Acta virologica **59**: 103 – 116, 2015 doi:10.4149/av_2015_02_103

REVIEW

Unravelling viral camouflage: approaches to the study and characterization of conformational epitopes

T. AUGUSTIN[1], O. CEHLAR[1,2], R. SKRABANA[1,2], P. MAJEROVA[1], J. HANES[1,2*]

[1]Institute of Neuroimmunology, Slovak Academy of Sciences, AD Centre, Dúbravská cesta 9, 845 10 Bratislava, Slovak Republic;
[2]Axon Neuroscience SE, Dvořákovo nábrežie 10, 811 02 Bratislava, Slovak Republic

Received November 10, 2014; accepted May 4, 2015

Summary. – Antibodies are broadly used in clinical and basic research. Many of monoclonal antibodies are successfully adopted for therapeutic and diagnostic targeting of viral pathogens. Efficacy of antiviral neutralizing or protective antibodies depends on their ability to recognize epitopes interfering with viral infection. However, viruses are able to incessantly change their antigenic determinants to escape surveillance of humoral immune system and therefore the successful antiviral therapies require continuous development. Characterization of interactions of antibodies with prevalently conformational viral epitopes is important for understanding antibody mode of action and can help to identify conserved regions that may be exploited in designing new vaccines and virus neutralizing antibodies. In this article, we are reviewing techniques in use for characterization of conformational epitopes of monoclonal antibodies with focus on viruses.

Keywords: monoclonal antibody; conformational epitope; X-ray crystallography; NMR; display technologies; mass spectrometry

Contents:

*Corresponding author. E-mail: Hanes@axon-neuroscience.eu; phone: +421-2-54788100.

Abbreviations: DenV = dengue virus; ESI = electrospray ionization; HA = hemagglutinin; HDX = the hydrogen/deuterium exchange; IDR = intrinsically disordered regions; MAb = monoclonal antibody; MALDI = matrix-assisted laser desorption/ionization; MS = mass spectrometry; NMR = nuclear magnetic resonance spectrometry; NOE = nuclear Overhauser effect; PHF = paired helical filament; STD-NMR = saturation transfer difference-NMR

1. Introduction

Proteins are an immensely diverse group of biopolymers owing to their variable amino acid sequence and plentiful residue modifications, which result in the most disparate structures and properties. There are at least 30,000 different proteins in human cells (Claverie, 2001) intertwined in complicated networks. Protein-protein interactions are taking place in most biological processes such as metabolism, signalling, biopolymer synthesis and also in immunity.

The interactions between an antibody and antigen are the basis of the immune humoral response. Antibodies are highly specific for the structures that they recognize and their immune activity is directed against discrete site on the antigen. Antigens are mainly proteins, carbohydrates, lipids or nucleic acids. For protein or peptide antigens, the interaction involves up to 20–30 amino acid residues on the surface of the antigen,

known as the epitope, but usually only minimum number (4–5 amino acids) is critical for antibody binding (Van Regenmortel, 1989). Depending on whether the residues are linear in primary sequence, epitopes can be continuous (linear fragment in primary amino acid sequence) or discontinuous, also known as conformational. Conformational epitopes are mediated through the tertiary structure of the antigen and the distant segments of the primary sequence are brought together via protein folding. Several studies of antibody-antigen complexes with known X-ray structures indicate little or no structural changes induced by complex formation (Mumey *et al.*, 2003; Skrabana *et al.*, 2010), but other authors reported a rearrangement of antibody combining site to various extent after complex formation with their antigen (James *et al.*, 2003; Nair *et al.*, 2002).

Antibodies play an important role in the response of organism immune system to viral infection. In order to block or mitigate viral infection, they can (1) directly block virus attachment site on the cellular receptor by binding one of the partner interacting surface, (2) inhibit the conformational changes of viral surface proteins required for their dissociation into host cytoplasm from endosomes, for fusion of cellular membranes before the entry to the cell or for the release of progeny virus from the host cell membrane, or (3) opsonize the virus particles or make them aggregate before they attach to the percipient cell (Marasco and Sui, 2007). The majority of antiviral antibodies developed as diagnostic or therapeutic agents have a conformational, discontinuous epitope on the viral or cellular antigens (DiMattia *et al.*, 2013). Successful application of an antibody therapy can be hampered by the fact that viruses have broadly developed strategies to escape antibody recognition, by mutating their antigenic determinants and by changes in their glycosylation pattern (Dorner and Radbruch, 2007). Fast and efficient methods of epitope mapping are therefore essential for a continuous development of viral synthetic vaccines. For example, in case of severe respiratory illnesses such as H5N1 avian influenza or severe acute respiratory syndrome (SARS), determination of the molecular determinants of the epitope was critically important in the development of an efficient vaccine (Oliphant *et al.*, 2005; Smirnov *et al.*, 2004; Sui *et al.*, 2004). Vaccinating with the epitope only could be much safer and as effective as using the entire organism or the isolated antigen (Unsold *et al.*, 1980). Epitope mapping has additional applications in the field of drug design (Irving *et al.*, 2001), disease diagnosis and immunointervention (Westwood and Hay, 2001).

To date, several different techniques are being adopted for antibody epitope mapping. They are based either on the elucidation of the structure of the antibody-antigen complex (X-ray crystallography and, in part, NMR spectrometry) or on the detection of amino acid sequence of antigen in close interaction with antibody (display technologies, MS-based methods, solution NMR, Geysenpepscan). There are other methods like ELISA or solution interaction kinetics by surface plasmon resonance, contributing data about antibody complexes affinity, which can indirectly map important residues for antibody recognition.

This review article is focused on the methods which are most commonly used for identification of the conformational epitopes of the monoclonal antibodies and are able to preserve native conformation of the target antigen during the characterization process. Each of the methods provides its advantage under different circumstances, and all of them were successfully used for determination of discontinuous epitopes. Examples of approaches for characterization of conformational epitopes of viral antigens are shown in Table 1 and further discussed in this review.

2. X-ray crystallography

The most powerful technique for the determination of three dimensional structures of the conformational epitopes

Table 1. Overview of methods used for characterization of conformational epitopes of monoclonal antibodies and examples for viruses mentioned in this review

Method	Virus	Antigen	Antibody	Reference
X-ray crystallography	Hepatitis B virus	HVBeAg	e6	DiMattia *et al.*, 2013
	Group 2 influenza A viruses	Hemagglutinin	CR8043	Friesen *et al.*, 2014
	Dengue virus	Domain DIII of envelope (E) protein	4E11	Cockburn *et al.*, 2012
NMR	Dengue virus	Domain DIII of envelope (E) protein	DV32.6	Simonelli *et al.*, 2013
Phage display	SARS coronavirus	Spike protein	80R	Tarnovitski *et al.*, 2006
Yeast display	West Nile virus	Domain DIII of envelope (E)protein	E16	Nybakken *et al.*, 2005
	Influenza virus H5N1	Hemagglutinin	H5-2A	Han *et al.*, 2011
Mass spectrometry	HIV	p24 inner core protein	5E2.A3	Hochleitner *et al.*, 2000
		gp120 envelope protein	559/64-D	Hager-Braun *et al.*, 2010

of antibodies is the X-ray crystallography. In contrast to NMR, X-ray crystallography is not limited by the size of studied antibody complexes. As the diffraction of X-rays directly depends on the 3D distribution of electron density in the crystal, final models confer discrete atomic position of antibody and antigen. Determining the structure of biological macromolecules by X-ray crystallography involves a series of steps as purification, crystallization, collection of diffraction data, solution of structure phase problem and refinement of atomic positions. However, even when pure soluble protein is available, producing high-quality crystals remains a major bottleneck in structure determination (Chayen and Saridakis, 2008). The first published three dimensional protein structure was that of myoglobin (Kendrew *et al.*, 1958) and since this time the pace of structure determination has accelerated during the last quarter century owing to automation of protein production and crystallization, the increasing power of synchrotron and XFEL X-ray sources, as well as the introduction of new algorithms and computer software for diffraction data collection, structure solution and refinement.

The crystallization of biological macromolecules involves a phase transition of protein molecules from the solution that has been brought into supersaturated state by the addition of mild precipitating agents such as neutral salts or polymers, and by the manipulation of various parameters that include temperature, ionic strength and pH. The factors affecting the structural state of the macromolecule such as metal ions, inhibitors, cofactors or other conventional small molecules are also important in the crystallization process. Among the most widely used crystallization techniques are the vapour diffusion, dialysis, batch and liquid–liquid diffusion (Bolanos-Garcia and Chayen, 2009; McPherson and Gavira, 2014). As the intact molecule of an antibody is intrinsically flexible due to the presence of pliable hinge region connecting antigen binding region (Fab) with the Fc region, for the crystallization experiments one needs to use shorter molecules containing the antigen combining site, namely Fab or scFv antibody fragments. Sufficient amounts of protein are prepared by limited proteolysis (Fab) or by recombinant technologies (Fab, scFv). Structure solution of antibody complexes from X-ray diffraction data is obtained in the majority of cases by the molecular replacement method (Abergel, 2013). Selection of appropriate model structure for molecular replacement is facilitated by the fact that nearly 2,000 antibody structures are available in the Protein Data Bank (PDB) and their number is continuously growing.

