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Characterization of epitopes recognized by monoclonal antibodies: experimental approaches supported by freely accessible bioinformatic tools

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Monoclonal antibodies (mAbs) have been used successfully both in research and for clinical purposes. The possible use of protective mAbs directed against different microbial pathogens is currently being considered. The fine definition of the epitope recognized by a protective mAb is an important aspect to be considered for possible development in epitope-based vaccinology. The most accurate approach to this is the X-ray resolution of mAb/antigen crystal complex. Unfortunately, this approach is not always feasible. Under this perspective, several surrogate epitope mapping strategies based on the use of bioinformatics have been developed. In this article, we review the most common, freely accessible, bioinformatic tools used for epitope characterization and provide some basic examples of molecular visualization, editing and computational analysis.

Introduction

The development of technologies for the selection of monoclonal antibodies (mAbs) represents a significant advance not only in differing research fields but also in clinical practice. Several mAbs are now being used in cancer therapy and immune response modulation for the treatment of autoimmune diseases, graft-versus-host diseases and allograft rejection. Their use in the treatment of infectious diseases is currently limited to the prevention of severe Human respiratory syncytial virus (RSV)-associated neonatal bronchiolitis, but their possible use is currently being considered in the treatment of other infections, including those of bacterial and fungal etiology [1]. Indeed, the possibility for selectively targeting microbial pathogens without side effects is of great interest to researchers and clinicians, especially considering the wide diffusion of resistance to available anti-infectious drugs [2]. Another important aspect related to the availability of mAbs directed against microbial pathogens is the possibility of using them as a 'probe' for the identification of protective epitopes in the targeted microbial proteins. This could facilitate the development of novel vaccine approaches based on the administration of recombinant molecules (mimotopes) capable of eliciting a protective response against regions selectively identified in a given protein (epitope-based vaccines). It is also evident that the development of epitope-based vaccines requires a deep knowledge of mAb/antigen (Ag) interactions. In fact, because many protective mAbs are directed against conformational epitopes, the effectiveness of mimotopes used in further immunizations is often strictly related to their 3D conformation; this is also the case of the shared protective epitopes identified by our group among different hepatitis C virus (HCV) genotypes or highly divergent influenza virus subtypes [2–8]. The identification of these regions is not usually straightforward, and is typically achieved through the resolution of an antigen–antibody crystal complex, which is not always, as in our experience, easy or possible to obtain. The main purpose of this article is to review some of the alternative surrogate epitope mapping strategies based on experimental data combined with the use of freely available bioinformatic tools. Particular attention will be paid to those tools useful in the identification of protective B-cell epitopes on relevant microbial pathogens, such as bacteria, viruses and fungi.

X-ray diffraction structure: the best method for epitope characterization

X-ray resolution of the mAb/Ag complex is the most reliable approach for definition of the epitope recognized by a protective mAb. This approach is based on the measurement of the intensity

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and angle of X-ray beams diffracted by the mAb–Ag co-crystal and the subsequent *in silico* determination of its atomic coordinates, thus allowing the complete visualization of the 3D conformation of the complex [9–12]. This allows the identification of specific amino acid residues, both in the Ag and in the mAb, involved in the docking. Unfortunately, for a variety of reasons including cost, the level of expertise needed, the difficulty in setting up functional crystallization conditions and poor yields in the production of certain bacterial or viral proteins, the crystal structure approach is not always viable. For example, the crystal structure of the HCV/E2 protein, the main surface glycoprotein of HCV, is not available at this time.

Alternative surrogate epitope mapping approaches

When the crystallization process is not successful or cannot be performed, several alternative surrogate strategies are available. Below we provide examples of three different epitope mapping approaches, the final results of which may be interpreted using freely available bioinformatic tools: peptide panning, alanine scanning and amide hydrogen/deuterium exchange mass spectrometry. In addition, we also describe the *in silico* prediction of the docking of a mAb to its antigen. While the first three approaches combine the use of bioinformatics with experimental data generation, the last one, even though it uses empirical data to predict the complex structure, is based solely on computational analysis.

These are only some of the experimental approaches that can be used in combination with freely available bioinformatic tools. Other possible strategies (i.e. cell-surface displayed libraries, ribosome-displayed libraries, several other pepscanning approaches, epitope excision by protease protection assays followed by mass spectrometry and nuclear magnetic resonance spectroscopy for solution-phase epitope mapping), have been extensively described elsewhere [13–17] and, for the sake of brevity, will not be reviewed here.

