic genetic techniques to improve the construct biophysical and functional features and their derivatization is simple and

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reproducible [6]. Furthermore, their physical dimension remains limited also when polymers are constructed, they have low production costs and, at least when nanobodies are used, have reduced immunogenicity [7,8]. The circulation half-life of recombinant antibodies can be tuned by varying the overall dimension and/or promoting the binding to albumin and IgGs [9,10]. At least in the case of nanobodies, there is the option to choose between libraries obtained from immunized animals and pre-immune libraries, naïve or synthetic. The first option exploits the *in vivo* somatic maturation to recover clones with increased affinity and specificity for a particular antigen, whereas pre-immune libraries can be challenged against any antigen and have the advantage to meet the expectations of the regulative authorities that, especially in the European Union, incentivize protocols that avoids the exploitation of lab animals and spare their life [11–13]. The quality of a pre-immune library depends on several features, but it is accepted that larger functional diversity facilitates the identification of better binders. Consequently, the adopted display system might significantly contribute to the possibility of identifying effective binders because it determines the library theoretical dimension as well as the panning efficiency [14,15]. In most of the cases, the process of selection starting from antibody fragment pre-immune collections results in the isolation of clones that are considered "hits" and require further *in vitro* maturation to achieve the desired biophysical characteristics. This procedure, that comprises an extensive quality control step [13], is laborious and time-consuming but necessary to reach reagents with standards suitable for therapeutic applications [16].

If affinity is a key factor for antibody evaluation, productivity, low aggregation propensity and stability are other critical elements to consider to optimize the overall antibody developability and this implies the necessity to try optimizing all these features [17]. The major issue of this step is that the improvement of different biophysical parameters often requires conflicting actions, namely the optimization of a parameter can induce a negative effect on another. From this point of view, nanobodies proved being particularly valid candidates for optimization because their often high initial stability can better stand structural modifications introduced to improve other features, such as affinity [18]. Various *in vitro* strategies have been developed to identify improved candidates inside a pool of mutants of the initial hit [19]. These "blind" approaches rely on the empiric capacity to select clones with increased biophysical characteristics by adopting progressively more stringent conditions during the screening of the mutant libraries. By applying such procedures, it is possible, for instance, isolating clones with higher affinity with respect to the original binder but still maintaining the structural stability that enable correct display as soluble molecules on the phage surface. The drawback of the method is that it does not "explain" the rationale that provides the improved characteristics. Alternative approaches exploit strategies based on sequence comparison to identify key residues potentially involved in stability and aggregation. Finally, structural models and the analysis of their biophysical parameters can be used to design binders, from both initial hits or totally *ex novo,* and introduce specific variants [20]. The advantage of using antibody fragments such as nanobodies is represented by their short sequence that requires less computational effort for any kind of analysis with respect to large molecules such as IgGs [21].

In recent years, computer-assisted *in silico* affinity maturation technology has critically progressed taking advantage of the rapid development of protein structure-based rational design, algorithm optimization, and higher computing power availability. In parallel, our overall knowledge of antibody-antigen complex structures has grown [22,23]. Different strategies for antibody fragment rational design have been developed, which include homology modelling [24], molecular dynamics simulation [25], molecular docking [26], mutation hotspots design [27], and interfaces residues analysis [28]. Altogether, computer-assisted antibody fragment rational design is maturing into an effective tool for the optimization of their biophysical properties such as affinity, specificity, and stability [29,30]. Both *in vitro* and *in silico* optimization approaches applied to scFvs and nanobodies have been described and commented [14,31–33] but the analysis remained confined to specific topics and applications. At the same time, this research field evolves very rapidly and updates require to be integrated to provide a complete picture (Fig. 1). In this review, we have summarized the potential and the challenges of the different available approaches by comparing the methods used to mature recombinant ligands *in vitro* and *in silico* and to predict antibody-antigen interactions. Mature technologies have been mentioned briefly and illustrated by few references, whereas more attention was dedicated to fast evolving research fields to outline emerging trends and applications.

2. Approaches suitable for *in vitro* affinity maturation

In humoral immunity, somatic maturation is responsible for the progressive affinity increase of antibodies towards antigens [34]. *In vitro* maturation tries to reproduce such mutagenesis process by introducing more or less controlled mutations in the original sequences. The resulting mutant libraries are then screened using conditions selected to favor the isolation of clones with improved characteristics, such as binding capacity or structural stability. The process does not require any preliminary knowledge about structures and antigen-antibody complex characteristics, has proved being effective in several cases and can be implemented adopting alternative mutagenesis strategies. Given the random nature of the approach, *in vitro* mutagenesis can introduce sequence liabilities that can affect the antibody developability and this issue must be acknowledged and addressed [35].

2.1. Point mutation

The point mutation approach aims at introducing random mutations of few amino-acids in each newly generated clone corresponding to the initial target sequence [36]. The effective mutation rate should be sufficient to provide structural alternatives, but not too high, to avoid complex analysis of clones in which mutations introducing positive and negative effects might compensate. Mutations are often limited to the CDRs because these sequences contribute to most of the paratopes, rather than be induced randomly along the whole antibody sequence with the risk of challenging the molecule stability [37]. However, mutations of the frameworks can be beneficial, as demonstrated experimentally. By progressively introducing random point mutations in both the VH and VL domains, a scFv increased its affinity for estradiol-17β almost 250-fold with respect to the parental Fab, to a final *K*D of 1.3×10^{-10} nM [38]. The affinity of the camelized murine antibody AFB1increased more than 20 times by combining point mutations in FR2, FR4 and CDR3 [39].