The hen egg white lysozyme (HEL) and the anti-HEL antibody HyHEL10 is one of the first studied antibody-antigen pair involving a conformational epitopes. The HyHEL10 epitope on the lysozyme is discontinuous, composed of residues from four different regions of the linear sequence of lysozyme. All six complementarity-determining regions of the HyHEL10 Fab contribute to the binding and also one residue from the framework is in contact with the lysozyme (Padlan *et al.*, 1989). The X-ray structural analysis enabled to distinguish the binding contacts of the antibody-lysozyme complex, that are mediated through 20 direct hydrogen bonds and another hydrogen bonds are mediated through 12 water molecules present in the combining site of HyHEL10 that were visible in the X-ray structure (Kondo *et al.*, 1999).

A distinct structural entities in proteins are intrinsically disordered regions (IDRs) (Forman-Kay and Mittag, 2013). Interestingly, viruses use short IDRs to form conformational epitopes, mimic cellular proteins and hijack cell regulation (Davey *et al.*, 2011). Determination of structure of IDRs is challenging, however, monoclonal antibodies could provide indirect aid to it. Monoclonal antibody MN423 recognizes a conformational epitope on the Alzheimer's disease core paired helical filament (PHF) subunit terminated at Glu391, formed by IDRs of tau protein (Novak *et al.*, 1989). The co-crystallization of MN423 Fab with a tau polypeptide derived from the PHF core led to the structure determination of the C-terminal PHF core hexapeptide ^{386}TDHGAE^{391} at 1.65 Å resolution and the structural analysis has suggested a role of the core PHF C-terminus in the PHF assembly (Sevcik *et al.*, 2007).

The chronic hepatitis B virus (HBV) infection that causes cirrhosis and liver cancer chronically infects 360 million people (Zlotnick *et al.*, 2013). It has a unique ability to establish virus-specific immune-tolerance while continually producing infectious virus particles. The infection results in the expression of three protein antigens known as surface antigen (HBsAg), core antigen (HBcAg) and e-antigen (HBeAg). The 21 K HBcAg, which forms dimers that assemble as multimegadalton capsids, and 17 K HBeAg, which also forms dimers but that do not assemble, are closely related. They share a sequence of 149 residues but have different amino- and carboxyl-termini (Watts *et al.*, 2010). Out of the anti-HBV antibodies, the monoclonal antibody (MAb) 3120 was found to be specific for assembled capsids (Fig. 1) and MAb e6 for unassembled dimers. DiMattia and co-workers have recently solved a structure of MAb e6 with HbeAg clarifying the structural switch that precludes the capsid assembly and engenders a distinct antigenic repertoire (DiMattia *et al.*, 2013).

Annual influenza epidemics cause 3–5 million cases of severe illness and up to 0.5 million deaths and periodic pandemics are able to kill millions. The hemagglutinin (HA), a surface glycoprotein on influenza viruses, mediates viral entry into host cells and is accessible to antibody recognition. HA is highly variable and classified into 18 divergent subtypes, which cluster phylogenetically into group 1 or group 2. The discovery and characterization of broadly neutralizing antibodies against influenza viruses have raised

hopes for the development of monoclonal antibody-based immunotherapy and the design of universal influenza vaccines. The recently crystallized antibody CR8043 has heterosubtypic neutralizing activity against influenza A group 2 viruses. X-ray and EM structures of CR8043 Fab in complex with H3 HAs reveal that the antibody targets a conserved conformational epitope on the HA stem (Friesen *et al.*, 2014) that consists of the fusion peptide and the β-sheet of processed HA2 preceding the A helix. This antibody has a different approach angle and uses different contact residues than the only previously characterized influenza A group 2 broadly neutralizing antibody CR8020, which has neutralizing activity against H3, H7 and H10 viruses (Ekiert *et al.*, 2011) and has a similar epitope. CR8043 has *in vitro* neutralizing activity against H3 and H10 viruses and protects mice against challenge with a lethal dose of H3N2 and H7N7 viruses. The binding of these antibodies prevents HA conformational change and thus blocks the membrane fusion and viral entry.

Dengue virus (DenV) is responsible for 20,000 deaths and 500,000 hospitalizations annually with economic impact rivalling that of malaria (Gibbons and Vaughn, 2002). Four antigenically-related serotypes of DenV circulate in tropical and subtropical regions of the world. Although DenV infection induces lifelong immunity against viruses of the same serotype, the raised antibodies appear to contribute to severe disease in case of subsequent infection by a different serotype, what's called the antibody-dependent enhancement and leads to dengue hemorrhagic fever (Cockburn *et al.*, 2012; Halstead, 2003; Pierson and Kuhn, 2012). No cure or vaccine for DenV is currently available mostly due to above mentioned facts. Development of vaccine recognizing all DenV serotypes could be a solution to this problem. Recently, the murine MAb 4E11 that neutralizes all four DenV serotypes has been crystallized together with its antigen DIII protein domain (Cockburn *et al.*, 2012). DIII is a small immunoglobulin-like 8-stranded domain part of the envelope (E) protein, whose homodimers are the main components of the viral surface and a dominant target for the human antibody response against DenV. The structures of 4E11 scFv antibody fragment in complex with DIII domain of the E protein from all four serotypes revealed the determinants of cross-reactivity and highlighted the mechanism by which the antibody disrupts the architecture of the mature virion.

Binding of Fab fragment of 1A1D-2MAb (Lok *et al.*, 2008), which has a similar epitope as 4E11 and shares 85% amino acid identity, to the mature DENV-2 virion was shown to be temperature-dependent. Proteins incorporated into flaviviruses are in constant motion as they sample related conformations at equilibrium. 1A1D-2 and 4E11 bind and stabilize the A-strand epitope as it becomes accessible on the surface of a "breathing" virion (Pierson and Kuhn, 2012).

3. Nuclear magnetic resonance spectrometry (NMR)

NMR is powerful, versatile and relatively fast technique for study of biological systems. This method is widely used for characterization of protein-ligand interactions and can also be suitable tool for characterization of epitope of monoclonal antibody. It is able to provide detailed information about structure, dynamics and kinetics of protein complexes at atomic resolution, even if the interactions are transient or weak, together with a numerical value of dissociation constant (Fielding, 2003). Since NMR is able to characterize protein structure in near physiological conditions, it offers a suitable alternative to crystallographic studies. Unfortunately, successful determination of all atomic coordinates of biological macromolecules by NMR is limited by the size of the system (currently <80 K in solution).

Despite this, the contact sites of antibody-antigen complexes can be studied using various NMR protocols, which are briefly characterized below (Fernández and Wider, 2006; Tugarinov *et al.*, 2005; Wilkinson *et al.*, 2009).

Binding of proteins influences measured chemical shift by changing the nuclear environment (distance from neighbouring groups, anisotropy and electronegativity), which are used for characterization of qualitative and quantitative properties of interaction. This made the chemical shift perturbation one of the most attractive methods for probing ligand-protein interaction by measuring its changes during titration with ligand (Zhang *et al.*, 2006). Generally, amino acid residues which show progressive chemical changes are located on the surface of binding site and effect of interactions to chemical shift is dependent on distance from epitope. Also dissociation constant (Kd) can be derived from chemical-shift changes as function of ligand concentration using the fast exchanging model (Cui *et al.*, 2003).

Relaxation rate (time taken by nuclei to return to equilibrium after a radiofrequency pulse) is altered by forming of complex between protein and ligand. Large molecules often exhibit fast relaxation due to slow molecular motion, whereas small molecules exhibit slower relaxation rates with rapid molecular motion. When small molecule binds to larger one, the nuclei in the proximity of the binding site can restore its equilibrium via new macromolecular neighbour. Nuclei involved in interaction will now exhibit higher relaxation rates, whereas those not involved slower relaxation. Such change can be used for identification of the residues which are potentially involved in binding process (Simpson *et al.*, 2011).

Nuclear Overhauser effect (NOE) can be used for measurement of interactions through space. It results from dipole-dipole interaction of atomic spin, which is inversely related to interspin distances. Protein-ligand interaction properties are obtained through magnetization transfer from protein to bound ligand or from bound ligand to protein by dipole-dipole interactions (Zhang *et al.*, 2006).