Approaches combining experimental data generation and bioinformatics

Peptide panning

One possible epitope mapping strategy is the so-called 'peptide panning' technique, consisting of the screening of commercial peptide libraries [18–21]. This approach is based on the affinity selection of short phage-displayed peptides against the mAb of interest [22,23]. Specifically, a phage population expressing a defined number of random peptides is amplified in bacteria and repeatedly 'panned' on the mAb, potentially allowing the selection of peptides to bind to it [22]. This is possible only after several 'panning' rounds on the mAb, leading, after each round, to a phage population enriched in mAb-binding peptides. Similar selection approaches could also be performed using cell-surface displayed and ribosome-displayed libraries.

The sequencing of the selected peptides and their subsequent analysis through dedicated, freely accessible bioinformatic tools, such as 'Pepitope server' [24], allows the identification of 'consensus motifs', consisting of the key amino acid residues conserved among the different selected peptides. On the basis of their characteristics (i.e. overall charge and hydropathicity profile) it is possible to infer the position of the key residues on the solvent exposed surface of the antigen [if its structure is available in *RCSB-Protein Data Bank* (http://www.rcsb.org/pdb/home/home.do)] as reported in Fig. 1. However, peptide-based approaches can also lead to the identification of consensus sequences outside of the real epitope. The erroneous prediction may be due to the presence of amino acid motifs on the antigen surface similar to those actually present in the mAb-bound region. Alternatively, this can also occur when the number of selected peptides is low and the consensus sequence does not accurately represent the amino acids of the 'real' epitope.

It is important to note that all of the approaches described above could potentially lead to the identification of 'mimotopes', that is, molecules capable of mimicking (without necessarily sharing any primary amino acid homology) the epitope recognized by the mAb of interest, and thus, if administered *in vivo*, could be potentially capable of eliciting a humoral response with similar features [25–27].

Alanine scanning

Another possible strategy for the epitope mapping is alanine scanning. This technique consists of the evaluation of possible alterations affecting the mAb/Ag binding after the introduction of alanine mutations in the Ag amino acid primary sequence template. The common use of alanine, as a mutating amino acid, in this approach is due to its small molecular weight and its neutral charge, even if, in certain circumstances (i.e. epitopes naturally containing alanine, or in the presence of glycosylation sites) other amino acid mutations can be used just as well [6]. This technique can also be used to experimentally confirm the role of residues identified through the peptide panning approach or by other approaches (e.g. competition with a mAb directed against an already characterized epitope) and also to broaden the number of investigated residues possibly involved in the mAb/Ag interaction (Fig. 1).

Once the antigen recognized by a given mAb is cloned onto an appropriate expression vector and the protein product is properly folded, it is possible to introduce alanine substitutions in the antigen amino acid sequence to check whether the different mutations can affect the binding to the mAb. After the generation of a large panel of alanine mutants in different amino acid positions, all proteins carrying alanine substitutions can be tested in binding assays. Mutants showing a decreased binding of the mAb carry a mutation is possibly involved in the binding with the mAb, and therefore part of its epitope [6,7]. However, in certain cases, an alanine mutation lying outside of the epitope can also affect the binding of the mAb. This can be due to possible conformational changes, even outside of the epitope, that can affect the epitope itself.

Once the experimental session is completed, the *in silico* analysis of the results obtained by alanine scanning is fundamental to the prediction of the putative mAb epitope. To check the 3D position of the residues involved in the mAb–Ag interaction, all the mutations that decrease the binding of the protective mAb to the antigen can be visualized directly on several antigen crystal structures available on line for free (RCSB-Protein Data Bank). This can be achieved using free molecular rendering computer programs such as UCSF Chimera package, RasMol, Cn3D, DeepView/SPDBV [28] or VMD-Visual Molecular Dynamics (Table 1) [29]. Generally, these software programs allows identification, through graphical editing and mapping on the 3D antigen structure, of the