The CDR3 domain is the major target of random mutagenesis for VH/VHH domains [40,41] and, despite its usually long sequence, this strategy still reduces drastically the number of potential variants and the downstream characterization workload. There is a general trend to address preferentially "binding hotspots" with mutagenesis and computational methods resulted useful in their identification [42,43].

2.2. DNA shuffling

This technique requires the generation of random DNA fragment libraries of selected sequence portions that are then reassembled into full-length sequences [44]. It can be used with longer DNA sequences than point mutation approaches based on error-prone PCR [44,45]. Harmsen et al. employed DNA shuffling to improve the characteristics of a llama single-domain antibody fragment, obtaining a mutant with increased stability (3-fold) and affinity (10-fold) [46]. The same method enabled the recovery of a scFv mutant against SARS-CoV with an equilibrium dissociation constant 270-fold lower than the original clone [47] and to improve significantly the affinity of an IgM antibody against pro-gastrin-releasing peptide [48]. Staggered extension process (StEP) is a

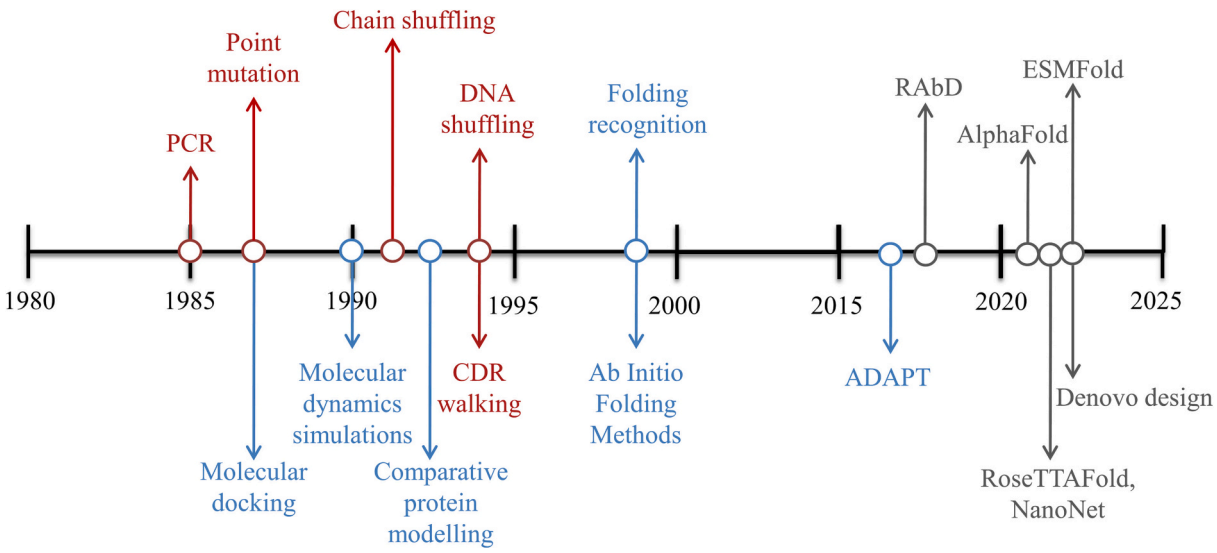

Fig. 1. Timeline of method availability that are instrumental for obtaining protein affinity maturation

mutagenesis option based on repeated cycles of template denaturation and very short annealing/polymerase-catalyzed extension steps that has been used for promoting protein evolution [49]. Although not commonly exploited for recombinant antibody affinity maturation, in combination with chain shuffling it proved being suitable for such application by introducing mutations spanning uniformly throughout the length of the antibody construct in an unbiased way. The approach allowed both inter- and intra-chain recombination and simultaneous mutations in all CDRs, generating variants that were screened by ribosome display [50]. Recently, the potency of nucleotide insertions and deletions (indels) to expand the antibody diversity during somatic hypermutation has been described [51] and successfully applied to the *in vitro* affinity maturation of some antibodies [52,53]. In parallel, an autoregressive generative model has been envisaged to evaluate indel effects on nanobody variant performance [54].

2.3. Chain shuffling

Chain shuffling is a method used for diversification of recombinant binders originated from conventional antibodies. In this approach one of the two antibody chains is replaced by a repertoire while the other chain is kept constant [55]. Chain shuffling has been used to generate high-affinity antibodies starting from genetic material obtained from immunized animals [56] and has been applied to both scFvs and Fabs. Chain shuffling allowed isolating scFv mutants with 58-fold higher binding affinity to Arginase 2 [57] and a monovalent antigen-binding fragment mutant specific for cobratoxin with affinity 8-fold higher than the original clone [58].

2.4. CDR walking

CDR walking exploits an iterative method to introduce variants exclusively in the CDR regions. Generally, different sub-libraries are prepared and mixed before undergoing panning to identify improved mutants [59]. Next, the best antibody resulting from the maturation of one CDR is used as the reference for the optimization of the other CDRs [60]. The modification of CDR-H1 and CDR-H2 after a preliminary step of CDR-H3 saturation mutagenesis (see 2.5), resulted in the 167-fold affinity improvement with respect to the parental BAK1 antibody [50]. The approach is faster and more efficient when the optimization is performed in parallel on both scFv domains. Steidl et al. conducted simultaneous CDR-L3 and CDR-H2 diversification using trinucleotide consensus cassettes, followed by the domain combination to obtain a mutant with 5,000-fold improved affinity [61]. The same strategy was successful in increasing 420-fold the affinity of Fab b4/12 for the HIV-1 envelope protein gp120 [62].

