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Review Article

B-cell epitope mapping for the design of vaccines and effective diagnostics

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ABSTRACT

The increasing resistance of many microbial strains to antibiotics, delayed laboratory results, and side effects of many chemotherapeutics has raised the need to search for sensitive diagnostics and new prophylactic strategies especially prevention by vaccination. Understanding the epitope/antibody interaction is the key to constructing potent vaccines and effective diagnostics. B-cell epitope mapping is a promising approach to identifying the main antigenic determinants of microorganisms, in special concern the discontinuous conformational ones. Epitope-based vaccines have remarkable privilege over the conventional ones since they are specific, able to avoid undesirable immune responses, generate long lasting immunity, and are reasonably cheaper. This up-to-date review discusses and compares the different physical, computational, and molecular methods that have been used in epitope mapping. The role of each method in the identification of potent epitopes in viruses, bacteria, fungi, parasites, as well as human diseases are tagged and documented. Simultaneously, frequent combinatorial methods are highlighted. The article aims to assist researchers to design the most suitable protocol for mapping their B-cell epitopes.

Contents

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1. Introduction

Immunology has a great impact on improving global health through the synthesis of rapid diagnostics. Simultaneously, it paved the road towards the protection, as well as the complete eradication, of many pathogens via the art of vaccination [1,2]. Antibodies (Abs), also known as immunoglobulins (Igs) are essential components of the immune system of all vertebrates. They are able to identify and neutralize foreign immuno-stimulating objects such as viruses, bacteria, parasites, fungi, cancer cells, and some toxins by binding to specific parts on their surface, that are usually called antigens (Ag) [3]. In fact, the paratope, a specific part of an antibody, binds to a particular region on the antigen that is called the epitope or the antigenic determinant [3,4]. Unlike the T-cell epitopes, the majority of the functional B-cell epitopes are discontinuous non-linear epitopes having 3D-conformational structures [5]. The studies of paratope–epitope interaction are considered recent [6]. Their study offers benefits to the fields of research in immune response, vaccines and diagnostics design, passive immunization, allergens, and auto-immunity [7,8].

The production of diagnostics and immunotherapeutics first depended on the use of the whole antigen upon *trial and error* methods or virulence studies guidance [2,9]. However, the degree of success of any epitope depends on its ability to induce the most specific and detectable rapid immune response in the case of diagnostics. While it lies on its capability to confer a neutralizing safe response for vaccines (B-cell dependent response) [10]. Moreover, its ability to stimulate cytotoxic or long-lasting potent immune response (T-cell dependent response) for vaccine production as well [11]. Therefore, epitope mapping has developed in order to focus on the selection of the most potent epitopes that could serve as potential targets for the production of epitope-based diagnostics and vaccines [1,4,12]. A special concern is dedicated for DNA vaccines that are built upon short peptide chains [13]. It was noticed that the presentation of non-protective epitopes deviates the immune system potency [1,14,15], and decreases the antigen–antibody affinity [16]. Therefore, epitope-based vaccines aim to provide protective focused immunity without mimicking the host self-antigens, which render them safe regarding autoimmune disease induction [1,14,15]. Vaccines' mapping aid in reducing the cost, complexity, and time of synthesis [1].

Although B-cell epitope mapping is the corner stone-step in the production of diagnostics, it is only the first step to design potent vaccines [1,10,12,17]. Epitope mapping will not lead to the straight forward identification of highly protective monoclonal antibodies (mAbs), as some antibodies that showed neutralizing activity *in vitro* were not able to do so *in vivo*. Moreover, they can give rise to *in vivo* antibody dependent enhancement (ADE), a phenomenon that increases the infectivity of host cells to viruses in the presence of some antiviral antibodies [18]. In addition to this, several epitope mapping techniques cannot differentiate between conformational and linear epitopes. Thus, careful considerations have to be taken when analyzing the results acquired by different methods used in mapping [1,4,19]. Furthermore, results obtained could be enhanced and confirmed by combining two or more techniques of epitope mapping [20,21]. These results may be consolidated by the T-cell ones, especially for the vaccine development against cancer [22,23] or intracellular pathogens [24–26].

2. The different methods for B-cell epitopes' mapping

The following paragraph documents the different physical, computational and binding methods used in B-cell epitope mapping for vaccines and diagnostics production.

2.1. Crystallography-based methods

Co-crystallization of antigen–antibody complex is one of the first techniques that were used in epitope mapping. In this technique, the highly purified antigens are obtained and allowed to co-crystallize with their corresponding antibodies. Then, the atomic structure of the complex is solved using X-ray diffraction analysis. The structure of the epitope is obtained by solving the three dimensional coordinates that represent the electron densities of the amino acids of the antigen–antibody complex [1,10,27]. The amino acids that are within a distance of 4 Å of each other are considered to be counteracting [6]. Unlike several techniques of B-cell epitope mapping, the co-crystallization method is able to detect continuous linear epitopes as well [4,10]. However, the technique is obviously complex and expensive, as it requires large amounts of highly purified protein-mAbs (monoclonal antibodies) complexes and the structure of the mAbs should be known [1,28]. Obviously the antigen–antibody complex must be crystallizable [29]. Thus some antigens are still mapped by this technique to reveal the B-cell epitopes of malaria [30] and bilharzias [31] parasites.