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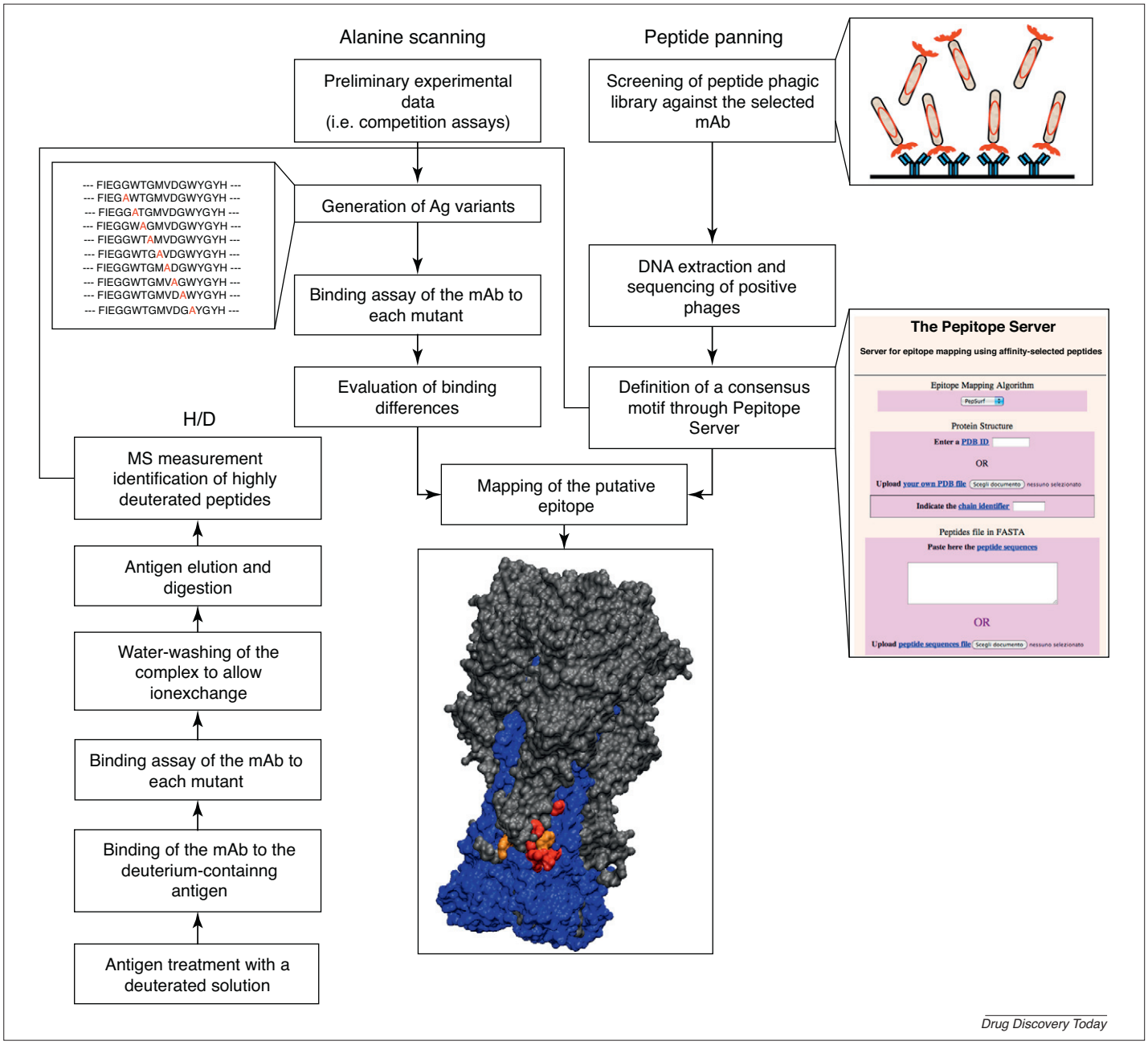

FIGURE 1

Epitope mapping workflow describing the different experimental approaches reported in the text and followed by *in silico* analysis. Both peptide-panning and amide hydrogen/deuterium exchange mass spectrometry require the analysis of the experimental results through dedicated web servers, potentially leading to the identification of amino acid residues involved in the mAb/Ag binding. The alanine scanning approach, followed by the *in silico* mapping of the residues able to inhibit mAb/Ag binding, may be considered the quickest and easiest strategy to confirm or implement the *in silico* analysis. *Abbreviations*: Ag: antigen; H/D: hydrogen/deuterium; mAb: monoclonal antibodies; MS: mass spectrometry.

positions of the amino acid residues that decrease the binding of the mAb to the antigen. It is possible to only visualize the Ag surface residues exposed to the solvent to check if the alanine substitutions that are able to affect the binding with the mAb are effectively exposed on the Ag surface and are sufficiently accessible for antibody binding.