2.5. Saturation mutagenesis

Saturation mutagenesis is a semi-rational approach that foresees the mutation of all the amino acids in a short region of a gene. Commonly used saturation mutagenesis techniques include cassette mutagenesis [63], PCR amplification [64], gene splicing by overlap extension [65], and mutagenic plasmid amplification [66]. It can be considered an extension of the point mutation approach used when there are indications about potential target amino acids. Since this information might be not sufficiently precise, the method can provide very deceiving results [67]. It is suitable for single loci because, when it is applied to more loci contemporarily, the number of possible sequences increases exponentially, making difficult performing the experimental downstream analyses. This drawback can be reduced by exploiting bioinformatics tools to remove clearly defective clones from mutant libraries [67,68]. Site-saturation mutagenesis of a protease-sensitive sequence was successful in reducing the proteolytic propensity of a MMP-14 inhibitory antibody [69] and in increasing 10-fold the affinity of a nanobody directed against *Clostridium difficile* toxin A [70].

3. Affinity maturation by rational *in silico*-based approaches

Despite *in vitro* methods were demonstrated to be effective, they are extremely time consuming and it cannot be expected that this bottleneck will change significantly in the future. Fig. 2 reports the number of PubMed publications since 2000 relative to antibody affinity maturation and clearly shows that *in silico* methods are becoming popular. In recent years, computer-aided methods have been applied to antibody research and drug discovery [71] for improving affinity, specificity [72]' thermostability [73], humanization [74] and limiting aggregation propensity [75]. Examples of different *in silico* affinity maturation software applied to antibody fragments are reported in Table 1. Such rational approaches rely on algorithms the efficacy of which is progressively improved and on the constant increase of computational capacities. It means that *in silico* methods will be more and more reliable, rapid and precise, even though at the present they are not always more effective than wet-lab optimization options in terms of required time and outcome. Conventional protein rational design methods usually refer to thermodynamic principles and try to identify the conformation with

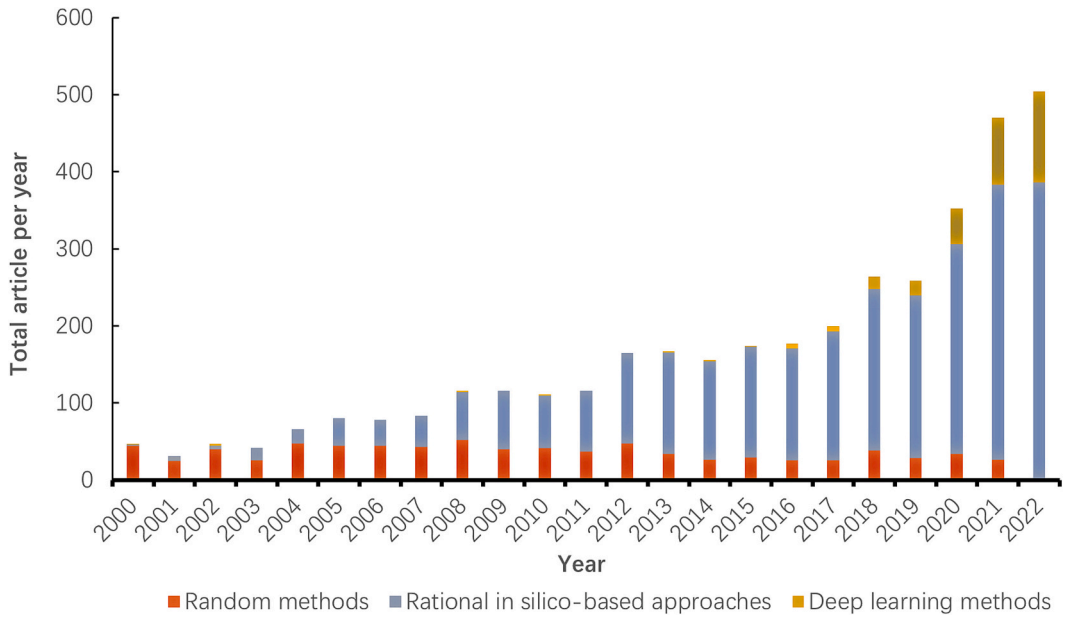

Fig. 2. Number of articles listed in PubMed describing antibody affinity maturation methods

Table 1
Examples of effective *in silico* antibody fragment affinity maturation obtained with alternative software