Thoughts to apply electron-microscopy (EM) to reveal the antigen–antibody interaction were not practical, since the complex-molecule was subjected to degeneration and dryness [32]. Since the beginnings of 2013 the progress in cryo-EM analysis technique re-introduced the use of EM to study the freezed antigen–antibody complex in a non-crystalline amorphous thin layer, especially when coupled with X-ray crystallography [33]. The new technique requires less amount of complex, do not necessitate high purity of the complex's components, and the ability of the complex to crystalize is not a must [32]. This technique was recently used to map the B-cell epitopes of HIV-1 [34] and HPV [35], or in combination with X-ray crystallography to map poliovirus type-1 and 2 [36,37] and to study the structure of the rabbit hemorrhagic virus [38] for vaccines' production.

2.2. Mass spectrum-based methods

The application of mass spectrum (MS) in epitope mapping had positively influenced the identification and characterization of discontinuous epitopes [39]. In general, there are two main methods to use MS in epitope mapping. The limited proteolysis method, in which different proteases are applied to the antigen of interest. The fragments released from the different cleavage-sites in the presence and absence of the antibody are detected by MS to reveal the bound fragments to the antibody [40]. While in the epitope excision method extensive proteolytic digestion is applied to the antigen incubated with antibody coated beads. The beads are then washed for several times to eliminate the non-epitope fragments leaving the epitope fragments bound to the antibody. The epitopes are then liberated by acid washing for further identification by LC-MS or MALDI/MS [41,42]. One of the major limitations of this method is the resolution which is, the ability to accurately determine a peptide fragment. The reason is that the limited proteolysis and epitope excision approaches require proteolytic digestion with trypsin. Therefore, epitope mapping is confined only to cleavage sites which results in the identification of long peptides (typically 30–60 residues) that poorly define the epitopes. In addition, the method is more concerned by the location of the reactive residues on the antigen of interest rather than the spacing between them [39], that shares in the conformational structure of the epitope.

The antigen–antibody complex placed in deuterated solvent, will exchange the deuterium atoms with the free non-bound sites. After digestion with pepsin the level of deuteration may be estimated by MS [43]. Hydrogen–deuterium exchange method coupled to MS (HD-MS) technique was used to overcome the defects

of regular MS methods. This method identify the interaction sites between the antigen and antibody. The antigen can be used with moderate purity, and polyclonal antibody may be applied [6]. However this method is still a rough mapping method, since it cannot identify epitopes of less than 10–20 residues long. Although the occasions when MALDI/MS was used to map the epitopes have been quite limited, the approach was used in mapping the epitopes of human thrombin [39], and HIV-1 gp41 [44]. Later on the HD-MS method was used as well to study the epitopes of the antibody against the coagulation factor to prevent Hemophilia symptoms [45]. While more recently Temporini et al. [42] mapped the glycoproteins of tuberculosis using the LC-MS technique.

2.3. Nuclear magnetic resonance (NMR)

When a high magnetic field is applied to a sample, the protons associated with each amino acid absorb electromagnetic radiation. The 3D-structure of the target protein could be constructed by measuring distances and angles between amino acids residues. NMR analysis provides a detailed structure analysis for epitope–Fab (fragment antigen binding) interaction, therefore the nature of epitope recognition could be accurately explained [1]. In this technique, the protons of the Ag-Ab are subjected to the effects of magnetic field and pulsed electromagnetic radiation in order to obtain a dynamic picture of the protein complex in solution and to construct the atomic definition of the Ag–Ab interface [1,46,47]. However, this technique has some significant drawbacks such as a high degree of sophistication, demand for technical expertise, expensive instrumentation, and sometimes gives contradicted results with X-ray results [1,19]. Furthermore, this technique is only applicable to continuous small molecular weight proteins that are below 30 kDa [48]. Moreover, the structure of the antigen should be known, Ag-Ab should be highly pure, and present in relatively high concentration [6]. NMR was successful in mapping the epitopes of MUC1, a breast cancer associated antigen [49,50], and E7 protein of Human Papilloma Virus-16 (HPV) [51]. Simultaneously, the technique was used to study of mechanism of action and binding sites of heparin [52]. More recently, NMR was used to identify the appropriate epitope of Tau protein associated with Alzheimer to be used as diagnostic tools for the disease [53]. In other cases, NMR was combined with surface plasmon resonance to identify the epitopes of *Legionella pneumophila* [54].