The alanine scanning approach used for the epitope mapping can be hindered by the limitations described above; however, the importance of its role as a surrogate technique in epitope definition has been demonstrated in several recent works confirming with a crystallization approach what already observed with the alanine scanning approach [6,30,31].

Amide hydrogen/deuterium exchange mass spectrometry

The amide hydrogen/deuterium (H/D) exchange method, followed by proteolysis and mass spectrometry (MS), is another surrogate method used for the study of protein–ligand interactions and protein–protein interactions [32]. The rationale for this technique is the comparison, through MS, of the amount of deuterium incorporated by the antigen when it is bound or unbound to the

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TABLE 1

Examples of molecular visualization and editing computer programs freely available

Logo	Program name	General features	Source (URLs)	References
	UCSF Chimera	Program for interactive visualization and analysis of molecular structures and related data such as density maps, supramolecular assemblies, sequence alignments, docking results, trajectories, and conformational ensembles. Good graphical rendering.	http://www.cgl.ucsf.edu/chimera/	Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, supported by the National Institutes of Health
	Ras Mol	RasMol molecule representations include depth-cued wireframes, 'Dreiding' sticks, spacefilling (CPK) spheres, ball and stick, solid and strand biomolecular ribbons, atom labels and dot surfaces. Allows editing of the models or crystals through a command line.	http://rasmol.org/	Based on RasMol 2.6 by Roger Sayle Biomolecular Structures Group, Glaxo Wellcome Research & Development Stevenage, Hertfordshire, UK
	Cn3D	Structure and Sequence Alignment viewer for NCBI databases that simultaneously displays structure, sequence, and alignment.	http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml	NCBI
	DeepView/SPDBV	Program that provides a user friendly interface useful for the analysis of several proteins at the same time. Endowed with an intuitive graphic and menu interface that allows one to easily identify amino acid mutations, H-bonds, angles and distances between atoms.	http://www.expasy.org/spdbv/	The SIB (Swiss Institute of Bioinformatics)
	VMD	Designed to allow on to visualize and analyze the trajectory of molecular dynamics (MD) simulation, it also allows modeling, visualization, and analysis of biological systems. It is pliant, endowed of different analysis tools and is fully customizable by users through script interface.	http://www.ks.uiuc.edu/Research/vmd/	Humphrey, W. *et al.*

mAb. Notwithstanding the presence of several methodological variants, a possible experimental approach clearly illustrating this technique consists basically of six major steps: (i) antigen treatment with a solution containing deuterium; (ii) binding of the deuterium-treated Ag with the mAb of interest; (iii) water washing of the treated-Ag in complex with the mAb, to allow ion exchange; (iv) mAb elution by a low pH buffer; (v) Ag elution followed by pepsin digestion; (vi) MS measurement [33]. After the experimental session, the Ag fragments containing deuterium can be analyzed *in silico* to find a possible consensus motif on the Ag crystal structure present in protein databases available on line, as previously described in 'peptide panning'. Interestingly, Pandit *et al.* demonstrated that the prediction obtained from such a medium-resolution technique could be a useful starting point to guide subsequent computational docking prediction analysis (discussed in the next paragraph), by firstly excluding antigen regions not involved in the binding and focusing the *in silico* analysis on the MS-identified amino acid residues. The work designed by Pandit *et al.*, also demonstrated a good correlation between the H/D-MS generated results and the crystal structure of the molecular complex analyzed (Fig. 1). Similar results can also be achieved by analyzing *in silico* the experimental data generated by other approaches such as epitope excision followed by mass spectrometry [16] and using nuclear magnetic resonance spectroscopy for solution-phase epitope mapping. Interestingly, this last approach has also been combined with the use of explicit-solvent molecular dynamics (MD) simulations [17].

Approaches based solely on computational analysis

mAb–antigen docking prediction

One of the most important goals of bioinformatics is to enhance understanding of biological processes where experimental strategies are not viable or conclusive. Bioinformatic strategies differ according to the final purpose of each project: sequence alignment, gene finding, drug design, protein structure alignment, protein structure prediction and protein–protein interactions.