Antibody	Target	Type of antibody	Structure of antibody	Affinity Maturation Software	Increased affinity	Year	Ref
cAb-CA05	HEWL	VHH	Model by MOE	GROMACS, MolFeat-EC	20x	2013	[173]
Bevacizumab	VEGF-A	Fab fragment	Crystal structure, Model by Modeller	PyMOL v1.3, GROMACS 4.0.7	18x	2013	[174]
11K2	MCP-1	ScFv	Crystal structure	GROMACS	4.6x	2014	[175]
bH1	VEGF-A HER2	Fab fragment	Crystal structure	SIE-SCWRL v4.0, FOLD X v3.0, Rosettav3.5	104x 46x	2017	[165]
D8	HBV	ScFv	Model by Modeller	PatchDock, FOLD X v3.0	10x	2018	[176]
A26.8	TcdA	VHH	Crystal structure	SIE-SCWRL, FOLD X, Rosetta	9x	2018	[167]
NbC18	PIGF	VHH	Model by Modeller	Cluspro, HADDOCK, GROMACS v 5.1.2	2.1x	2018	[177]
V_HNAC1	Human α-synuclein	VHH	Model by Swiss-Modeller	GROMACS, ClusPro, DelPhi software	48x	2018	[79]
AB1	muCCL20	ScFv	Model by SabPred	Discovery Studio 2016, Schrödinger Biologics Suite2016-3, Rosetta	4x	2019	[178]
Nb02	CD47	VHH	Model by Modeller v9.19	SIE-SCWRL, FOLD X, Rosetta, HADDOCK, Schrodinger	87.4x	2019	[140]
D2-L29	HEL	VHH	Crystal structure	MOE	12x	2020	[179]
VHH212	HIF-1α	VHH	Model by Modeller	GROMACS v4.5.5, HADDOCK, InterProSurf , mCSM-AB, OSPREY , FoldX	17.5x	2020	[150]
Adalimumab	TNF-α	Fab fragment	Model by PIGSPro v2 and ABodyBuilder	PatchDock, FireDock, ClusPro 2.0, HADDOCK 2.2, PRODIGY, YASARA, mCSM-AB etc.	82x	2021	[180]
E53	DENV	scFv	Crystal structure	Discovery Studio 4.0	100x	2021	[181]
AP2M21	hPCSK9	ScFv	Model by ABodyBuilder server	HawkDock, PyMol v2.3.0	24.2x	2021	[182]
KD035	VEGFR2(D2-3)	Fab fragment	Crystal structure	MOE, NAMD	8.2x	2021	[183]
CR3022	SARS-CoV-2 RBD	Fab fragment	Crystal structure	Discovery Studio 4.5, GROMACS 5.1.2	15x	2022	[184]
VHH2	TNF-α	VHH	Model by Alphafold2	GROMACS 2019, HADDOCK, FOLD X	3.9x	2023	[151]

minimal free energy [76]. The approach intrinsic limitation is that the actual free-energy difference between the target state and all other possible states must be larger than the error introduced by the used approximations [77]. Despite these constrains, the mutants originated by such simulations, once validated experimentally [78], were instrumental in understanding several mechanisms regulating protein stability. Computer-aided methods already enable a better understanding of antibody-antigen interactions [79], even in the absence of a crystal structure of the antibody-antigen complex [80,81]. However, thanks to deep learning methods, structure modelling has become extremely faster and more reliable, filling in what was the most limiting step of protein rational optimization.

Summarizing, the logical procedure guiding rational mutagenesis follows the scheme reported in Fig. 3. First, it is necessary to acquire detailed information about the threedimensional structure of the macromolecules involved in the antigen-antibody complex. The successive step (molecular docking) aims at identifying the contact surfaces between the macromolecules (paratope and epitope) and, more specifically, the critical residues involved in the interactions. This information will guide the mutagenesis phase. The resulting mutant candidates will be first evaluated *in silico* and then validated experimentally.

3.1. Building 3D models

The availability of the 3D structure of both antigen and antibody critically increases the effectiveness of antibody optimization by *in silico*

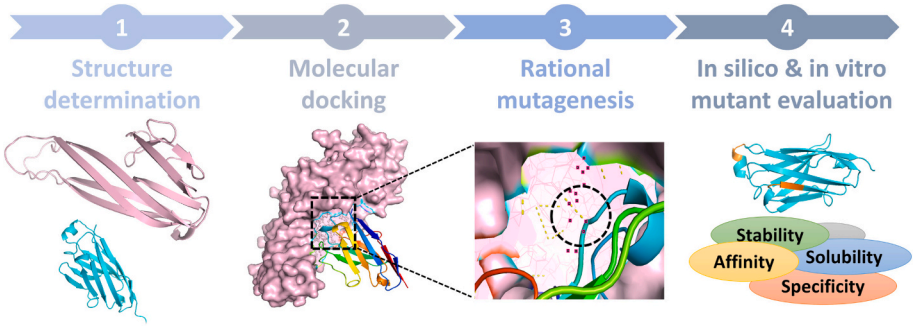

Fig. 3. Logic step sequence for protein/antibody rational optimization

approaches. X-ray crystallography has been the standard method for dissecting the interactions between antigens and antibodies [82], but the limitation is that the structures of a large number of molecules still need to be fully solved [83]. NMR is difficult to apply to decipher complex protein structures because of their large mass but can be exploited to identify the antibody residues contributing to build contact surfaces when, as it is the case of nanobodies, the dimension of the ligand is small enough to be compatible with the technology [84]. Cryo-EM represents the newest option to study antigen-antibody interactions and the increased availability of such technology is boosting the process of protein (and protein complexes) structure resolution [85]. Furthermore, hydrogen-deuterium exchange mass spectrometry (HDX-MS) is an effective method to identify the residues involved in the epitope-paratope interaction surface. As other technologies requiring both expensive equipment and specific expertise, HDX-MS approach is still confined in a relatively restricted number of labs, but it proved being extremely successful to study the interaction of proteins with both small molecules and other proteins, such as in the case of antigen-antibody complexes [86]. It demonstrated being particularly useful for the identification of conformational epitopes, as those recognized by nanobodies binding to junctional epitopes [87], and to discriminate antigenic sites that induce different neutralization response [88]. HDX-MS has the advantage of being a measurement in solution, thus preventing artefacts due to crystallization or freezing, and allows the study of complex systems such as membrane proteins and protein aggregates. Its main limitation is that it does not provide information about which antibody residue interacts with which antigen residue, as compared to crystallography or cryo-EM. Therefore, similarly to the procedure applied when epitope mapping is performed with NMR [84], HDX-MS data should be combined with docking experiments to refine the binding model and identify the residues that are rearranged exclusively because long-distance allosteric conformational changes [89]. As an alternative to experimental methods, computational-based structure prediction methods have demonstrated an astonishing level of reliability [90] for constructing atomic-resolution models of target proteins starting from their amino acid sequences [32]. Some of them are suitable for any protein class and others specifically designed for dealing with antibodies.