2.4. Surface plasmon resonance (SPR)

SPR is a complex physical technique that has wide variety of applications, one of which is epitope mapping. SPR is a powerful method for the analysis of low affinity-protein/protein interactions. The physical principles of the technique is very complex, however a suitable working knowledge of the technique does not involve extensive theoretical background. The instruments are appropriate for the evaluation of the binding of recombinant-proteins to natural ligands and mAbs. SPR immunoassay resembles the concept of the ELISA technique, but it is label free [55,56]. It was first used in 2002 by Kooistra et al. [54] to map an important outer polysaccharide that serves as an epitope for *L. pneumophila* using NMR and SPR as discussed before.

However, SRP has shared practically in mapping the epitopes of the active form of vitamin B12 (holo-transcobalamin) [57], the manganese transport protein C (MntC) of *Staphylococcus* sp. [58], the epitopes of Hemophilia disease [59], and ricin toxin [60]. In other cases, SPR was combined with phage display technique for epitope mapping to identify the major epitopes of Goodpasture auto-immune disease [21]. It was combined as well with binding and *in silico* techniques to identify the cross-reactivity between the milk and soy bean allergens [61].

2.5. Computational-based methods

Computational docking is a computer-based method for epitope mapping that depends on the generation of two distinct crystal 3D-structures for the antigen and the antibody and subsequently creating a battery of computer algorithms in order to dock *in silico* one structure onto the other. In 1982 Kuntz et al. created DOCK, which was the first docking program [62]. Docking programs are assuming that the structure of the antigen–antibody complex is representing the lowest free energy state accessible to the system [63]. The major drawback in docking algorithms is the scoring functions, since high scoring functions do not ensure achieving a near native predicted complex [64,65]. However, this technique is able to predict the small protein molecules complexes such as enzyme-inhibitor/ligand complexes and antibody-hapten complexes, since large molecules undergo many conformational changes that makes docking procedure extremely difficult [66,67]. In essence, little was focused on using this approach in epitope mapping [66], however the method was used to map the H1N1 epitopes [68], and was combined with mutagenesis method to map HIV-1 [69].

Recently the *in silico* computer-based software used to predict the possible Ag–Ab interactions greatly developed. Those new bioinformatics products were used to screen protein epitopes of several antigens in order to reduce further studies by molecular methods, hence reduce cost, time and efforts. *In silico* products are usually used to screen the epitopes of newly studied antigens that have a defined genetic profile or those antigens of emerging uncontrollable microbes [70]. Nowadays several B-cell epitopes databases are available for many antigens [71–73]. Those include the two protein–protein docking methods, DOT [74] and PatchDock [75]. Together with the one-structure-based epitope prediction tools such as CEP [76], DiscoTope [77], and ElliPro [78]. As well as the two structure-based methods for protein–protein binding site prediction such as ProMate [79], COBEpro [80], BCpred [81], BepiPred [82], BePpro [83], and Seppa [84]. Moreover, commercial techniques such Epiquest™ 2014 software sold by APTUM Biologics Ltd, and online web-based software such as Epitopia [85], and B-pred [86] appeared. Simultaneously combinatorial methods able to detect both T- and B-cell epitopes appeared such as Jameson-Wolf DNAStar [87].

The technique was used to unravel the target epitopes of the transforming growth factor (TGF)-β1 to control hepatic fibrosis [88], Hemophilia control factors [89], *Mycobacterium avium* (MAP) that correlates with diabetes disease in humans [90–94], anthrax [95], the emerging nosocomial bacteria *Acinetobacter* [96] and *Klebsiella* [97], HIV [98], Epstein–Barr virus (EBV) [99], foot and mouth disease virus [100], Crimean–Congo hemorrhagic virus [101], blue tongue virus [102], Chikungunya virus [103], and to detect the allergens of soybean [104], eggplant [105], peanut [106], and the under-researched onion [107]. Some studies combined the *in silico* approaches with binding analysis to identify the epitopes of snake venom [108], and soybeans cross-reactivity with milk allergen [61].

2.6. Binding assays

Binding assays depend on the ability of an antibody to bind to different fragments of an antigen that may be either a protein or a polysaccharide. They have the advantage of being able to quantify the immune response towards a specific epitope [1].

Western blot is a technique, used to screen individual epitopes in a mixture, and may be applied to semi-quantify the relative

responses of each epitope. First, the mixture is applied to a gel-electrophoresis in a carrier matrix (polyacrylamide gel) in order to separate them according to size, charge, or other differences in individual bands. Then, the separated bands are transferred to a carrier-membrane (e.g. nitro-cellulose, nylon or PVDF), where they are allowed to interact with antibodies specific to the target antigen(s). The antigen could be detected with radio-active, or fluorescent labels, or enzymes that give a subsequent reaction with an applied reagent, resulting in a coloring or emission of light [109,110]. The western blot method is simple, reliable, and sensitive. In addition, it is able to scan the whole epitopes of a pathogen all at once, either being conformational or linear. It is used to detect both macromolecules and hapten epitopes [111]. Also the technique managed to map the epitopes of almost all antigens such as bacteria [112], fungi [113], human diseases [114], and parasites [115]. ELISA usually integrates with immunoblot to enable the accurate quantification of a specific identified epitope [1,109]. The techniques applied to perform ELISA in one-step evolved [116].