Amino acid sequence analysis in the microbiological field is most commonly focused on pairwise or multiple sequence alignment. This kind of analysis generally utilizes highly diffuse freely available computer alignment tools. However, for epitope-based vaccine *in silico* design, the 'scenario' is completely different, and, as mentioned above, the researchers' interest moves to structural bioinformatics. This bioinformatic branch encompasses the use of computational tools for molecular rendering and structural alignment, as well as structural superposition. Essentially, molecular rendering of crystals allows the understanding of both 3D localization and the physical propriety of a molecule or amino acid residues of interest to occur. Whereas, the structural alignment attempts to establish homology between two or more polymer structures based on their shape and 3D conformation, which are not strictly related to the linear amino acid sequence. This is the process usually applied to protein tertiary structures. To perform these analyses, many databases and software tools have been developed following different approaches. These approaches can essentially be divided into those allowing the prediction of a

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protein structure and in those studying the molecular interactions between proteins.

mAb–antigen docking prediction: protein structure prediction

Prediction of the structure of a protein from its linear sequence is an important problem in computational biology that has not yet been fully resolved. Prediction of protein structure consists in the calculation of the 3D structures of a protein (i.e. its secondary, tertiary, and quaternary structures) starting with its amino acid sequence [34]. It is generally observed that the native structures of proteins correspond to minimum-energy states. For this reason there are several software tools applying different calculation methods.

For Ab modeling, different informatic strategies and tools can be applied, such as PIGS Server (Prediction of Immuno Globulin Structure), WAM (Web Antibody Modeling), RosettaAntibody (Fv Homology Modeling Server) and several others, such as those employing the so-called '*ab initio*' methods.

PIGS (http://www.biocomputing.it/pigs) and WAM (http://antibody.bath.ac.uk/) are web servers relying upon a database of immunoglobulins structurally known and aligned; they perform the automatic prediction of the Ab structure based on the canonical structure method [35–37]. Canonical structure methods are generally effective but are burdened by their relative imprecision in predicting loop structures; to overcome this limitation, both PIGS and WAM use different algorithms to build 'non-canonical' complementarity determining regions (CDRs) residues.

The RosettaAntibody program (http://antibody.graylab.jhu.edu/) represents an advance in homology modeling-based tools, as it uses simple energy functions to optimize the first model obtained by selecting the best template for each Ab-framework (FR) and CDR. In other words, it predicts antibody Fv (variable domain) structures through homology modeling and simultaneous optimization of model conformations through the Monte-Carlo-plus-minimization algorithm to generate many putative structures and scoring functions leading to the selection of the ten best Ab models (Fig. 2b).

The so-called *ab initio* methods [38] differ from these other methods because they are essentially based on physical and chemical principles that do not require a template. Even if useful in the absence of canonical structures, they often fail to predict the correct structure and are additionally burdened by high computational costs.

mAb–antigen docking prediction: protein–protein interaction prediction

The study of molecular interactions by bioinformatics is mostly focused on the prediction of the interactions between different proteins. This bioinformatic branch combines bioinformatics with structural biology to find possible interactions between proteins (i.e. Ag/mAb interactions).

Empirically, the interactions between pairs of proteins can be inferred from several experimental techniques, such as alanine scanning, peptide panning, yeast two-hybrid systems [39], affinity purification/mass spectrometry [40], protein microarrays [41] and fluorescence resonance energy transfer [42]. However, a lot of computational methods for interaction prediction have been developed in the recent years [43,44]. As already reviewed by others [45,46], they can, in the main, be clustered in Bayesian network modeling, 3D template-based protein complex modeling, phylogenetic profiling, prediction of co-evolved protein pairs based on similar phylogenetic trees, identification of homologous interacting pairs, identification of structural patterns and supervised learning problems.