3.1.1. Template-based approaches: comparative/homology modeling and threading methods

These protein structure prediction methods, including homology modeling and threading methods, rely on detectable similarity between target sequence and known structures. In the case of (conventional) antibodies, homology modeling exploits the characteristics of conserved antibody sequences corresponding to frameworks and CDRs determined by applying the standardized numbering methods (Kabat [91], Chothia [92] and Abnum [93]). The framework sequences are highly homologous and easy to model, while the CDR loops are highly variable and require additional constraints for accurate modeling. The greatest challenge is predicting the conformation of CDR-H3, which is the most variable in length and sequence and usually contributes mostly to paratope formation [31,71]. PIGS/PIGSpro [94] and Rosetta Antibody [95] are typically used for this modeling approach. Whereas the evaluation of nanobody thermodynamic characteristics is significantly simplified by the low residue number of such ligands, the advantage vanishes when nanobodies must be modelled. This is because there are relatively few available structures, with respect to conventional antibodies, and their conformation significantly differ from that of VH, a domain with shorter CDR3 sequences and display affected by the co-presence of light chain loops [32]. Consequently, the main challenge is to adapt platforms designed for VH domains of conventional antibodies to the specific structural characteristics of VHHs. A modified Rosetta Antibody for nanobodies has been dismissed, whereas the antibody structure prediction algorithm ABodyBuilder [96] in combination with the Structural Antibody Database (SAbDab) [97] was successfully tested to model nanobodies. Despite the promising characteristics and the confirmation of its capacity to predict antibody 3D structures, ABodyBuilder has not been used frequently and in a comparative work exhibited less accuracy in modeling the H3 loop than RoseTTAFold and SWISS-MODEL [98].

Modeller and SWISS-MODEL are still the most common software used for nanobody modeling[99,100]. Specifically, Modeller is an easy-to-use homology modeling software that is very effective when suitable homologous templates with high similarity and coverage can be retrieved from the protein database. It was exploited to obtain structural models of nanobodies specific for several antigens, such as VGFR2 [99], TNFR1α [100], PLA2 [101,102], MMP8 [103], DARC [104], BMP4 [105] and various toxins.

Threading methods were developed to recognize proteins with low sequence homology but similar folds and operate matching the query sequence directly onto the 3D structures of solved proteins [106], decomposing the template structure into local structural features and iteratively update sequence profiles of multiple sequence alignment to identify distantly homologous templates [107]. This approach has been applied by different platforms, such as GenThreader [108], ORION [109], FINDSITE [110], I-TASSER [106]. GenThreader is suitable when the target is relatively complex and its flexibility and dynamics need to be considered. ORION aims at identifying local structural information (protein blocks) corresponding to patterns that can accurately describe protein modules and predict the structure of globular rather than membrane proteins. FINDSITE is useful when the target sequence is not very similar to the template, but the two share some conserved regions. I-TASSER can be applied to combine the results of various structural prediction tools and improve the accuracy of the final model.

The threading-based server Raptor-X succeeded in modeling VHHs against different toxins [111] and I-TASSER web server was used to model the structures of VHHs against the HCV non-structural protein NS3/4A and Protein A [112,113]. C-I-TASSER maximizes contact-map prediction and optimized I-TASSER force field which can handle structural regions lacking accurate spatial restraints. Early method versions rely excessively on the manual settings of scoring parameters and their search space were relatively fixed. Currently, they all provide easy-to-

use web servers for simple submission and result recovery and have integrated deep learning modules that overcome the limitation of standardized manual setting. The implementation of automatic parameter and method integration for target maturation allows carrying out deep sampling and search in a global conformation space, two factors that contribute raising optimization by avoiding to be trapped into local optimal solutions. This approach allows obtaining models as accurate as those built by traditional homologous methods even when structural data are particularly scarce [114].

3.1.2. Ab initio methods and deep learning

Ab initio methods are used for predicting protein structures in the absence of related homologue structures. Their approach is based on the evaluation of the protein minimal native free energy and the large-scale search of conformational space. These methods are not commonly used in antibody modeling because of the inaccurate force field availability and the limited conformational search power [115]. Accuracy and sensitivity can be improved by combining multiple approaches. For instance, CASP10 exploits I-TASSER together with QUARK (the real *ab initio* platform) and succeeded in providing the first structure of two proteins with more than 150 residues, a result that represented the length record at the publication time [116].

The present availability of an unmet amount of data and the advancements in machine learning algorithms has very rapidly mixed up the trends briefly depicted in the above paragraphs and made deep learning the apparent most successful approach for protein modeling. This innovative method has been successfully applied to antibodies as well [117,118]. Using antibody-antigen binding datasets of synthetic antibody libraries [119], public databases [97,120] and deep mutational scanning to train models, machine learning can precisely predict the characteristics of antibody-target interactions and propose antibody sequences instrumental in producing binders with improved biophysical characteristics [121,122].