However, the dot blot technique requires spotting of the purified bio-molecule to be detected directly on the membrane. In this technique, there is no need to separate the macromolecules by electrophoresis. Although used mainly for qualitative detection, it might also be used as a semi-quantitative method [1]. Whereas, the microarray methods are emerging as one of the major techniques that are used in multiplexed detection of DNA [117,118], proteins [119,120], peptides [121–124], antibodies [125], and carbohydrates [126], which are immobilized on planar supports such as glass/coated slides [127]. Cells, as well as, tissues are examined by microarrays that have wide spread applications such as plasmid transfections, RNA interference, and serological assays [128–130]. The peptide microarrays (PMAs) is a promising tool that has a wide variety of applications such as epitope-mapping, substrate profiling, and probing peptide–ligand interactions [121,131]. SPOT synthesis techniques developed for the profit of peptide microarray assays [132–135]. Recently, the technique developed through a next-generation application using a high-density peptide microarray, that can perform thousands of tests at the same time [136]. In general, the technique has several advantages such as high-throughput, improved reproducibility, high-density and low sample consumption. However the technique has limitations including the difficulty of getting highly purified peptides, and the orientation of the spotted proteins because of the limited number of the reactive sites of a protein [137]. Moreover, PMAs are only capable of detecting linear epitopes. Thus, PMA is an excellent method for mapping protein binding sites that were solved before, and not for discovering new interaction between proteins [138].

The applications of the binding techniques to screen the potent epitopes are rich; however, the majority of applications were directed towards western blot and ELISA. ELISA was a pioneer tool in epitope mapping by binding procedures. It was used in mapping the epitopes of HIV-1 envelop subunit vaccines, recombinant gb120 and rgb160 [139,140], HCV [141], the Japanese Encephalitis Virus (JEV) [142,143], hepatitis E virus (HEV) [144], EBV [145], *Klebsiella pneumoniae* [146], gonococcal saccharides [147], *Enterocytozoon bieneusi* [148], gastrointestinal cancer [149], MAP similarity with proinsulin [150], and plectin (a major component of the cytoskeleton) [151,152]. Western blot/ELISA combinatorial system was successful in mapping the uropathogenic *Escherichia coli* [153], Staphylococcal α-hemolysin [154], and *Brucella melitensis* periplasmic protein [155]. The combinatorial methods were used by several researchers since 1989 to map the epitopes of the emerging nosocomial pathogen *K. pneumoniae* [156]. Sample specifications and screened antigens varied among trials [157–160], till a potent epitope-based vaccine and diagnostic against the pathogen was patented [161].

Furthermore, western blot/ELISA techniques were also used in mapping several viral epitopes such as the epitopes of hepatitis B virus (HBV) [162], JEV [163], Dengue fever virus [164,165], Cytomegalovirus (CMV) [166], bovine leukemia virus glycoprotein [167], and Beet Necrotic Yellow Vein virus [168–170]. Moreover, western blot was practiced in mapping the epitopes of the human sperm [171,172], systemic lupus erythematosus (SLE) [114], and allergens [173,174]. Epitopes of the parasites such as malaria [175], and *Treponema pallidium* [176] were also mapped by the same technique.

Simultaneously several diagnostic tools were identified using immunoblot/EILSA techniques such as the MAP antigens in diabetic patients [177–179], Parkinson disease [180], and Leishmania's parasite [181].

PMAs appeared as a useful tool for epitope mapping of Rubella Virus, CMV, *Toxoplasma gondii*, and Herpes Simplex Virus type I and II [182,183], protein MPB70 of *Mycobacterium bovis* [184,185], bovine leukemia virus [186], polymorphic protein of *Plasmodium falciparum* [187,188], murine and human anti-hTSHR antibodies [189], Lupus auto-antibodies [190], bovine β-lactoglobulin [191], Atlantic code in Spanish people [192], brain cancer diagnostics [193], and Potato Y virus diagnostics [194]. *Trypanosoma cruzi* epitopes were re-studies by high density peptide microarray in order to develop a vaccine and a diagnostic tool against the parasite [195,196]. ELISA was combined with PMAs to screen the epitopes of foot and mouth disease virus [197], HEV [198–200], the Tau protein in Alzheimer's disease [201]. Western blot was combined with ELISA to map the epitopes of *Pneumocystis carinii* [113], and the main antigenic protein in *Bullous pemphigoid* [202].