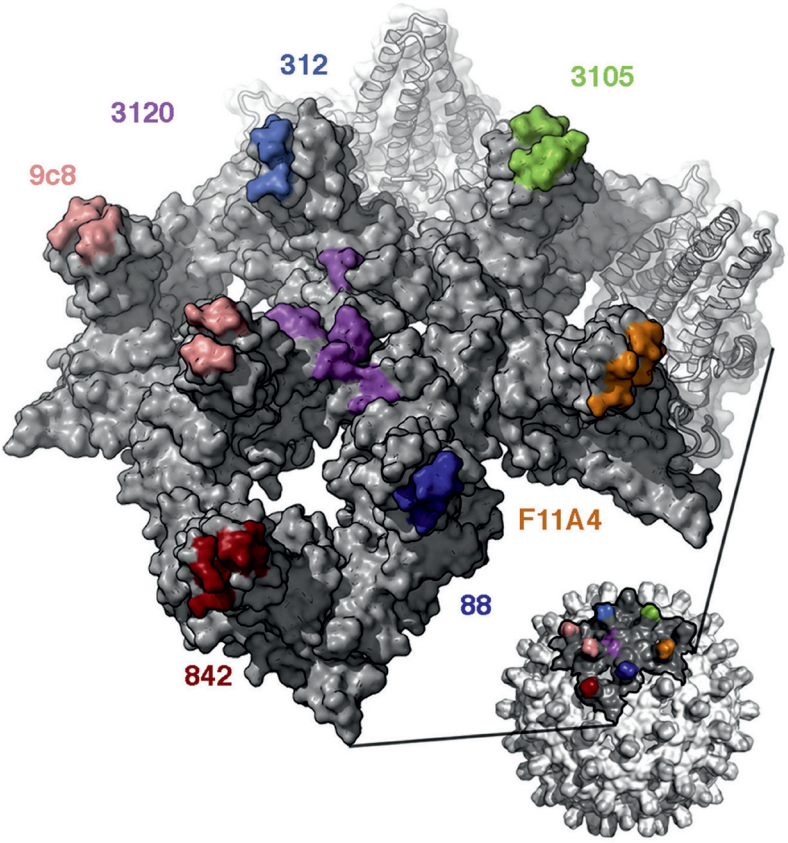
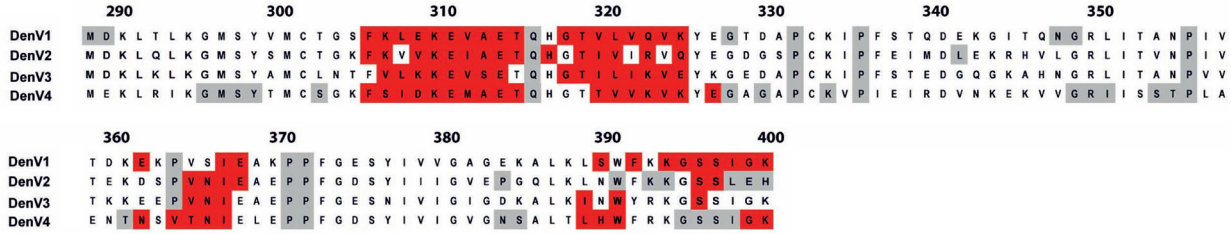


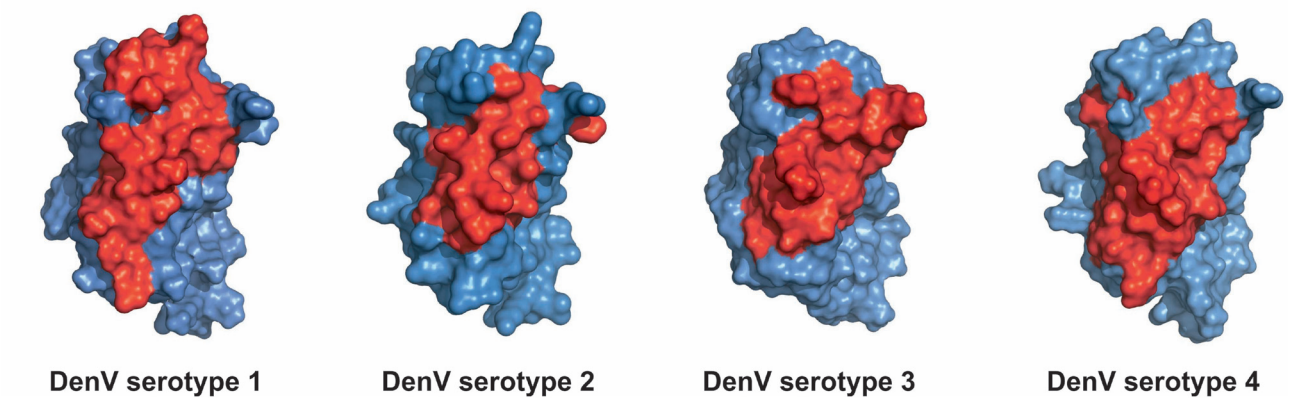
Fig. 1
Partial surface of an HBcAg capsid of hepatitis B virus with the epitopes for several anti-HBc/eAg antibodies mapped in colors
HBcAg epitopes are typically discontinuous consisting of two or more loops from different subunits or discontinuous regions of the same subunit. Most of these epitopes reside around the spike tips of capsid except MAb 3120 (purple) that binds to the conformational epitope at the floor region (Conway *et al.*, 2003). Adopted from DiMattia *et al.* (2013) with permission from Elsevier.

Fig. 2
NMR epitope mapping of antibody DV32.6 recognizing DIII domain of dengue virus (DenV) from four DenV serotypes
Residues in DIII whose NMR signal is affected by antibody binding are indicated in red on the sequence (a) and on the surface representation (b) of DIII domain of each DenV serotype. Residues for which no NMR information is available are colored in grey in the sequence. The discontinuous epitope shows slight variations amongst serotypes both in sequence and structure, including some conserved residues. Presence of conserved residues explains why DV32.6 Can bind to all four serotypes, and other residues are likely to be responsible for the different binding and neutralization properties. Adopted from Simonelli *et al.* (2013).

(a)



(b)



Saturation transfer difference NMR (STD-NMR) is commonly used method for characterization of epitope of monoclonal antibodies (Johnson and Pinto, 2004; Murata *et al.*, 2003). This technique is based upon the traditional 1D steady-state NOE experiment and can easily reveal interactions between small and large molecules (for example antigen-antibody) (Mayer and Meyer, 2001; Neuhaus and Williamson, 2000). If spin diffusion within the protein is efficient, saturation of a single protein resonance can be rapidly spread over the entire protein and leads to uniform NMR spectrum. Binding of the ligand to the target protein can allow saturation transfer by NOE. The ligand protons which are located in the proximity of the interaction should be saturated to the highest degree and therefore have a strongest signal in the STD spectrum. Similarly, the ligand protons, which are located further from surface of target protein, possess lower degree of saturation transfer and their STD intensities will be weaker. Since degree of ligand saturation reflects their proximity to target surface, it can be used as an epitope method to describe antigen-antibody interaction (Bhunia *et al.*, 2012; Takeuchi and Wagner, 2006; Yan *et al.*, 2003). STD-NMR in combination with trNOE (see below) was successfully used for characterization of epitope of anti-gibberellin A4 MAb (Murata *et al.*, 2003).

The transferred NOE (trNOE) spectroscopy provides detail information about three-dimensional structure of the ligand in the bound state (Clore and Gronenborn, 1983). It refers to observation of negative NOE effects in bound conformation, in contrast to positive effects normally present in absence of target protein. Since magnitude of NOE enhancement is exponentially related to the interspin distance, NOE experiments are commonly used for assignment of the dimensional structure of protein and ligand-target complexes, orientation of ligand and target domains towards the binding site and dynamic information for the ligand-target interactions (Zhang *et al.*, 2006).

Characterization of the NMR structure is dependent on successful assignment of NMR signals, which relies on resolving signals from correlation maps. The abundance of the NMR signals, which results in spectral crowding is the biggest obstacle in NMR spectroscopy of large protein. Therefore, higher resolution spectrometers are required to diminish signal overlaps present in NMR spectroscopy of large proteins (Frueh *et al.*, 2013). Some methods have been developed to overcome this limitation using advanced pulse sequences, deuteration or creative labeling such as TROSY, methyl TROSY, CRIPT and CRINEPT. Thus, it has become possible to obtain well-resolved spectra and structural information up to 82 K for monomeric proteins and up to 900 K for complex systems (Fiaux *et al.*, 2002; Takeuchi and Wagner, 2006; Tugarinov *et al.*, 2005).

In order to obtain and compare the three-dimensional structure of a large number of different complexes, it is important to have faster methods than traditional experimental techniques. The computational docking with RosettaDock (Gray *et al.*, 2003), validated subsequently by the solution NMR chemical shift mapping identifying the interface residues, was shown to be suitable for the structural characterization of a large panel of different antibodies bound to the same antigen and was demonstrated on the complex of DenV DIII domain of DenV serotype 4 (DenV4) with antibody DV32.6 (Simonelli *et al.*, 2010) and DV32.6 complexes with DIII of remaining serotypes (Simonelli *et al.*, 2013). The DV32.6 antibody, which was isolated from a donor recovered from DenV serotype 2 infection, recognizes a conformational epitope on DIII of all DenV serotypes (Fig. 2) but fails to neutralize the DenV4. The obtained structural data have enabled the rational design of antibody mutants with selectively altered binding specificity or improved neutralization properties even in the absence of the high resolution X-ray structure.

4. Display technologies

Display technologies are techniques for screening of peptide or polypeptide libraries for ligands whereby proteins are connected with the genetic information that encodes them. There are many screening display tools available dependent on the type of fused unit. Proteins can be linked to phage (Pande *et al.*, 2010), bacterial (Daugherty, 2007), yeast (Gai and Wittrup, 2007) or mammalian cells (Zhou *et al.*, 2010). Ribosome display (Hanes *et al.*, 2000; Hanes and Pluckthun, 1997) and mRNA display (Lipovsek and Pluckthun, 2004; Roberts and Szostak, 1997) are cell-free systems, where proteins are physically linked to its coding mRNA through the ribosome or puromycin adaptor, respectively. From all display technologies the phage display is the most commonly used technique for epitope mapping (Huang *et al.*, 2006).

Phage display is a cheap, rapid and powerful analytical tool for discovery of protein-protein interactions in various fields. It has been widely used in discovery of peptide ligands such as enzyme substrates (Yoshida *et al.*, 2003), inhibitors (Huang *et al.*, 2003), receptors (El-Mousawi *et al.*, 2003), DNA-binding peptides (Cheng *et al.*, 1996) and also for development of new drugs, diagnostics and vaccines (Riemer *et al.*, 2005; Wang and Yu, 2004). Phage display has also become a promising method for epitope mapping because of its ability to mimic the genuine epitope in terms of its physiochemical properties and spatial organization.