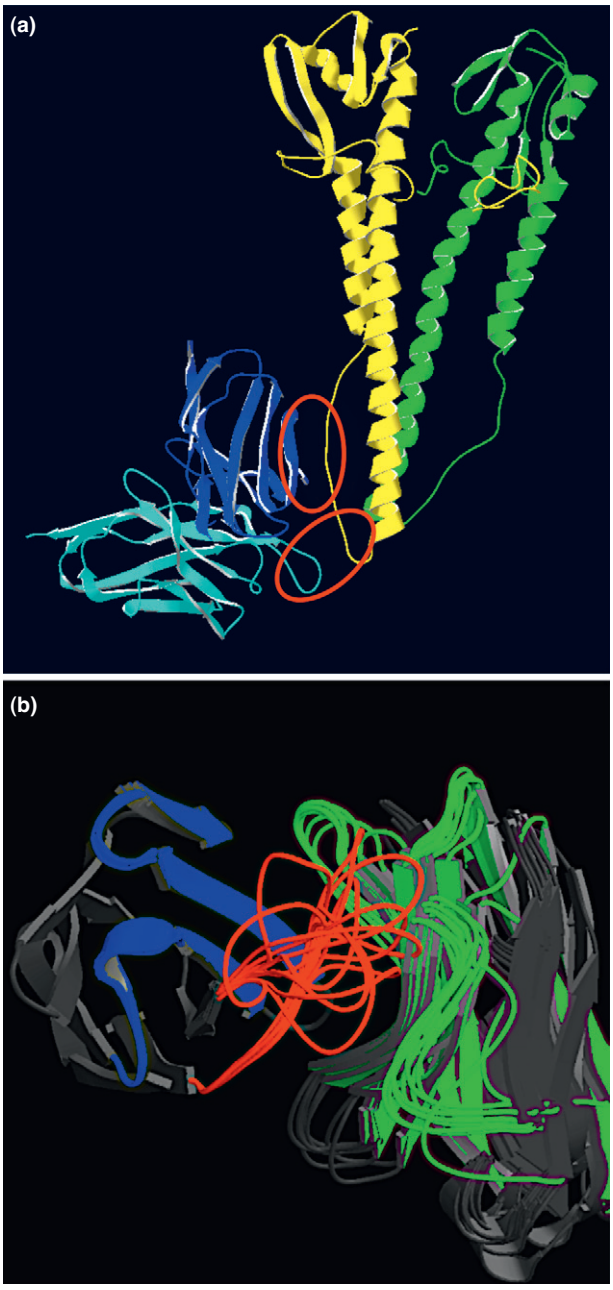

FIGURE 2

(a) Example of mAb/Ag docking prediction performed by the RosettaDock server. Graphical editing performed using 'RasMol' suite. Putative contact sites between Ab fragment (blue and light blue) and Ag crystal structure (yellow and green) are highlighted by red circles. **(b)** Prediction of the ten best possible Ab fragment variable domain 3D structures through homology modeling and optimization of model conformations performed by Rosetta (CDR1, CDR2, CDR3 possible conformations are respectively represented in blue, green and red).

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Docking studies between two molecules can best be addressed when the binding between two macromolecules is well known and characterized by experimental evidence (as is the case with antibodies) with several exclusions based on *a priori* knowledge allowing to restrict the possible regions involved in the docking [47,48]. This kind of analysis can be performed using some scoring functions (mathematical methods based on the prediction of the strength of non-covalent interactions between two molecules after their docking) that need some prerequisites, ascertained or predicted by computational biology, such as protein tertiary structure, ligand active conformation and binding mode [49–56]. Given this, we report an example of docking analysis between a mAb and its target antigen.

Firstly, the primary mAb sequence is loaded into computer programs for secondary structure prediction and then, as described above, computer servers and/or local programs such as the RosettaAntibody create models for tertiary structure. Once this analysis step is completed, further mAb/Ag docking interaction studies can be addressed using scoring functions adopted from web servers or programs such as RosettaDock server [57,58], ZDock [59] or HADDOCK [60,61]. RosettaDock predicts the structure of protein complexes given the structure of the individual components and an approximate binding orientation (Fig. 2a); as it performs a local docking search, reliability of the docking largely depends on the initial positioning of the structures, both in terms of proximity and orientation of the interacting surfaces. ZDock does not require *a priori* knowledge of the putative interacting surfaces, because it searches all possible binding modes in the translational and rotational space. It also includes the option of manually selecting specific amino acid residues that must be included or excluded in the interacting surfaces. Both RosettaDock and ZDock rely on algorithms that consider the interacting proteins as rigid bodies, lowering their predicting accuracy for flexible domains. HADDOCK overcomes this limitation by performing a semi-flexible refinement step after the first docking prediction. It also has the functionality to include restraints on the basis of experimental data. The differing computational analysis tools present various advantages and disadvantages as recently described by Pedotti *et al*. [62]. However, even if it is unable always to produce reliable results when compared to crystallography, *in silico* docking prediction analysis shows possible docking between mAb and target antigen where the main approach is not possible. From this, one is able to select amino acid residues involved in the predicted interaction and perform further experiments, such as alanine scanning, to confirm the computational analysis prediction.