Binding methods proved to be successful for epitope mapping when combined with pepscan as in the mapping of the epitopes rotavirus-A [203], the recombinant adeno-associated virus type 2 [204], the outer membrane proteins of *Chlamydia pneumoniae* [205], and *Bacillus anthracis* [206]. The application of binding methods was sometimes combined with the mutagenesis technique as in the identification of several potent protein epitopes of *Plasmodium malaria* [115], and the staphylococcal enterotoxin B [112]. Additionally, a combination of several methods of epitope mapping, specifically western blot, ELISA, and phage display highlighted a potent epitope of *Mycoplasma hyopneumoniae* [20]. Moreover, in 2012 Maksimov et al. [207] demonstrated the potential of eight epitopes of *T. gondii* using PMA and a bioinformatics–based approach. The immunoblot/ELISA was used sometimes in combination with SPR and *in silico* techniques to detect the common epitopes between the soybean and milk [61], or the phage display to study the mycobacterial complicated protein epitopes [208].

2.7. Mutagenesis

Mutagenesis is a vital technique whereby DNA mutations are deliberately engineered to produce mutant proteins. The mutant protein (antigen) can be examined to determine the effect of the mutation on its ability to bind to a target antibody and determine the genuine epitope of that antigen [1,209–213]. The method is popular as it is relatively simple [211], however results obtained from the technique might be confusing because the mutated contact amino acids can either stimulate binding, inhibit binding, or show no effect [1]. Mutagenesis could be classified according to the technique used to induce the mutation into three main categories the site directed mutagenesis, the shotgun mutagenesis, and the site directed masking [110].

Site directed mutagenesis is one of the simplest methods of epitope mapping [211], in which a highly specified mutation is introduced into a specific sequence of the antigen of interest and then the antigen is tested for its ability to bind to the corresponding antibody. Failure of binding signifies that a particular amino acid

is critical to the functioning of the antigen being tested, and therefore constructing a map for the epitope of the antigen being tested. Site directed mutagenesis could be classified as either alanine scanning mutagenesis (ASM) or saturating mutagenesis [1]. In ASM, every single residue in a specific peptide sequence is subsequently replaced by alanine one at a time and at the same time, and glycine usually replace alanine residues that are present in the original sequence [209,210]. Therefore, the central amino acids in the studied antigens that are necessary for antigen–antibody recognition can be identified. However, this technique has some significant drawbacks since the method involves great exertion due to the necessity of producing many mutants, purifying them, and evaluating their structural integrity and binding capacity. Furthermore, the results obtained by ASM are not usually matched with the physical binding site identified by crystallographic methods [1]. On the other hand, saturating mutagenesis depends mainly on inducing random mutations in the DNA sequences [214,215] by using several chemical reagents such as nitrous acid [216] and hydroxylamine [217]. Other chemical reagents such as formic acid [218,219] and hydrazine [220] have the ability to damage the bases of DNA causing incorrect base pairing [214]. In this technique, a low fidelity DNA polymerase is used in order to create random mutations in the amplicon (the product to be amplified) in PCR amplification [221]. In essence, site directed mutagenesis is a confusing tool for epitope mapping due to the fact that many mutations that have significant effect on the degree of binding between the target being tested and the monoclonal antibody could be misleading as they are not part of the real epitopes [1].

Shotgun mutagenesis uses a high throughput technology for the identification of the binding sites between antigens and their corresponding antibodies. This technique enables the expression and analysis of large libraries of mutated target proteins by introducing systemic mutations that nearly cover all the amino acids in the target protein in order to determine the amino acids that play an essential role in the binding process. Using a high throughput "Shotgun Mutagenesis" approach for epitope mapping, one can rapidly construct comprehensive epitope maps across the entire sequence of difficult target proteins such as G protein-coupled receptors [212].

A more recent methodology to map epitopes by mutagenesis, called site directed masking, was introduced by Paus and Winter in 2006. The technique is only applicable when the 3D-structure of the protein is available [213]. In this approach, the protein antigen surface is occupied by a panel of single cysteine mutations that is used, along with a linker to solid phase, to block accessing of antibody to this location [222]. Therefore, the mutations which prevent antibody binding specify the location of the antibody epitope interaction, which can be further enhanced by alanine scanning mutagenesis [222].

Mutagenesis techniques had shared practically in the mapping of the epitopes of the hen egg-white lysozyme allergen [223], the house-dust-mite allergen [224], HIV-1gp120 [225], the E2 envelope protein of hepatitis C virus (HCV) [226], Foot and mouth disease virus [227], Dengue's virus [228], and *Plasmodium's* parasite [229]. Occasionally, site directed mutagenesis technique was shown to be successful when combined with the phage display method of epitope mapping. This was shown in the mapping of the epitopes of the human plasminogen activator inhibitor-1 [230], and the alpha subunit of the nicotinic acetylcholine receptor [231]. While, the mutagenesis approach was combined with pepscan in the mapping of the human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas [232]. The application of mutagenesis techniques of epitope mapping was combined sometimes with other binding methods [112,115] or with SRP [21] as previously discussed and phage display [233,234]. High-throughput mutagenesis was usually paired with next generation *in silico* sequencing. A genome wide library of HCV and mutagenesis succeeded to map the epitopes of HCV [235], as well as different antibodies targeting Pertussis Toxin, TNF, and the cancer target TROP2 [236].