In phage display, exogenous (poly)peptides are expressed and presented on the surface of phage particle to bind to various target molecules. The foreign DNA fragment, random part of gene or oligonucleotide, is incorporated into the genome of filamentous phage. This linkage of displayed peptide on the surface of viral particle combined with combinatorial

power of peptide libraries have established phage display as a selection tool for characterization of epitope of monoclonal antibody. Phage display peptide libraries are made by shotgun cloning of random fragments into the N-termini of coat proteins (Williams *et al.*, 1998). Such a library is a heterogeneous mixture of phages carrying a different foreign DNA inserts and therefore display a different peptides on their surfaces. Interactions between fusion peptides and target monoclonal antibody select only such phage particles which are able to mimic original epitope. This interaction process is called biopanning. Biopanning involves 3 steps: 1) *Phage binding*: Target antibody is immobilized on the solid surface and phage library carrying random peptides is added to the immobilized antibody in a solution that allows minimal non-specific binding. First round of biopanning should be performed with large and diverse peptide library to ensure better chance of isolating peptides of interest, especially for antibody recognizing conformational epitope. 2) *Removing unbound phages:* The first round of biopanning requires less stringent washes and higher yield of phage clones of interest over the background. Washing during later rounds of biopanning can be done under more stringent conditions to isolate phages binding to the target antibody with higher affinity. 3) *Phage elution:* Due to the stability of phages, non-specific elution can be performed under extreme conditions such as low pH, ionic strength, denaturants, sonication or limited proteolysis. Recovery of bound phages can be carried out also by antigen itself. It is important to note that antigenic elution recovers only phages interacting specifically with target antibody (Smith and Petrenko, 1997). Eluted phages are amplified using transduction of *Escherichia coli* as a host cell and the biopanning process is repeated three to six times. Antibody binding peptides presented on phages are analyzed using DNA sequencing of phage clones.

M13 filamentous phage is the most commonly used phage system for display of peptides/proteins. The peptides are presented on M13 phage coat proteins, either pIII or pVIII. The size of the foreign peptides displayed on every copy of coat protein limits the use of phage display because larger peptides or gene fragments interfere with coat protein function in viral packaging and bacterial infectivity (Smith, 1985). This could be overcome by a hybrid virion system in which random peptide sequence is displayed only on a fraction of the endogenous coat protein (Pande *et al.*, 2010). In another hybrid virion system the phage genome includes two copies of the coat protein, one as a wild type and the other as a fusion gene (Pande *et al.*, 2010). The conventional N-terminal fusion of foreign amino acid sequence to the pIII is frequently used in phage display since it is more tolerant to larger insertion. As peptide is fused to N-terminus of coat protein, this approach cannot be used for identification of epitope with absolute requirement for a free carboxyl-terminus. However, peptides cloned at the C-terminus of engineered variants of pIII and pVIII proteins can also be efficiently displayed to overcome this problem (Khuebachova *et al.*, 2002), as well as peptide library displayed at the C terminus of gpD protein of lambda phage (Gupta *et al.*, 2003).

Part of gene of interest (gene-fragments) or random oligonucleotide can be incorporated into genome of phage. The gene-fragment libraries are very useful in longer epitopes which adopt structural conformation (Fack *et al.*, 1997). But in some cases part of antigen cannot include full amino acid sequence of conformational epitope necessary for recognition by antibody. To avoid this problem random peptide libraries are more convenient for characterization of such discontinuous epitope. The random oligonucleotides are incorporated between the coding sequence for the signal peptide and N-terminus of the coat protein pIII. The length of random peptides can vary from 6 to 43 amino acids (Burritt *et al.*, 1995; McConnell *et al.*, 1996). This allows screening of residues which are either continuous or widely separated in primary sequence of antigen. Libraries with loop scaffold have been also developed. Amino acid sequences of peptides are flanked by a pair of cysteine residues that form a cross-bridge. Such approach has been successfully used for targets that did not bind ligand from linear libraries (Clackson and Wells, 1994).

Mapping of epitope of antibody is usually accomplished by comparing the sequences of antibody-selected peptides to the antigen. Sometimes the peptides are identical or very similar to amino acid sequence of the antigen, thereby pointing the location of the native epitope (Scott and Smith, 1990). However for conformational epitopes such case is rare, and usually peptides have little, if any, similarity with the amino acid sequence on the antigen. Mimotopes are random peptides affinity selected by, for example phage display, which do not reflect primary sequence of antigen and mimic only structure of discontinuous epitope. As mimotope represents only physiochemical properties and spatial organization of epitope, it may not have similarity to any amino acid sequence of the antigen, what makes it almost impossible to find consensus sequence among different mimotopes manually. Several analytical tools have been developed to analyze native epitope based on sequences of the mimotopes and the three dimensional structure of the antigen. Existing programs for phage display based epitope mapping can be divided to four categories according to their dependency on antigen structure. Algorithms in first category such as FINDMAP compare only sequences from mimotopes and antigen (Mumey *et al.*, 2003). Programs such as SiteLight (Halperin *et al.*, 2003), 3DEX (Schreiber *et al.*, 2005), PepSurf (Mayrose *et al.*, 2007) or MIMOX (Huang *et al.*, 2006) which work with both, the sequence data and the antigen structure belongs to the second category. MIMOX is freely available online service making evaluation of the phage display data easily accessible for community. Program

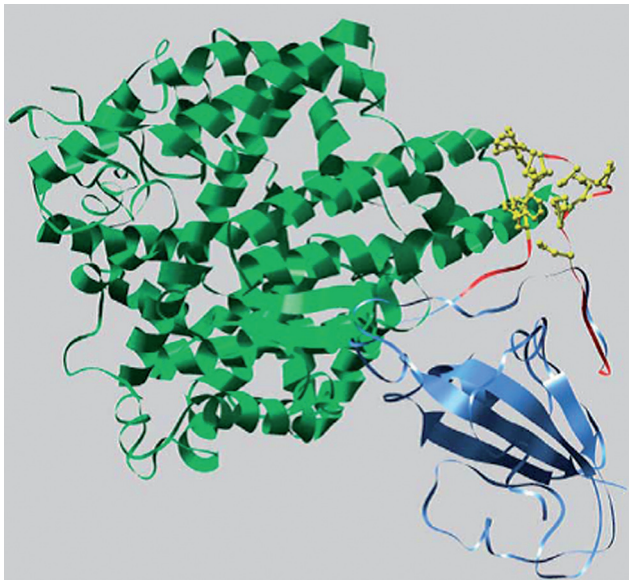


Fig. 3
Presentation of the potential conformational epitope site of the 80R antibody (colored yellow) on the surface of spike protein of SARS-CoV (marked in blue) calculated by Mapitope algorithm
Picture shows interaction between spike protein and receptor ACE2. Neutralizing effect of the 80R antibody is based on interaction with receptor binding site on spike protein. Adopted from Tarnovitski *et al.* (2006) with permission from Elsevier.

aligns mimotopes and derives consensus sequence which is subsequently mapped on to the antigen structure. Potential epitopes are determined by spatial clustering of the mapped residues (Huang *et al.*, 2006). MIMOP (Moreau *et al.*, 2006) and Mapitope (Tarnovitski *et al.*, 2006) belong to the third category of algorithms which combine two different approaches and can work with or without the structural data of the antigen. The unique principle of the Mapitope is that the simplest meaningful part of the epitope is an amino acid pair (AAP) of residues that lie within the footprint of the epitope. The AAPs need not to be consecutive residues on the antigen, but can be brought together in near proximity via folding of the polypeptide chain. Mimotopes are first deconvoluted into the AAPs, statistically significant pairs are identified and then mapped in the crystal structure of the antigen. Such a strategy was successfully used for characterization of conformational epitope of 80R antibody (Fig. 3) recognizing spike protein of SARS coronavirus (Tarnovitski *et al.*, 2006).

Pepitope belongs to the last category which combines different algorithms. This approach implements three programs for phage display epitope mapping: PepSurf, Mapitope and a combination of them. Thus allowing user to compare predictions based on different methodological approaches via single web platform (Mayrose *et al.*, 2007).

Filamentous phages are not only option as carrier of random peptides. Yeasts are also commonly used for displaying of peptides. Each yeast cell can display ~ 50,000 copies of the target protein fused to yeast cell wall protein. Yeasts can be easily grown on a large scale, offer post-translational modifications lacking in bacterial phage display (Boder and Wittrup, 1997) and technique is compatible with FACS. However, yeast display provides lower diversity of library of mutant proteins during experiment compared to phage display (Gera *et al.*, 2013). The yeast display was successfully applied for identification of linear and conformational epitopes of complex proteins, for example hemagglutinin of highly pathogenic H5N1 influenza virus (Han *et al.*, 2011), epidermal growth factor receptor (Cochran *et al.*, 2004), or botulinum neurotoxin type A (Levy *et al.*, 2007). West Nile virus (WNV) is zoonotic pathogen with a wide range of hosts, including humans. It is able to cause neuroinvasive diseases such as meningitis or encephalitis. Since there is still no vaccine for a human use (Ishikawa *et al.*, 2014), characterization of neutralizing antibodies against WNV could be essential for development of the efficient prophylaxis. Yeast display in combination with X-ray crystallography was used for characterization of the conformational epitope of the E16 antibody recognizing protein E of the West Nile virus (Nybakken *et al.*, 2005).