Instead of using physics-based simulations that require immense computational time and cannot capture the overall complexity of macromolecular interactions, deep learning exploits for its modeling multiple complex neural networks that combine distant evolutionary searches. Biophysical implications are irrelevant and therefore effective modeling is feasible even in the absence of any structural or functional information [31]. At present, AlphaFold is the most known and best-performing example of an artificial intelligence-based program for protein modeling [123,124] and an evaluation of the prediction quality is provided by the AlphaFold Protein Structure Database. Other neuronal network-based programs such as RoseTTAFold and DeepAb provide insight into protein function in the absence of experimentally determined structures, can rapidly generate models of protein-protein complexes and were successfully applied to antibody modeling [98,125,126]. The few available comparative analyses indicate that AlphaFold outperformed the competitors [127], but most of the programs are too new for having sufficient data that could indicate their limits and peculiar strengths. So far, DeepAb was exploited to provide reliable prediction of the structures of conventional antibody variable domains [126]. Among these recent proposals, a particular interest should be dedicated to NanoNet because it is the first AI program specifically dedicated to nanobody optimization [128]. NanoNet uses deep learning for accurate prediction of the 3D coordinates for the whole VHH sequence without separate modeling of framework and CDR regions. In contrast to deep learning methods such as RoseTTAfold and DeepAb, NanoNet is applicable to high-throughput modeling of large databases since it generates very rapidly (millisecond to seconds, when side chains are added) high-quality structures suitable for docking and epitope mapping [128]. Its model quality is not biased by the CDR3 length which poses a serious challenge to other common comparative/homology modelling (Swiss Model-E, ModWeb, Modeller) and to AlphaFold2 [33]. In the few available comparative studies, NanoNet performed as good as alternative modelling approaches [32,129] and in a structure-function analysis of VHHs targeting the receptor binding domain (RBD) of the SARS-CoV-2 spike, it enabled to optimize the CDR3 loop conformation and generate models that fit better in the cryo-EM map than those proposed by alternative platforms [130]. Nevertheless, a direct comparison with DeepH3, an algorithm specifically developed to model the CDR H3 loop [131], is missing so far.

Recently, the results obtained with the deep learning method for antibody structure prediction programme (IgFold) have been published [129]. Instead of using the few thousands of available structures, IgFold has been trained using embeddings from AntiBERTy [132], a language model pretrained on 558 million natural antibody sequences to directly predict the atomic coordinates corresponding to the antibody structure. As a result, by exploiting graph networks IgFold can directly predict backbone atom coordinates. The available validation data indicate that structure predictions obtained by applying such deep learning procedure can be extremely precise. Specifically, the accuracy of IgFold for Fv structures is at least as good as that offered by AlphaFold, DeepAb and ABlooper and remains elevated also for the highly variable CDR H3 loops. In the case of nanobodies, IgFold predictions had accuracy comparable to those of AlphaFold and NanoNet and significantly better than those recovered applying DeepAb, even though no method obtained RMSD values below 4 Å for CDR3. In the described simulation with nanobodies, AlphaFold showed the best average performance, a quality attributed to its wider training set. Nevertheless, IgFold requires significantly shorter time to generate a structure (15 s, as much as for NanoNet, instead of 6 min for the ColabFold [133] implementation of AlphaFold) and gives a reliable error estimation of the prediction that is useful to evaluate the output robustness. IgFold developers expect that these method characteristics will be particularly beneficial for antibody-antigen docking applications. The illustrated results lead again to the issue represented by the datasets used for training (see [31] for an exhaustive list), since their composition can strongly affect the learning capacity of the methods.

3.2. Molecular docking and identification of interaction surfaces

The driving principle of molecular docking is that the complementarity between the interacting partners is mainly regulated by two key components: geometry (steric compatibility) and energy (interactions affect the system free energy) [134]. A variety of docking software has been originally developed to study the interactions with small-molecule ligands and then expanded to analyze interfaces between large macromolecules. Different docking programs, such as DOCK [135], AutoDock [136], AutoDock Vina [137], FlexX [138] and GOLD [139] use unique search algorithms and scoring functions for the analysis of the potential binding sites and the conformations of ligands to the target molecule. Search algorithms generate sets of potential binding mechanisms for the target site, considering the ligand rotation and internal freedom degree. Scoring functions assess the stability of the different generated complexes, attribute a score to each combination and finally select the most stable combinations according to their score [134]. AutoDock Vina was successfully used in combination with Modeller for nanobody structural studies [140].

Molecular docking is particularly useful for both predicting and describing antigen-antibody interaction mechanisms and recently has been widely used to analyze the binding mechanisms and the key residues involved in the antibody recognition of SARS-CoV-2 and its variants [141,142]. Molecular docking is critical during the process of *in silico* ligand optimization to screen, among the several mutants proposed by the simulation, those suitable for experimental validation. High-affinity variants of the VHH against Bap antigen of *Acinetobacter baumanni* have been selected using such a strategy [143].