2.8. Pepscan

In 1984, Mario Geysen launched a new method for epitope mapping, in which the desired antibody is investigated against a library of peptide sequences from the protein of interest (antigen) to test their ability to bind to the desired antibody [110,237]. The segments of peptides that have the ability to bind to the antibody of interest demonstrate a significant part of the antigen [226]. The presence of two distant peptides that share the ability to bind to an antibody of interest due to having structural similarity signifies that these two peptides are contributing to the intact epitope of interest [1].

Geysen and coworkers' technique is very suitable for the *in vitro* identification and characterization of T-cell epitopes, as well as B-cell epitopes [237,238]. B-cell epitope analysis procedure by pepscan involves four major steps, which are the chemical synthesis of peptides immobilized on polypropylene pins, then the use of ELISA as a method for binding analysis, followed by bioinformatics-based data analysis, and finally constructing initial results [127].

In general, there are two types of the chemical peptide synthesis, solid phase peptide synthesis and multiple synthesis on pins. In the solid phase technique, peptides are constructed on an insoluble polymeric support by sequentially adding amino acids. In multiple synthesis on pins technique, amino acids are synthesized on pins that are fixed to a plastic support and then incubated with antibodies of interest [127,239,240]. Binding analysis methods such as ELISA are often used for the evaluation of the binding capacity of the synthesized peptides with the antibodies being tested [127,239,241].

Usually, there is confusion between the two terms pepscan and peptide microarrays (PMAs). In general, the use of the term to refer to a pepscan implies the use of a binding analysis technique such as ELISA to confirm the binding capacity of a desired antigen/antibody towards different synthetic peptides, while the PMAs is just a collection of different peptides displayed on solid surface such as plastic or glass chip [123,242]. In some experiments, the pepscan approach was combined with the peptide microarray technique in order to achieve better results [122,243,244].

The advancement in the field of bioinformatics assisted the development of peptide microarrays techniques. The pepitope server is a very promising bioinformatics tool used in the prediction of discontinuous epitopes based on affinity-selected peptides. It is composed of three algorithms for epitope mapping: PepSurf, Mapitope, and a combination of the two in order to run and compare between two epitopes that use different methodological approaches through a single web platform [245–248].

In general, the method has a wide variety of applications, ranging from the identification of epitopes present on monoclonal antibodies [249] to the screening and detection of epitopes present on bacteria [250–252], viruses [253,254], parasites [255,256], tumor markers [257–259], and enzymes [260]. Moreover, the technique has been extensively used in the design of vaccines [261–263]. However, the technique is limited to the identification of linear epitopes [1,264] and requires a confirmatory binding method [122]. The method proved to be successful when combined with the phage display method. In 2001, Yip et al. [265] identified the several epitopes of ErbB-2, a self onco-protein that is highly expressed in several types of cancer. Furthermore, as discussed before, pepscan can combine with mutagenesis approach [232], and binding methods [203,204,266].

2.9. Surface display methods

One of the most powerful techniques is based on testing the binding capacity of a variety of peptides displayed on the surface of a bacteriophage to the monoclonal antibody of interest. In 1985, Smith and Petrenko managed to express a variety of peptide sequences on the surface of non-lytic filamentous bacteriophage [267,268]. In this technique, when a foreign DNA was inserted into the genome of M13 filamentous bacteriophage, viral peptides including the foreign DNA were synthesized and displayed on the amino terminal portion of the viral coat protein. Smith stated that phage particles are representing a link between the genotype i.e., encapsulated nucleic acid) and the phenotype (i.e., expressed capsid proteins) [269,270]. Furthermore, in 1990, McCafferty et al. [271] showed that an antibody fragment could be displayed on the phage surface, and even a hormone could be displayed on the surface of the bacteriophage [272]. The creation of vast arrays of slightly different viral peptides is accomplished by allowing each position in the viral vector oligonucleotide to acquire any of the four possible nucleic bases. As a result, nucleic bases randomization allows the creation of all possible combinations to be produced. Each phage is capable of producing unique peptides that correspond to the specified code in its genome [1,273]. Recently, automated phage display software appeared [274]. Nevertheless, several surface display systems were recently developed using yeast and bacteria [275–278], and cell-free display systems such as mRNA display [279] and ribosome display [280,281].

There are four main steps that are critical for the selection of a specific phage peptide from a phage display library. First, capturing of binding phage on immobilized antigen from the library of proteins displayed on the phage surface using ELISA. Second, washing to remove unbound particles. Third, elution of the bound phage. Fourth, sequencing the target peptide and amplifying the gene coding for it in a suitable host organism such as *E. coli* [282].

