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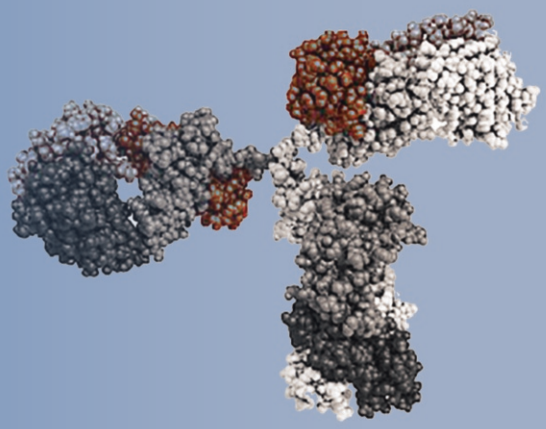

Gunnar Houen *Editor*

Peptide Antibodies

Methods and Protocols

Second Edition

Humana Press

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Peptide Antibodies

Methods and Protocols

Second Edition

Edited by

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Cover Illustration Caption: Space-filling model of Infliximab, the first therapeutic antibody to be approved for human use, in ”open” conformation. Infliximab binds tumor necrosis factor (TNF) and is used for treatment of rheumatoid arthritis and several other inflammatory diseases. From Maibom-Thomsen SL, Trier NH, Holm BE, Hansen KB, Rasmussen MI, Chailyan A, Marcatili P, Højrup P, Houen G. Immunoglobulin G structure and rheumatoid factor epitopes. PLoS One. 2019;14:e0217624.

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Preface

Almost 10 years have passed since the writing of the first edition of Peptide Antibodies: Methods and Protocols. Today, such antibodies are an integrated part of essentially all areas of molecular biology; however, progress is still being made in the production and use of such antibodies. This second edition volume contains chapters and protocols on all aspects of peptide synthesis and analysis, peptide-carrier conjugation, epitope and paratope prediction and identification, as well as improved assays and other uses of peptide antibodies.

Thanks to the series editor, John Walker, and to all contributors and editorial staff.

I dedicate this volume to my son, Søren.

Glostrup, Denmark *Gunnar Houen*

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Chapter 1

Peptide Antibodies: Current Status

Gunnar Houen

Abstract

Peptide antibodies have become one of the most important classes of reagents in molecular biology and clinical diagnostics. For this reason, methods for their production and characterization continue to be developed, including basic peptide synthesis protocols, peptide-conjugate production and characterization, conformationally restricted peptides, immunization procedures, etc. Detailed mapping of peptide antibody epitopes has yielded important information on antibody-antigen interaction in general and specifically in relation to antibody cross-reactivity and theories of molecular mimicry. This information is essential for detailed understanding of paratope-epitope dynamics, design of antibodies for research, design of peptide-based vaccines, development of therapeutic peptide antibodies, and de novo design of antibodies with predetermined specificity.

Key words Peptides, Antibodies, Epitopes, Paratopes, Three-dimensional, Conformational, Linear, Continuous, Contact residues, Therapeutic antibodies, Peptide vaccines

1 Peptide Antibodies

Peptide antibodies, defined as antibodies induced by immunization with a synthetic peptide (coupled to a carrier protein), were first described in 1980 [1, 2]. Subsequent studies revealed the utility of such antibodies in essentially all aspects of molecular biology and immune diagnostics [3–9]. Today, production and use of peptide antibodies is routinely done by many laboratories and commercial companies. However, several aspects of peptide antibody production and use continue to be part of active research and development projects.

2 Peptide Antibody Design

The selection of peptides for production of protein-reactive peptide antibodies remains an important task. Surface-exposed epitopes are mandatory, if the antibodies are intended to react with the native

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protein. Many programs exist, which can predict possible continuous (linear) epitopes on protein surfaces [10–14] (*see* **Note 1**). Flexible linker regions and N-, C-terminal sequences will most likely induce antibodies reacting with the unfolded protein and may also result in peptide antibodies reacting with the native protein [5, 7] (*see* **Note 2**). However, the design of peptide vaccines inducing conformation-specific antibodies reacting with the native protein remains a challenging task, which may be approached with cyclic peptides (*see* **Note 3**).

3 Improvements in Peptide Synthesis

Production of peptides up to 20–30 residues is routinely done by solid-phase peptide synthesis, employing fluorenylmethoxycarbonyl (Fmoc) protection strategy in combination with specific side chain protection and deprotection strategies. This technique is continuously being developed to increase yields and minimize formation of side products and to allow synthesis of peptides with posttranslational and other modifications [15] (*see* **Note 4**).