Docking efficiency is improved when the search space is reduced. Since the process requires a consistent computational input when it deals with molecules such as nanobodies which possess highly flexible regions, the performance of docking software (ClusPro, LightDock,

ZDock and HADDOCK) highly correlates with the accuracy of epitope information [144]. The platforms have different strengths and weaknesses and no one clearly outperforms the others. ClusPro analyzes structural changes in combination with sequence information to achieve high accuracy but is very slow [145]. It was successfully used to guide the maturation of an intrabody nanobody against alpha-synuclein [79]. LightDock is faster and can handle structural flexibility, but its precision may be lower [146]. ZDock is mostly exploited to produce initial models to be refined by other software [147]. HADDOCK integrates multi-source information to define active residues and constraints to recover binding sites [148] and has been often applied to analyze nanobody-antigen binding interfaces, alone or in combination with InterProSurf, a software dedicated to the identification of the interacting amino acid residues [149–152]. A specific effort has been provided to identify tools that can reduce the workload of docking, such as the sequence-based probabilistic machine learning algorithm named Parapred that predicts antibody paratope conformation to improve both speed and accuracy of the downstream docking algorithm [153,154]. When an epitope is known, but no corresponding antibodies exists, it is possible to use the structure-based deep learning software DLAB that exploits a convolutional neural network for rapid modeling of putative binding antibodies against that target antigen which are then screened by applying ZDock [155]. Recently, a machine learning-guided method for the re-ranking of nanobody-antigen binding poses (NbX) has been proposed that has the potential of improving the specific docking process of VHHs [156]. It comprehensively considers the energy, contact and interface features of the Nb-Ag interface and seems to outperform prediction software originally developed for generic protein complexes.

AlphaFold and RoseTTAFold were initially not very accurate to model protein-protein complexes [157] but their performance was improved when used in combination with other platforms, for instance with the physics-based docking server ClusPro [158] or by incorporating mass spectrometry results obtained by covalent labeling [159]. More recently, AlphaFold-Multimer has demonstrated an impressive ability to model protein complexes [160] and a tool for the interpretation of these predictions is now available online at Predicted Aligned Error (PAE) Viewer webserver (http://www.subtiwiki.uni-goettingen.de/v4/paeViewerDemo) [161]. Other packages can be useful to integrate the AlphaFold-Multimer functions, such as AlphaPulldown, dedicated to the study of protein-protein interactions [162], and ABlooper, that predicts CDR loop structures in an end-to-end fashion [163]. This opportunity might become particularly important considering that the major limitation for deep-learning approaches is still the accurate modelling of the CDR3 [129].

3.3. Strategies to enhance affinity

The availability of structural information, obtained experimentally or from accurate modeling systems, enables the design of site-directed mutants predicted to have improved biophysical characteristics. In the optimal situation, the analysis of an experimentally available structure of antigen-antibody complexes shows what residues are involved in the binding, determines the relative role of each residue [59] and the chemical features of the other proximal amino acids. As we have just seen, artificially generated structures are more and more reliable and can represent a valid alternative. Next, mutations can be introduced to optimize interaction surfaces or ligand stability and the assessment of the mutation effects can be performed by predictive methods that evaluate and score the variants thermodynamically. This is a meaningful preliminary screening step to save time and resources before moving to mutant production and experimental validation [164]. Several computing platforms based on alternative principles have been proposed to evaluate protein characteristics and the effects of mutations (Table 2). This effectiveness of such procedure is confirmed by the success of specific optimization projects [150,165–167] and some very recent options deserve a specific description.

Table 2
Computational tools suitable for the prediction of antibody biophysical characteristics and of mutation effects

Methods	URL	Modalities used for assessment	R-values	Ref
Aggrescan3D	http://bioc omp.chem.uw.edu.pl/A3D2/	Structure-based prediction of aggregation properties	/	[185]
AUTO-MUTE	http://proteins.gmu.edu/automute	Utilization of cutting-edge supervised classification and regression algorithms with energy-based and machine learning approaches	/	[186]
BeAtMuSiC	http://babylone.ulb.ac.be/beatmusic/index.php	Coarse-grained predictor with statistical potentials derived from protein structure	0.48	[187]
Binding affinity	http://bmm.crick.ac.uk/chalei01/binding_affinity/	Consensus approach based on more than 10 structural parameters	0.68- 0.75	[188]
BindProf	http://zhanglab.ccmb.med.umich.edu/BindProf/	Shape complementarity and high complementarity with physics-based scores	<0.5	[189]
CCharPPI	http://life.bsc.es/pid/ccharppi	Calculation of up to 108 parameters, including models of electrostatics, desolvation and hydrogen bonding, as well as interface packing and complementarity scores, empirical potentials at various resolutions, docking potentials and composite scoring functions.	/	[190]
CuPSAT	http://cupsat.uni-koeln.de	Structural environment specific atom potentials and torsion angle potentials	0.7	[191]
DeepDDG	http://protein.org.cn/ddg.html	Prediction of changes in the stability of proteins due to point mutations	0.48−0.56	[192]
DUET	http://structure.bioc.cam.ac.uk/duet	Consolidation of two complementary approaches (mCSM and SDM) in a consensus predictor with Support Vector Machines (SVM)	0.74	[193]
ELASPIC	http://www.kimlab.org/software/elaspic	Combination of semi-empirical energy terms, sequence conservation and SGBDT	0.75	[194]
ENCoM	http://bcb.med.usherbrooke.ca/encom	Coarse-grained normal mode analysis method	0.6	[195]
Eris	http://eris.dokhlab.org/	Use of physical force field with atomic modeling, fast side-chain packing and backbone relaxation algorithms to predict the effect of small-to-large sidechain size mutations	0.75	[196]

(continued on next page)

Table 2 (*continued*)