Although, the technique is very powerful in mapping the main epitopes of viruses, bacteria, and cancer antigens, it is sophisticated and needs high expertise. The technique seems to be more suitable for the screening of the epitopes of viruses and cancer antigens due to the extreme need for the precise identification of target epitopes [1]. Moreover, it requires the use of a binding analyses technique such as ELISA.

The technique was applied on several occasions to screen the epitopes of the intrinsic membrane protein neutrophil cytochrome b [283], rituximab, a chimeric monoclonal antibody against the protein CD20, which is found on the surface of B-cells [284], buffalo β-lactoglobulin allergen [285], and EBV [286]. Yeast surface display was applied to map the hemagglutinin of H5N1 influenza virus [287], while it was used more recently to map the HIV-gp120 epitopes [288]. As discussed before, surface display technique was combined with several mapping techniques [20,21,208,230,231,233,236,265,289] to resolve the epitopes of different antigens.

3. Comment

The first approaches to produce immunotherapies and diagnostics depended on the use of the whole antigens. However, the advancement in immunology proved that the pre-production design of those preparations has an apparent clear positive impact on the potency, specificity, and safety. The ability to detect the pure immunogen that interacts specifically with the first, obvious, and selective immune signal has a great interest in the design of diagnostics. However, the possibility of identifying the safe antigenic determinant, which triggers the humoral immune response against a specific antigen, ensures the design of a potent vaccine. Collectively, the detection of those responses is performed by the B-cell epitope mapping techniques.

The B-cell epitope mapping has undergone a series of evolution and development steps. The physical methods for epitope mapping preceded the binding ones. Although X-ray was applied almost a century ago, it was complex and ineffective in identifying B-cell conformational epitopes. Therefore, MS developed to characterize discontinuous epitopes after being randomly fragmented. Later on, MALDI/MS was followed by the expensive NMR technique that requires high degree of expertise and sophistication, and only can resolve small continuous low molecular weight proteins. However, cry-electron microscopy and HD-MS techniques reintroduced the use of those techniques to an extent. These techniques were followed by docking computational method that depends on the prediction of the lowest affinity energy between low molecular weight 3D-protein structures, while the most recent physical technique was the SPR that has some applications for epitope mapping in the last decade. In general, physical techniques were not ideal ones for epitope mapping, unless coupled with pepscan or phage display methods. Even though the advances in *in silico* computational methods were effective in predicting the possible antigenic determinants, they requested a confirmation step by molecular techniques.

In a parallel line, the use of the first binding technique, ELISA arose by the 1960's, and developed rapidly to mutagenesis, western blot, dot blot and microarray that synchronized the same time of using SPR. Although PMA and dot blot have high throughput, high reproducibility, low sample consumption, together with the necessity to purify the epitopes limited their use against the western blot. Moreover, dot blot and PMA can only detect linear epitopes, and hence cannot be used alone to investigate new epitopes. During the mid-1980's pepscan and surface display methods appeared for epitope mapping. Those latter two methods depend on binding and bioinformatics approaches. Later on the *in silico* software evolved to scan the genetically mapped antigens. It is apparent that all techniques possess advantages and defects (Table 1). Therefore, their use depends on the case to be studied and the type of available data if any. For the same reason, combinatorial methods appeared to reduce time, expenses, and to give better results.

Although the majority of B-cell epitopes are non-linear discontinuous folded amino acid sequences, some may be polysaccharide (PS) molecules. NMR, SPR, ELISA, PMA, dot blot and western blot techniques have the advantage of detecting polysaccharide epitopes, hence they were used to detect the PS of *Legionella*, *Shigella*, *Klebsiella*, and breast cancer cells. However, western blot owned the over-privilege for the first step of screening mixtures or whole antigens with no need for purification like in all other techniques. Therefore, this method was used to screen unknown epitopes in mixtures. Although *in silico* software were used to screen new unknown eptiopes, a complete genetic map for the antigen was necessary to apply the method. All reported molecular mapping methods are mainly able to detect epitopes qualitatively; however the ELISA, dot blot, and western blot methods are able to make quantitative analysis. Despite mutagenesis, surface display, cryo-EM and ELISA were able to detect the discontinuous conformational epitopes; the X-ray, NMR, pepscan, dot blot and PMA were restricted to continuous linear ones, whereas the MS-dependent methods and western blot were able to determine both types of epitopes (Table 1).

The systematic review of the B-cell epitope mapping used to map different antigens, highlighted the correlation between specific methods and certain antigens. Pepscan and the different binding methods showed a remarkable superiority for epitope mapping of diagnostics for bacteria, viruses, tumor markers, and *Candida* sp. (Table 1). The epitope mapping for vaccines depended on the same

Table 1
Comparison between the different methods used for B-cell epitope mapping.