5. Mass spectrometry

For characterization of conformational epitopes of monoclonal antibodies, the mass spectrometry (MS) could be advantageous because of its sensitivity, speed and capability of sequencing peptides (Raska *et al.*, 2003). When coupled to liquid chromatography separation, MS can provide valuable information about target protein such as molecular weight (MW), amino acid sequence or post-translational modification. This information can be obtained with minimal disruption of the quaternary structure of the target system (e.g., complex antigen-antibody).

Due to the large size of target proteins, the epitope detection requires the use of suitable ionization technique. Two methods are most commonly used for ionization of large biomolecules-electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). MALDI technique is based on incorporation of analyte into the crystalline matrix (typically a small organic acid). Pulsed-laser irradiation causes rapid volatilization of the matrix/analyte solid mixture. Analyte molecules are volatilized as ions and accelerated using high potential electric field into the field-free flight tube where they drift to the detector. Because of preciously timed and pulsed nature of ions generation, the time of flight information can be converted into mass (Van de Water *et al.*, 1997). Time of

flight-type mass analyzers are widely used for detection of mass of the large molecules because of their height mass accuracy, mass resolution and wide mass to charge (m/z) detection range (Zhang *et al.*, 2009). ESI is technique that generates multiple charged ions from liquid stream, what effectively increases mass range of a mass spectrometer enabling coupling of quadrupole mass analyzer (Zhen *et al.*, 2004). Triple quadrupole analyzer could be used for fragmentation of multiple charged ions generated by ESI (Hunt *et al.*, 1989). The conjunction of quadrupole analyzer and ESI made possible rapid determination of amino acid sequence of a peptide. Tandem mass-spectrometry connected with liquid chromatography (LC/MS/MS) made it also possible to detect amino acid sequences from complex peptide mixture (Covey *et al.*, 1991).

Two approaches are widely used for determination of amino acid sequences of the protein by MS: ″bottom-up″ and ″top-down″. In the ″bottom-up″ approach, also known as peptide mapping, the target protein undergoes reduction, denaturation, alkylation and digestion. Digested peptides are subsequently separated by LC and precursor ions analyzed by MS. Selected precursor peptides are fragmented and amino acid sequences determined using tandem MS (MS/MS). Due to limited duty cycle not all ions detected in the MS mode can be selected for fragmentation in MS/MS mode. ″Top down″ approach sequences proteins directly in the gas phase by MS/MS. This method involves less sample handling and provides more reliable analyses since it can avoid artificial modification as AA rearrangement and deamidation, which can complicate ″bottom up″ analyses (Fodor and Zhang, 2006; Gaza-Bulseco *et al.*, 2008). However ″top down″ approach has limitation when sequencing large proteins. Since complete sequence coverage for traditional fragmentation method such as collision-induced dissociation (CID) can be achieved only for proteins less than 5 K, also ″top down″ approach meets this limitation of fragmentation method for sequencing large proteins (Mo *et al.*, 2012).

To date, MS epitope mapping experiments involving immunoprecipitation used two strategies based on proteolytic cleavage of antigen: epitope extraction (Zhao and Chalt, 1994) and epitope excision (Przybylski, 1994). In epitope extraction, antigen is first digested by proteolytic enzymes and then digested fragments are affinity captured by binding to immobilized antibody. The application of this method for identification of the discontinuous epitopes can be limited, as enzymatic cleavage of polypeptide chain between the residues composing the epitope can lead to loss of affinity. In epitope excision, native antigen is first incubated with immobilized antibody to form complex and then subsequently digested with protease. Unbound peptides are washed off, antigenic peptides detached from antibody and analyzed by mass spectrometry. This strategy is based on several factors – antibody is resistant toward proteolytic enzymes; in complex antibody-antigen epitope is protected against proteolytic cleavage; proteolysis of complex does not lead to dissociation of immune complexes (Van de Water *et al.*, 1997). Binding of antibody to antigen significantly decreases rate of digestion by protease, having greatest effect on regions involved in antibody contact. Thus, an epitope can be identified by protective effect of antibody to some sequences more than others (Jemmerson and Paterson, 1986). Peptides bound to the antibody can be analyzed directly after proteolytic cleavage and washing step increases speed and sensitivity (femtomole levels) of MS. Affinity beads are placed directly on the MALDI-MS/MS without prior separation using LC and potential sample loss during elution of peptides from affinity beads (Raska *et al.*, 2003).

Using immunoprecipitation method in MS has two drawbacks. Weak interactions between antigen and antibody could be missed when stringent wash conditions are used. In contrast, non-stringent conditions may lead to identification of more peptides, but many of them could be false positive. One approach to solve this problem is using covalent cross-linking to antigen-antibody complex and thereby stabilizing protein-protein interactions. Several cross-linkers have been developed varying in spacer arm length, reactive group and other properties. Chemical modification of the bi-functional cross-linkers consists of two steps. First, one reactive group of the cross-linker interacts with amino acid residue to attach cross-linker to protein by forming covalent bond. In the second step, the remaining functional group reacts with another amino acid residue and forms second covalent bond, which stabilizes interaction between amino acids (Sutherland *et al.*, 2008).

Formaldehyde seems to be one of the promising cross-linking reagents for MS analysis of weak protein-protein interactions. Formaldehyde is very small molecule, which contains only a single aldehyde group and exhibits relatively short spacer arm (in the range of 2.3–2.7 Å). The application of the formaldehyde as cross-linker provides several advantages. Only closely associated proteins can be covalently bound due to the short length of spacer arm (so called ″zero length″ cross-link), it allows very fast cross-linking and the stabilization of the transient interactions, covalent linkage is reversal and formaldehyde is available almost in every laboratory (Klockenbusch and Kast, 2010; Tang *et al.*, 2005). The side chains of cysteine, lysine, tyrosine, histidine, tryptophan, arginine, asparagine and glutamine as well as the amino termini of peptides have all been reported as major functional group reactive with formaldehyde (Heck *et al.*, 2001; Metz *et al.*, 2004, 2006; Toews *et al.*, 2008). In ″bottom-up″ approach cross-linked samples are subjected to proteolytic cleavage prior to LC and MS analysis. Formaldehyde seems to have no negative impact on enzymatic digestion, peptide fragmentation or accurate protein identification (Sutherland *et al.*, 2008).

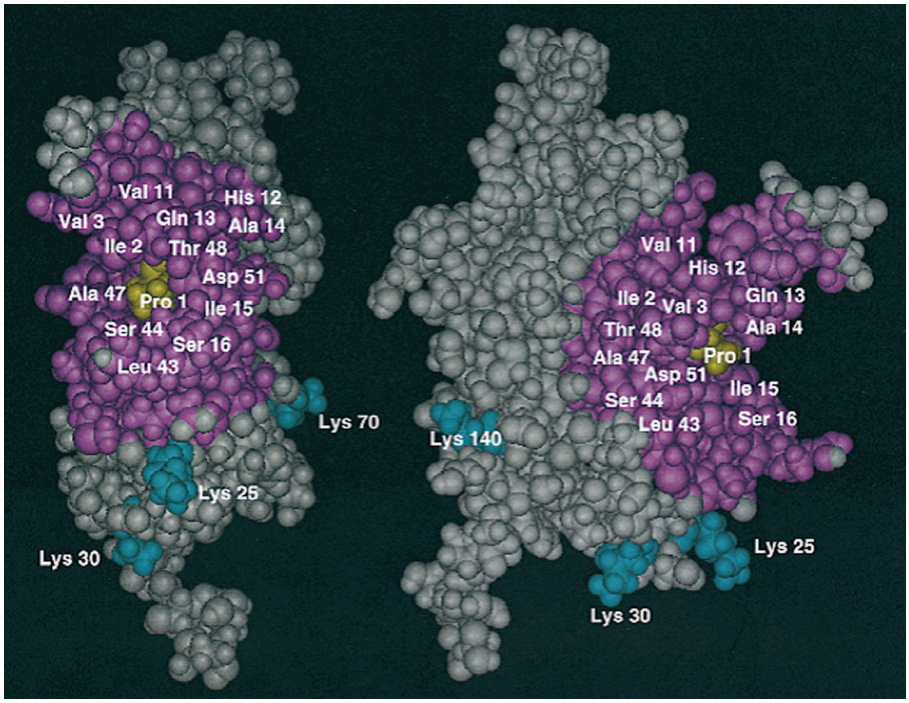


Fig. 4
Structure of HIV-p24 inner core protein

Pro1 (marked in yellow) was determined as a part of the epitope according to differences in relative reactivity of the free and affinity bound form of the antigen. Potential epitope area (marked in magenta) is calculated on average epitope surface, which can be covered by an antibody. Lysine residues (marked in turquoise) do not seem to be located within the epitope structure as they showed no reduction in relative reactivity. Adopted from Hochleitner *et al.* (2000) with permission from John Wiley and Sons.