Methods	URL	Modalities used for assessment	R-values	Ref
I-Mutant 2.0	https://omictools.com/imutant-tool	Support vector machine (SVM) tool based on structure and sequence	0.71	[197]
mmCSM-AB	http://biosig.unimelb.edu.au/mmcsm ab/	Graph-based signatures and atomic inter-action information	0.95	[43]
MutaBind	http://www.ncbi.nlm.nih.gov/projects/mutabind/	Use of molecular mechanics force fields, statistical potentials and fast side-chain optimization algorithms	0.57	[198]
Parapred	http://www.mvsoftware.ch.cam.ac.uk/	Using a deep-learning architecture to leverage features from both local residue neighborhoods and across the entire sequence	>0.5	[153]
pkCSM	http://structure.bioc.cam.ac.uk/pkcsm	Use of graph-based signatures	0.6-0.9	[199]
Platinum	http://structure.bioc.cam.ac.uk/platinum	Comprehensive database providing experimental information related to changes in protein–ligand affinities upon mutation and their three-dimensional structures	/	[200]
PoPMuSic	https://soft.dezyme.com/	Using a linear combination of statistical potentials to detect structural weaknesses	0.63	[201]
PPA-Pred	http://www.iitm.ac.in/bioinfo/PPA_Pred/	Sequence-based affinity prediction using functional information	0.739-0.992	[202]
SAAMBE	http://compbio.clemson.edu/saambe_webserver/	Analysis of energy minimized 3D structures	0.62	[203]
TAP	http://opig.stats.ox.ac.uk/webapps/sabdab-sabpred/TAP.php	Five developability guidelines	0.77	[204]

R-values correspond to the ratio between predicted and measured values.

RosettaAntibodyDesign (RAbD) reassembles the experimentally determined 3D structure of the antibody to redesign single or multiple CDRs. Its adoption allowed improving antibody affinity from 10 to 50 times [168]. A LSTM-based deep generative model exploiting the sampling of virtual sequences can prioritize the most promising candidates and successfully identified a mutant antibody against kynurenine with over 1,800-fold higher affinity with respect to the original molecule, a result significantly better than that obtained applying traditional high-frequency screening to the same dataset [119]. The next challenge will be a fully *ab novo* computational-based antibody design [118] instead of the optimization of already validated model antibodies recovered by panning. In this perspective, neural networks have been already successfully trained with antibody libraries to identify candidates with improved affinity [166,169]. Promising results were also obtained exploiting a fragment-based procedure in which the single CDRs (paratope components) complementary to a set of independent target epitopes belonging to the same antigen were first designed and then ranked according to the total number of favorable interactions. Finally, the top-ranking sequences were grafted into an antibody scaffold [21]. Experimental data confirmed that the design nanobodies bound to the expected epitopes with affinities in the 10^{-7} molar range, although no information was made available about their yields and stability. After having computed the solvent-accessible surface area of the structures corresponding to a protein dataset in the presence and absence of bound designed CDR fragments, the authors inferred that most (median value: 78%) of the surface of each antigen is typically targetable adopting the described strategy.

3.4. Open questions

The improvement of one nanobody biophysical characteristic (e.g. affinity) can induce the loss of another (e.g. solubility) [170]. Therefore, the challenge would be identifying a holistic approach able to improve the overall quality of a binder. Pragmatically, this aim can be achieved by means of successive steps in which only the mutations that do not compromise the initial features, and improve at least one of them, will be accepted. We previously applied this procedure to humanize a nanobody sequence and significantly increase its yield without affecting its affinity [75,171]. However, the simultaneous improvement of more parameters has been recently reported for a trained deep learning system that could predict mutations that co-optimized affinity and specificity [172].This success is promising and prompts to collect further demonstrations showing that a deep-learning platform can accomplish the optimization of multiple nanobody biophysical features simultaneously. A further level of complexity to explore in the future concerns the optimization of multi-specific constructs in which not only the binding characteristics of the single ligands must be considered, but also the 3D organization of one binding element with respect to the others. The present limit is the lack of *ad hoc* sets of data large enough for effective training of deep learning methods.

4. Conclusion and prospects

Due to ethical issues and administrative burdens, animal immunization as a means to obtain antibodies seems to become a running out model. Synthetic libraries of increasing quality in terms of structural and functional diversity have been progressively proposed and the selected clones often do not require further affinity maturation. Nevertheless, several other biophysical and functional features might require optimization for increasing the overall antibody developability and biological efficacy. In the last 30 years, *in silico*-based approaches based on structural information and basic thermodynamic principles were developed with the aim to provide rational antibody optimization protocols. Deep learning-based software has now the chance to generate very rapidly and accurately the missing experimental protein structures as well as improving both the docking step and the evaluation of the candidate mutants. The implementation of an approach totally guided by deep-learning methods has the potential to shorten dramatically the overall timeline of the *in-silico* maturation pipeline for both proteins and antibodies. This seems finally affordable also for nanobodies because their very specific features determined by their peculiar structural characteristics seem to be overcome by reliable *ad hoc* software. What is still missing at the present is a sufficient mass of data to understand whether the promising but still limited reports correspond to an already mature phase or whether specific improvements are still required to reach reliable standards. Therefore, community-based benchmarking works would be particularly useful to collect the data necessary to understand what the state-of-the art really is and identify the most critical points on which to concentrate the research effort.

Declaration of competing interest

Author P.W. was employed by Tianjin Modern Innovative TCM Technology Co. Ltd. Author M.Z. was employed by China Resources Biopharmaceutical Co. Ltd. Author Y.W. was employed by Tianjin Pharmaceutical Da Ren Tang Group Co. Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The authors declare no conflict of interest.

Data availability

No data was used for the research described in the article.

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No data was used for the research described in the article.

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