		X-ray/cryo-EM	MS-based	NMR	SPR	*In silico*	ELISA	Western	Dot blot	PMA	Mutag-enesis	Pepscan	Surface display
Epitope	Type	Pr.	Gl/Pr.	PS/Pr.	PS/Pr.	Pr.	PS/Pr.	PS/Pr.	PS/Pr.	PS/Pr.	Pr.	Pr.	Pr.
	Purity	High	Mod	High	High	–	High	mixture	High	High	High	High	–
	Concentration	High	High	High	High	–	Small	Small	Small	Small			
	Structure	Known	Known	Known	Known	Known	Depends*	Unknown	Known	Known	Known	Known	Known
	Genetically mapped	No	No	No	No	Yes	No	No	No	No	Yes	Yes	Yes
	Size	Variable	Variable	Small (⩽60 kDa)	Variable	Mod.	Variable	Variable	Mod.	Mod.	Mod.	Mod.	Mod.
	Conformation	Linear/Conf	Linear/Conf	Linear	Conf	Linear/Conf	Linear/Conf	Linear/Conf	Linear	Linear	Conf	Linear	Conf
	Quantification	No	No	No	Weak	No	Yes	Semi	Semi	Semi	No	No	No
Ab	mAb structure	Known	PL	Known	No	Known	Depends*	PL	PL	PL	Pure	Pure	Pure
Application	Number of samples	Lim.	Lim.	Lim.	Lim.	Large	Mod.	Mod.	Large	Large	Mod.	Mod.	Lim.
	Combination	X-ray/cryo-EM	–	SPR	NMR/SD/PC	MT/WT/PMA	PC/WT	PC/PP/EL	–	EL/WT/PC/SD	CS/PP/SPR/PC/WT	MT/PMA/SD	SPR/PMA/MT/PP
	Sophistication	Yes	Yes	Yes	Mod.	Mod.	Simple	Simple	Mod.	Mod.	Mod.	Mod.	Yes
	Defects	Expensive & complex	Rough (⩾20 kDa)	Expensive& expertise	Expensive	Predictive	–	–	Linear epitopes	Linear epitopes	Confusing results	Requires peptide library	Complex
	Previous uses	Pa & Vs	Bt; Ds. & Vs	Cs; Ds; Bt & Vs	Al; Bt; Ds; Tx & Vt	Al; Bt; Ds; Tx &Vs;	Bt; Cs; Ds; Pa; Vs & Dg	Al; Bt; Fg; Ds; Pa & Dg	Ba & Dg	Bt; Cs; Ds; Pa; Vs & Dg	Al; Cs; Ds. & Pa	Bt; Cs; Ds; Pa; Vs & Dg	Al; Ba; Cs & Vs

Key: Al = Allergens; Bt = Bacteria; Conf = Conformational; Cs = Cancer; Dg = Diagnostics (antibodies); Ds = Disease; EL = ELISA; Fg = Fungi; Gl = Glycoprotein; PC = *in silico*; Lim = Limited; Mod = Moderate; MT = Mutagenesis; Pa = Parasite; PL = Polyclonal; PP = Pepscan; Pr. = Protein; PS = polysaccharide; SD = Surface display; Tx = Toxins; Vt = vitamins; Vs = Virus; WT = Western.

* The structure of the Antibody or epitope should be known in case of ELISA.

methods together with surface display, and sometimes mutagenesis procedures. The mixed bacterial epitopes were mostly screened by western blot, and surface display. While those of the viruses depended mostly on microarray and/or pepscan, ELISA or phage display. Those of parasites were detected by ELISA and sometimes mutagenesis, however tumor markers were usually investigated by phage display, pepscan and mutagenesis, *Pneumocytes carnii* was mapped by ELISA with western blot. Finally, allergen and autoimmune diseases were revealed by mutagenesis and western blot.

Several combinations appeared where the pepscan was a basic technique together with binding methods, especially for viruses, or mutagenesis for cancer epitopes mapping; fewer methods appeared for mutagenesis with binding methods for cancer epitopes. Epitope's mapping revealed the major epitopes that may be used as vaccine and diagnostic building blocks for several tumor antigenic determinants such as PSA, CD-20 (rituximab), breast cancer, and pancreatic cancer. Screening of the *Mycoplasmsa bovis* epitopes by pepscan and PMA was fruitful. Moreover, several successful studies on *K. pneumoniae* using western blot technique highlighted the major precise blocks for vaccine and diagnostic production.

The primary screening of emerging pathogens were usually performed by western blot and pepscan, as well as, *in silico* software if the genetic material of the antigen was provided. Mapping showed a major role in screening the epitopes of the Rotavirus by pepscan and PMA; SARS by PMA, pepscan and ELISA; HCV by ELISA and mutagenesis; and HIV-1 gp41, gp120 and gp160 by pepscan, ELISA and western blot. It is obvious that the combinations of at least two methods ameliorate the results and refine them remarkably.

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