Differential chemical modifications and hydrogen/deuterium mass spectrometry are both based on the protective effect of antibody to antigenic sequences involved in antibody contact. The concept behind the strategy is that the reactivity of amino acid residues in antigen correlates with their surface accessibility (Glocker *et al.*, 1994). Thus, comparison of relative reactivity of amino acid residues in free antigen and of the residues in the antigen in complex with the antibody can reveal which amino acid sequences are involved in paratope-epitope interaction. When differential chemical modification is used, a free antigen and complex antigen-antibody are modified using specific reagents according to type of modified amino acid residue. Lysine (acetic anhydride or hexadeuteroacetic anhydride used as reagent) (Hochleitner *et al.*, 2000), arginine (hydroxyphenylglyoxal) (Hager-Braun *et al.*, 2010) and tyrosine (tetranitromethane or iodine) (Santrucek *et al.*, 2004) residues are most commonly used as target for amino acid modification. After removal of excess reagent, modified antigens are digested using specific endoproteases and molecular weight of resulting peptides of free antigen and complex with antibody are compared using MS. The site of modification can be identified by a characteristic molecular weight shift of respective peptides. Residues which are protected from modification by the antibody interaction are considered to be part of the epitope structure.

Using chemical modification of lysine and arginine residues followed by MS analysis, the epitope of monoclonal antibody 559/64-D on gp120 HIV protein was determined (Hager-Braun *et al.*, 2010). Similar approach was used for characterization of the epitope of 5E2.A3 (Fig. 4) recognizing p24 inner core HIV protein, where differences in relative reactivity of the free and bound antigen fragment determined Pro1 as a part of the conformational epitope (Hochleitner *et al.*, 2000).

In the hydrogen/deuterium exchange (HDX) technique the protein backbone amide hydrogens are exchanged with deuterium in solution. The backbone amide hydrogens located at the surface of the antigen or involved in weak hydrogen bonds can exchange rapidly, whereas those involved in stabilizing hydrogen bonds, buried in the protein interior or protected by antibody interaction exchange more slowly (Wei *et al.*, 2014). The exchange process is normally performed at room temperature by adding 10–20-fold excess of buffered D_2O at pH 7.0 to free antigen or complex antigen-antibody for predefined time. The reaction is quenched by

adding buffer at pH 2.5 and dropping temperature to 0°C to slow down deuterium exchange. For MS analysis at peptide level, quenching is followed by proteolytic digestion with acidic protease and fast chromatographic separation at low temperature to decrease reverse hydrogen/deuterium exchange (Chen *et al.*, 2011). Pepsin is widely used protease in HDX-MS analyses because it is stable and active under the quenching conditions. Incorporation of the deuterium into the antigen is measured by MS. Measurement can be done at protein level to determine total deuterium incorporation in antigen or at the peptide level to reveal localized exchange information. Peptides from the antigen-antibody complex that are involved in binding would demonstrate protection effect of antibody toward HDX (lower HDX rates) compared to the same peptides in the free form of antigen (Mo *et al.*, 2012). Interestingly, other amino acid sequences located far away from binding site may also alter HDX rates as result of allosteric conformational change induced by binding. However, such sequences exhibit less significant change in HDX compared to peptides involved in binding (Zhang *et al.*, 2011). When using gas-phase fragmentation method, especially collision-induced dissociation (CID), intramolecular migration of peptide amide hydrogen can occur (Jorgensen *et al.*, 2005). Non-ergogenic fragmentation methods with minimal vibrational excitation (electron transfer dissociation, electron capture dissociation or in-source decay MALDI) could be preferable activation methods because they minimize hydrogen migration (Pan *et al.*, 2008; Rand *et al.*, 2008).

6. Conclusions

Identification of the epitope of monoclonal antibody is essential for proper use of antibodies for research, diagnostic or therapeutic approaches. To date, many methods have been developed for mapping of the epitopes, but not all of them are suitable for identification of conformational ones. This review provides short insight into techniques suitable for characterization of conformational epitopes which are very often recognized by antibodies on viral surfaces. The summarized methods are able not only to directly analyze binding site on the antigen, but also to preserve native conformation of the antigen during characterization process. There is no universal rule, which could be used when picking the right method for epitope mapping, since each one provides its advantages and disadvantages under different circumstances. Generally, X-ray crystallography provides highest resolution for epitope mapping, but a crystallography project could be stalled by difficulty to obtained well-diffracting crystal of antibody-antigen complex. NMR spectroscopy is widely used for epitope characterization, but determination of atomic coordinates is often limited to antigens smaller than 25 K and requires large amount of purified, isotope labelled sample. Display techniques are generally easy to perform, fast and cheap methods, but the interpretation of gained data can be arduous, especially for conformational epitopes. Finally, MS is advantageous because of its speed and sensitivity, but it is complicated to gain relevant data for antibodies with low affinity to the antigen. The appropriate method for mapping of the conformational epitope should be chosen individually based on the variable properties of the antigen-antibody complexes.

Acknowledgements. This work was supported by Axon Neuroscience and Research grants LPP-0038-09, VEGA 2/0177/15, 2/0163/13 and APVV 0200-11.

References

Abergel C (2013): Acta Crystallogr D 69, 2167-2173. http://dx.doi.org/10.1107/S0907444913015291

Bhunia A, Bhattacharjya S, Chatterjee S (2012): Drug Discov Today 17, 505-513. http://dx.doi.org/10.1016/j.drudis.2011.12.016

Boder ET, Wittrup KD (1997): Nat Biotechnol 15, 553-557. http://dx.doi.org/10.1038/nbt0697-553

Bolanos-Garcia VM, Chayen NE (2009): Prog Biophys Mol Biol 101, 3-12. http://dx.doi.org/10.1016/j.pbiomolbio.2009.12.006

Burritt JB, Quinn MT, Jutila MA, Bond CW, Jesaitis AJ (1995): T J Biol Chem 270, 16974-16980. http://dx.doi.org/10.1074/jbc.270.28.16974

Clackson T, Wells JA (1994): Trends Biotechnol 12, 173-184. http://dx.doi.org/10.1016/0167-7799(94)90079-5

Claverie JM (2001): Science 291, 1255-1257. http://dx.doi.org/10.1126/science.1058969

Clore GM, Gronenborn AM (1983): J Magn Reson 53, 423-442.

Cockburn JJB, Sanchez MEN, Fretes N, Urvoas A, Staropoli I, Kikuti CM, Coffey LL, Seisdedos FA, Bedouelle H, Rey FA (2012): Structure 20, 303-314. http://dx.doi.org/10.1016/j.str.2012.01.001

Cochran JR, Kim YS, Olsen MJ, Bhandari R, Wittrup KD (2004): J Immunol Methods 287, 147-158. http://dx.doi.org/10.1016/j.jim.2004.01.024

Conway JF, Watts NR, Belnap DM, Cheng N, Stahl SJ, Wingfield PT, Steven AC (2003): J Virol 77, 6466-6473. http://dx.doi.org/10.1128/JVI.77.11.6466-6473.2003

Covey TR, Huang EC, Henion JD (1991): Anal Chem 63, 1193-1200. http://dx.doi.org/10.1021/ac00013a003

Cui Y, Wen J, Hung Sze K, Man D, Lin D, Liu M, Zhu G (2003): Anal Biochem 315, 175-182. http://dx.doi.org/10.1016/S0003-2697(03)00007-1

Daugherty, PS (2007): Protein engineering with bacterial display. Curr Opin Struct Biol 17, 474-480. http://dx.doi.org/10.1016/j.sbi.2007.07.004

Davey NE, Trave G, Gibson TJ (2011): Trends Biochem Sci 36, 159-169. http://dx.doi.org/10.1016/j.tibs.2010.10.002

DiMattia MA, Watts NR, Stahl SJ, Grimes JM, Steven AC, Stuart DI, Wingfield PT (2013): Structure 21, 133-142. http://dx.doi.org/10.1016/j.str.2012.10.017

Dorner T, Radbruch A (2007): Immunity 27, 384-392. http://dx.doi.org/10.1016/j.immuni.2007.09.002

Ekiert DC, Friesen RHE, Bhabha G, Kwaks T, Jongeneelen M, Yu WL, Ophorst C, Cox F, Korse HJWM, Brandenburg B, Vogels R, Brakenhoff JPJ, Kompier R, Koldijk MH, Cornelissen LAHM, Poon LLM, Peiris M, Koudstaal W, Wilson IA, Goudsmit, J (2011): Science 333, 843-850. http://dx.doi.org/10.1126/science.1204839

El-Mousawi M, Tchistiakova L, Yurchenko L, Pietrzynski G, Moreno M, Stanimirovic D, Ahmad D, Alakhov V (2003): J Biol Chem 278, 46681-91. http://dx.doi.org/10.1074/jbc.M308681200

Fack F, Hugle-Dorr B, Song D, Queitsch I, Petersen G, Bautz EK (1997): J Immunol Methods 206, 43-52. http://dx.doi.org/10.1016/S0022-1759(97)00083-5

Fernández C, Wider, G (Eds)(2006): Advanced Techniques in Biophysics, Vol. 10. Springer Berlin Heidelberg, pp. 89-128. http://dx.doi.org/10.1007/3-540-30786-9_5

Fiaux J, Bertelsen EB, Horwich AL, Wuthrich K (2002): Nature 418, 207-211. http://dx.doi.org/10.1038/nature00860

Fielding L (2003): Curr Top Med Chem 3, 39-53. http://dx.doi.org/10.2174/1568026033392705

Fodor S, Zhang Z (2006): Anal Biochem 356, 282-290. http://dx.doi.org/10.1016/j.ab.2006.06.023

Forman-Kay JD, Mittag T (2013): Structure 21, 1492-1499. http://dx.doi.org/10.1016/j.str.2013.08.001