Other different molecular docking tools utilized to probe protein–protein interactions have been described [50,63,64]. Some of the most widely used docking simulation software tools are UCSF DOCK (http://dock.compbio.ucsf.edu) or AutoDock 4 and AutoDock Vina (http://autodock.scripps.edu). Both UCSF DOCK and AutoDock (4 and Vina) have applications in X-ray crystallography, structure based drug design, virtual screening, combinatorial library design and protein–protein docking and use different scoring functions. DOCK is a bioinformatic tool that can examine possible binding orientations of protein–protein complexes. It can be used to search databases of molecular structures for compounds which bind to target receptors [65]; in addition, a Molecular Dynamics (MD) engine has been improved in its most recent program versions (6 and 6.5) particularly in the scoring functions' amber score. AutoDock is a suite of automated docking tools. It is designed to predict how small molecules, such as substrates or drug candidates, bind to a receptor of a known 3D structure. AutoDock's strongest feature is the range of powerful optimization algorithms available.

Clearly, the docking prediction analysis still presents some limitations [66] and can produce false prediction results. In fact, given that bioinformatic strategies used to predict protein–protein interactions corresponding with 'true molecular docking', are continually evolving [67], the full characterization of a mAb epitope is an open question to date [68]. Despite this, incorrectly predicted *in silico* results can, fortunately, often be confuted by experimental studies.

Beyond the static approaches and closer to the dynamic interactions: 'The Molecular Dynamics'

The interplay between experimental and computational approaches is highly relevant in better defining the epitope–paratope interaction from a dynamic point of view; both crystallographic structures and *in silico* docking techniques provide a static picture of the interaction, which might lead one to underestimate the role of some key residues. Moreover, a static model cannot be efficiently applied to highly flexible molecules. MD simulations, starting with experimental data, have recently been used to cover those technical limitations; this computational method is based on the resolution, at each time step, of the classical equation of motion for every atom of the system. Different platforms are now available to perform MD simulations, of which the two most commonly used and available as freeware are NAMD (http://www.ks.uiuc.edu/Research/namd/) and GROMACS (http://www.gromacs.org/). Each of these typically use the CHARMM force field, which takes into account both the bonded and non-bonded interactions, to compute the forces applied to each atom of the system [69,70]. MD has been used to better understand interactions between an antibody and its antigen, as it allows the investigation of the proteins motion on a range of timescales, revealing interactions, correlations and conformations that are important in protein–protein interactions. Thus, MD simulations can provide refined information on which residues are directly and stably interacting at the epitope–paratope interface; some of these in fact, might be under- or overestimated because the crystallizing conditions are often far from the physiological environment, favoring different conformations other than the native one [71,72]. Recently, a combination of NMR and MD has been applied to the characterization of the interaction of carbohydrates-specific antibodies to their target, the resulting crystallography only achievable with great difficulty due to its limitations in solving flexible structures [73,74]. Finally, attempts to predict the interaction of two proteins starting from the structures of the mAb and only its target using MD have been made, with a good prediction rate determined by comparing simulation results with Ab–Ag complexes already resolved through X-ray crystallography [75].

Concluding remarks

Several examples of epitope mapping strategies, including the use of freely available bioinformatic tools, have been briefly reviewed. To be able to study a mAb/Ag interaction through crystallization

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and X-ray diffraction of the complex certainly represents the gold standard for epitope characterization. However, several factors may interfere with the Ab/Ag complex crystal resolution. From this perspective, several surrogate approaches combining experimental procedures and bioinformatics have been developed. Informatic tools in the research field can be used either as a supporting tool or, on some occasions, as the only available tool for certain kinds of analysis. However, in our opinion, regarding mAb/Ag interaction characterization, the computational analysis cannot and should not be reduced to a meticulous *in silico* analysis of the sequences or protein prediction structures without considering the experimental data obtained from a 'real' study of such interaction. Meanwhile, even if empirical knowledge can circumscribe and address the computational analysis, an *in silico* study can be very helpful for the understanding of interactions between molecules that can represent the 'key-motif' of a possible 'epitope-based' vaccine design.

Conflicts of interest

The authors declare no conflict of interests.

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