Friesen RH, Lee PS, Stoop EJ, Hoffman RM, Ekiert DC, Bhabha G, Yu W, Juraszek J, Koudstaal W, Jongeneelen M, Korse HJ, Ophorst C, Brinkman-van der Linden EC, Throsby M, Kwakkenbos MJ, Bakker AQ, Beaumont T, Spits H, Kwaks T, Vogels R, Ward AB, Goudsmit J, Wilson IA (2014): Proc Natl Acad Sci USA 111, 445-450. http://dx.doi.org/10.1073/pnas.1319058110

Frueh DP, Goodrich AC, Mishra SH, Nichols SR (2013): Curr Opin Struct Biol 23, 734-9. http://dx.doi.org/10.1016/j.sbi.2013.06.016

Gai SA, Wittrup KD (2007): Curr Opin Struct Biol 17, 467-473. http://dx.doi.org/10.1016/j.sbi.2007.08.012

Gaza-Bulseco G, Li B, Bulseco A, Liu HC (2008): Anal Chem 80, 9491-9498. http://dx.doi.org/10.1021/ac801617u

Gera N, Hussain M, Rao BM (2013): Methods 60, 15-26. http://dx.doi.org/10.1016/j.ymeth.2012.03.014

Gibbons RV, Vaughn DW (2002): Br Med J 324, 1563-1566. http://dx.doi.org/10.1136/bmj.324.7353.1563

Glocker MO, Borchers C, Fiedler W, Suckau D, Przybylski M (1994): Bioconjug Chem 5, 583-590. http://dx.doi.org/10.1021/bc00030a014

Gray JJ, Moughon S, Wang C, Schueler-Furman O, Kuhlman B, Rohl CA, Baker D (2003): J Mol Biol 331, 281-299. http://dx.doi.org/10.1016/S0022-2836(03)00670-3

Gupta A, Onda M, Pastan I, Adhya S, Chaudhary VK (2003): J Mol Biol 334, 241-254. http://dx.doi.org/10.1016/j.jmb.2003.09.033

Hager-Braun C, Hochleitner EO, Gorny MK, Zolla-Pazner S, Bienstock RJ, Tomer KB (2010): J Am Soc Mass Spectrom 21, 1687-1698. http://dx.doi.org/10.1016/j.jasms.2010.03.031

Halperin I, Wolfson H, Nussinov R (2003): Protein Sci 12, 1344-1359. http://dx.doi.org/10.1110/ps.0237103

Halstead SB (2003): Adv Virus Res 60, 421-467.

Han T, Sui J, Bennett AS, Liddington RC, Donis RO, Zhu Q, Marasco WA (2011): Biochem Biophys Res Commun 409, 253-259. http://dx.doi.org/10.1016/j.bbrc.2011.04.139

Hanes J, Jermutus L, Pluckthun, A (2000): Methods Enzymol 328, 404-430.

Hanes J, Pluckthun A (1997): Proc Natl Acad Sci USA 94, 4937-4942. http://dx.doi.org/10.1073/pnas.94.10.4937

Heck AJ, Bonnici PJ, Breukink E, Morris D, Wills M (2001): Chemistry 7, 910-916. http://dx.doi.org/10.1002/1521-3765-(20010216)7:4<910::AID-CHEM910>3.0.CO;2-U

Hochleitner EO, Borchers C, Parker C, Bienstock RJ, Tomer KB (2000): Protein Sci 9, 487-496. http://dx.doi.org/10.1110/ps.9.3.487

Huang J, Gutteridge A, Honda W, Kanehisa M (2006): BMC Bioinformatics 7, 451. http://dx.doi.org/10.1186/1471-2105-7-451

Huang L, Sexton DJ, Skogerson K, Devlin M, Smith R, Sanyal I, Parry T, Kent R, Enright J, Wu QL, Conley G, DeOliveira D, Morganelli L, Ducar M, Wescott CR, Ladner, RC (2003): J Biol Chem 278, 15532-15540. http://dx.doi.org/10.1074/jbc.M212934200

Hunt DF, Zhu NZ, Shabanowitz J (1989): Rapid Commun Mass Spectrom 3, 122-124. http://dx.doi.org/10.1002/rcm.1290030408

Chayen NE, Saridakis E (2008): Nature Methods 5, 147-153. http://dx.doi.org/10.1038/nmeth.f.203

Chen G, Warrack BM, Goodenough AK, Wei H, Wang-Iverson DB, Tymiak AA (2011): Drug Discov Today 16, 58-64. http://dx.doi.org/10.1016/j.drudis.2010.11.003

Cheng X, Kay BK, Juliano RL (1996): Gene 171, 1-8. http://dx.doi.org/10.1016/0378-1119(95)00889-6

Irving MB, Pan O, Scott JK (2001):. Curr Opin Chem Biol 5, 314-234. http://dx.doi.org/10.1016/S1367-5931(00)00208-8

Ishikawa T, Yamanaka A, Konishi E (2014): Vaccine 32,1326-1337. http://dx.doi.org/10.1016/j.vaccine.2014.01.040

James LC, Roversi P, Tawfik DS (2003): Science 299, 1362-1367. http://dx.doi.org/10.1126/science.1079731

Jemmerson R, Paterson Y (1986): Science 232, 1001-1004. http://dx.doi.org/10.1126/science.2422757

Johnson MA, Pinto BM (2004): Bioorg Med Chem 12, 295-300. http://dx.doi.org/10.1016/j.bmc.2003.09.041

Jorgensen TJ, Gardsvoll H, Ploug M, Roepstorff P (2005): J Am Chem Soc 127, 2785-2793. http://dx.doi.org/10.1021/ja043789c

Kendrew JC, Bodo G, Dintzis HM, Parrish RG, Wyckoff H, Phillips DC (1958): Nature 181, 662-666. http://dx.doi.org/10.1038/181662a0

Khuebachova M, Verzillo V, Skrabana R, Ovecka M, Vaccaro P, Panni S, Bradbury A, Novak M (2002): J Immunol Methods 262, 205-125. http://dx.doi.org/10.1016/S0022-1759(02)00006-6

Klockenbusch C, Kast J (2010): J Biomed Biotechnol 2010, 927585. http://dx.doi.org/10.1155/2010/927585

Kondo H, Shiroishi M, Matsushima M, Tsumoto K, Kumagai I (1999): J Biol Chem 274, 27623-27631. http://dx.doi.org/10.1074/jbc.274.39.27623

Levy R, Forsyth CM, LaPorte SL, Geren IN, Smith LA, Marks JD (2007): J Mol Biol 365, 196-210. http://dx.doi.org/10.1016/j.jmb.2006.09.084

Lipovsek D, Pluckthun A (2004): J Immunol Methods 290, 51-67. http://dx.doi.org/10.1016/j.jim.2004.04.008

Lok SM, Kostyuchenko V, Nybakken GE, Holdaway HA, Battisti AJ, Sukupolvi-Petty S, Sedlak D, Fremont DH, Chipman PR, Roehrig JT, Diamond MS, Kuhn RJ, Rossmann MG (2008): Nat Struct Mol Biol 15, 312-317. http://dx.doi.org/10.1038/nsmb.1382

Marasco WA, Sui J (2007): Nat Biotechnol 25, 1421-1434. http://dx.doi.org/10.1038/nbt1363

Mayer M, Meyer B (2001): J Am Chem Soc 123, 6108-6117. http://dx.doi.org/10.1021/ja0100120

Mayrose I, Penn O, Erez E, Rubinstein ND, Shlomi T, Freund NT, Bublil EM, Ruppin E, Sharan R, Gershoni JM, Martz E, Pupko T (2007): Bioinformatics 23, 3244-3246. http://dx.doi.org/10.1093/bioinformatics/btm493

McConnell SJ, Uveges AJ, Fowlkes DM, Spinella DG (1996): Mol Divers 1, 165-176. http://dx.doi.org/10.1007/BF01544954

McPherson A, Gavira JA (2014): Acta Crystallographica Section F-Structural Biology and Crystallization Communications 70, 2-20. http://dx.doi.org/10.1107/S2053230X13033141

Metz B, Kersten GF, Baart GJ, de Jong A, Meiring H, ten Hove J, van Steenbergen MJ, Hennink WE, Crommelin DJ, Jiskoot W (2006): Bioconjug Chem 17, 815-822. http://dx.doi.org/10.1021/bc050340f

Metz B, Kersten GF, Hoogerhout P, Brugghe HF, Timmermans HA, de Jong A, Meiring H, ten Hove J, Hennink WE, Crommelin DJ, Jiskoot,W (2004): J Biol Chem 279, 6235-6243. http://dx.doi.org/10.1074/jbc.M310752200

Mo J, Tymiak AA, Chen G (2012): Drug Discov Today 17, 1323-1330. http://dx.doi.org/10.1016/j.drudis.2012.07.006

Moreau V, Granier C, Villard S, Laune D, Molina F (2006): Bioinformatics 22, 1088-1095. http://dx.doi.org/10.1093/bioinformatics/btl012

Mumey BM, Bailey BW, Kirkpatrick B, Jesaitis AJ, Angel T, Dratz EA (2003): J Comput Biol 10, 555-567. http://dx.doi.org/10.1089/10665270360688183

Murata T, Hemmi H, Nakajima M, Yoshida M, Yamaguchi I (2003): Biochem Biophys Res Commun 307, 498-502. http://dx.doi.org/10.1016/S0006-291X(03)01219-1

Nair DT, Singh K, Siddiqui Z, Nayak BP, Rao KV, Salunke DM (2002): J Immunol 168, 2371-2382. http://dx.doi.org/10.4049/jimmunol.168.5.2371

Neuhaus D, Williamson MP (2000): The Nuclear Overhauser Effect in Structural and Conformational Analysis. Wiley-VCH, second ed., New York.

Novak M, Wischik CM, Edwards P, Pannell R, Milstein C (1989): Prog Clin Biol Res 317, 755-761.

Nybakken GE, Oliphant T, Johnson S, Burke S, Diamond MS, Fremont DH (2005): Nature 437, 764-769. http://dx.doi.org/10.1038/nature03956

Oliphant T, Engle M, Nybakken GE, Doane C, Johnson S, Huang L, Gorlatov S, Mehlhop E, Marri A, Chung KM, Ebel GD, Kramer LD, Fremont DH, Diamond MS (2005): Nat Med 11, 522-530. http://dx.doi.org/10.1038/nm1240

Padlan EA, Silverton EW, Sheriff S, Cohen GH, Smithgill SJ, Davies DR (1989): Proc Natl Acad Sci USA 86, 5938-5942. http://dx.doi.org/10.1073/pnas.86.15.5938

Pan J, Han J, Borchers CH, Konermann L (2008): J Am Chem Soc 130, 11574-11575. http://dx.doi.org/10.1021/ja802871c

Pande J, Szewczyk MM, Grover AK (2010): Biotechnol Adv 28, 849-858. http://dx.doi.org/10.1016/j.biotechadv.2010.07.004

Pierson TC, Kuhn RJ (2012): Structure 20, 200-202. http://dx.doi.org/10.1016/j.str.2012.01.014

Przybylski M (1994): Adv Mass Spectrom 13, 257-283.

Rand KD, Adams CM, Zubarev RA, Jorgensen TJ (2008): J Am Chem Soc 130, 1341-1349. http://dx.doi.org/10.1021/ja076448i

Raska CS, Parker CE, Sunnarborg SW, Pope RM, Lee DC, Glish GL, Borchers CH (2003): J Am Soc Mass Spectrom 14, 1076-1085. http://dx.doi.org/10.1016/S1044-0305-(03)00405-7

Riemer AB, Kurz H, Klinger M, Scheiner O, Zielinski CC, Jensen-Jarolim E (2005): J Natl Cancer Inst 97, 1663-1670. http://dx.doi.org/10.1093/jnci/dji373

Roberts RW, Szostak JW (1997): Proc Natl Acad Sci USA 94, 12297-12302. http://dx.doi.org/10.1073/pnas.94.23.12297

Santrucek J, Strohalm M, Kadlcik V, Hynek R, Kodicek M (2004): Biochem Biophys Res Commun 323, 1151-1156. http://dx.doi.org/10.1016/j.bbrc.2004.08.214

Scott JK, Smith GP (1990): Science 249, 386-390. http://dx.doi.org/10.1126/science.1696028

Sevcik J, Skrabana R, Dvorsky R, Csokova N, Iqbal K Novak M (2007): FEBS Lett 581, 5872-5878. http://dx.doi.org/10.1016/j.febslet.2007.11.067

Schreiber A, Humbert M, Benz A, Dietrich U (2005): J Comput Chem 26, 879-887. http://dx.doi.org/10.1002/jcc.20229

Simonelli L, Beltramello M, Yudina Z, Macagno A, Calzolai L, Varani L (2010): J Mol Biol 396, 1491-1507. http://dx.doi.org/10.1016/j.jmb.2009.12.053

Simonelli L, Pedotti M, Beltramello M, Livoti E, Calzolai L, Sallusto F, Lanzavecchia A, Varani L (2013): Plos One 8, e55561. http://dx.doi.org/10.1371/journal.pone.0055561

Simpson AJ, McNally DJ, Simpson MJ (2011): Prog Nucl Magn Reson Spectrosc 58, 97-175. http://dx.doi.org/10.1016/j.pnmrs.2010.09.001

Skrabana R, Dvorsky R, Sevcik J, Novak M (2010): J Struct Biol 171, 74-81. http://dx.doi.org/10.1016/j.jsb.2010.02.016

Smirnov YA, Gitelman AK, Govorkova EA, Lipatov AS, Kaverin NV (2004): Virus Res 99, 205-208. http://dx.doi.org/10.1016/j.virusres.2003.11.012

Smith, GP (1985): Science 228, 1315-1317. http://dx.doi.org/10.1126/science.4001944

Smith GP, Petrenko VA (1997): Chem Rev 97, 391-410. http://dx.doi.org/10.1021/cr960065d

Sui J, Li W, Murakami A, Tamin A, Matthews LJ, Wong SK, Moore MJ, Tallarico AS, Olurinde M, Choe H, Anderson LJ, Bellini WJ, Farzan M, Marasco WA (2004): Proc Natl Acad Sci USA 101, 2536-2541. http://dx.doi.org/10.1073/pnas.0307140101

Sutherland BW, Toews J, Kast J (2008): J Mass Spectrom 43, 699-715. http://dx.doi.org/10.1002/jms.1415

Takeuchi K, Wagner G (2006): Curr Opin Struct Biol 16, 109-117. http://dx.doi.org/10.1016/j.sbi.2006.01.006

Tang X, Munske GR, Siems WF, Bruce JE (2005): Anal Chem 77, 311-318. http://dx.doi.org/10.1021/ac0488762

Tarnovitski N, Matthews LJ, Sui J, Gershoni JM, Marasco WA (2006): J Mol Biol 359, 190-201. http://dx.doi.org/10.1016/j.jmb.2006.03.008

Toews J, Rogalski JC, Clark TJ, Kast J (2008): Anal Chim Acta 618, 168-183. http://dx.doi.org/10.1016/j.aca.2008.04.049

Tugarinov V, Choy WY, Orekhov VY, Kay LE (2005): Proc Natl Acad Sci USA 102, 622-627. http://dx.doi.org/10.1073/pnas.0407792102

Unsold R, DeGroot J, Newton TH (1980): AJR Am J Roentgenol 135, 767-773. http://dx.doi.org/10.2214/ajr.135.4.767

Van de Water J, Deininger SO, Macht M, Przybylski M, Gershwin ME (1997): Clin Immunol Immunopathol 85, 229-235. http://dx.doi.org/10.1006/clin.1997.4434

Van Regenmortel MH (1989): Immunol Today 10, 266-272. http://dx.doi.org/10.1016/0167-5699(89)90140-0

Wang LF, Yu M (2004): Curr Drug Targets 5, 1-15. http://dx.doi.org/10.2174/1389450043490668

Watts NR, Vethanayagam JG, Ferns RB, Tedder RS, Harris A, Stahl SJ, Steven AC, Wingfield PT (2010): J Mol Biol 398, 530-541. http://dx.doi.org/10.1016/j.jmb.2010.03.026

Wei H, Mo J, Tao L, Russell RJ, Tymiak AA, Chen G, Iacob RE, Engen JR (2014): Drug Discov Today 19, 95-102. http://dx.doi.org/10.1016/j.drudis.2013.07.019

Westwood OMR, Hay FC (2001): Epitope mapping: A Practical Approach. Oxford University Press, Oxford UK.

Wilkinson IC, Hall CJ, Veverka V, Shi JY, Muskett FW, Stephens PE, Taylor RJ, Henry AJ, Carr MD (2009): J Biol Chem 284, 31928-31935. http://dx.doi.org/10.1074/jbc.M109.025304

Williams SC, Badley RA, Davis PJ, Puijk WC, Meloen RH (1998): J Immunol Methods 213, 1-17. http://dx.doi.org/10.1016/S0022-1759(98)00022-2

Yan J, Kline AD, Mo H, Shapiro MJ, Zartler ER (2003): J Magn Reson 163, 270-276. http://dx.doi.org/10.1016/S1090-7807(03)00106-X

Yoshida H, Yagi Y, Ikebukuro K, Sode K (2003): Biotechnol Lett 25, 301-305. http://dx.doi.org/10.1023/A:1022345200666

Zhang Q, Willison LN, Tripathi P, Sathe SK, Roux KH, Emmett MR, Blakney GT, Zhang HM, Marshall AG (2011): Anal Chem 83, 7129-7136. http://dx.doi.org/10.1021/ac201501z

Zhang X, Tang H, Ye C, Liu M (2006): Drug Discovery Today: Technologies 3, 241-245. http://dx.doi.org/10.1016/j.ddtec.2006.09.002

Zhang Z, Pan H, Chen X (2009): Mass Spectrom Rev 28, 147-176. http://dx.doi.org/10.1002/mas.20190

Zhao Y, Chalt BT (1994): Anal Chem 66, 3723-3726. http://dx.doi.org/10.1021/ac00093a029

Zhen Y, Xu N, Richardson B, Becklin R, Savage JR, Blake K, Peltier JM (2004): J Am Soc Mass Spectrom 15, 803-822. http://dx.doi.org/10.1016/j.jasms.2004.02.004

Zhou C, Jacobsen FW, Cai L, Chen Q, Shen WD (2010): MAbs 2, 508-18. http://dx.doi.org/10.4161/mabs.2.5.12970

Zlotnick A, Tan ZN, Selzer L (2013): Structure 21, 6-8. http://dx.doi.org/10.1016/j.str.2012.12.003