4 Peptide Characterization

Mass spectrometry (MS) and amino acid analysis (AAA) are commonly used for peptide characterization. Improvements in MS techniques have made this the preferred method for characterization of peptides and conjugates although being of a semiquantitative nature (*see* **Note 5**). AAA has the advantage of being quantitative and allowing quantitation of peptide carrier conjugation in some instances (*see* **Note 6**).

5 Peptide Antibody Production and Characterization

Traditional immunization and cloning techniques continue to be a preferred method of peptide antibody production, occasionally in combination with recombinant technologies [6, 16–20] (*see* **Note 7**). De novo design or selection of antibodies from various libraries may become feasible in the future but presently requires more knowledge of antibody-antigen interactions (*see* **Note 8**).

Whereas peptide antibodies usually are produced using standard procedures, their characterization optimally encompasses various parameters such as primary and three-dimensional structure and paratope-epitope specificity and affinity.

Determination of the primary structure of a monoclonal antibody is routinely done by MS and/or is inferred from sequencing of B cell/hybridoma DNA [21–24]. Antibody three-dimensional

structure may be determined by a variety of techniques, which are still in development, including nuclear magnetic resonance (NMR) spectroscopy, X-ray crystallography, cross-linking (XL) MS, and cryo-electron microscopy (EM) [25–28] (*see* **Note 9**). These techniques may also yield information on paratopes and epitopes, when applied to antibody-antigen complexes.

The epitopes of peptide antibodies are often described as continuous or linear, as opposed to three-dimensional or conformational epitopes [6]. All peptide antibodies, however, react with a three-dimensional epitope, which usually must be present in a defined context to adopt the correct conformation. Detailed mapping of epitopes of several peptide antibodies, using solid phase and solution assays, has revealed important aspects of antibody-antigen interactions, which are generally applicable to the understanding of antibody interactions with continuous epitopes [29, 30]. Such antibodies rely on a set of contact residues (side chain interactions) in combination with backbone contacts for stable interaction. The number of residues/peptide bonds involved may vary from one to several, for example, in the form of a central motif or as separate residues along the peptide epitope (*see* **Note 10**). Peptide-based immune assays, however, do not give information on the paratopes of antibodies. X-ray crystallography of antibody-peptide complexes yields detailed information of paratope-epitope interactions [31, 32] but does not give information on the relative contribution of individual interactions for overall affinity and specificity. NMR spectroscopy may in principle yield the same information as may also Cryo-EM [33, 34]. Different forms of MS, for example, deuterium exchange MS in combination with proteolytic peptide mapping, may also yield information on paratope-epitope interactions [35, 36].

Antibody affinity can be determined by a variety of techniques, including surface plasmon resonance (SPR) enzyme-linked immunosorbent assay (ELISA) or similar techniques [37–40]. The versatility of these techniques makes it possible to carry out studies with substituted peptides, allowing estimation of the relative contribution of individual amino acid residues to on-rates and off-rates as well as overall affinity. This, however, has not yet been realized for any peptide antibodies, although several studies have indicated that most contact residues are critical for overall affinity, that is, a highly cooperative interaction (*see* **Notes 10** and **11**).

6 Peptide Antibodies in Clinical Diagnostics

Antibodies, including peptide antibodies, are some of the most important reagents in clinical diagnostics [8]. The relative ease of production and characterization, together with their high specificity and prior knowledge of epitopes, allows development of highly

specific assays and detailed studies of target structures and modifications in various clinical samples by, for example, immunohistochemistry and immunocytochemistry [6, 8]. As an example, peptide antibodies to calreticulin and its C-terminally modified forms, which are seen in myeloproliferative diseases have been used for differential double immune-blotting, for immune-histochemistry, and for immune-cytochemical diagnostics [30, 41–44] (*see* **Note 12**). Another example is the role of citrullination of Epstein-Barr Virus antigens in rheumatoid arthritis, demonstrating the essential role of EBV in this disease [45, 46]. Finally, the detection of cancer-associated mutations and phosphorylations of Tyr, Ser, or Thr residues are classical example of the ability of peptide antibodies to detect disease-associated alterations of cellular proteins in clinical samples [8, 47, 48].

7 Discussion and Conclusion

Peptide antibodies are one of the most successful and important classes of reagents in molecular biology and clinical diagnostics. For this reason, they continue to be further developed and refined. Several peptide antibody epitopes have been characterized in much detail, yielding insight into the high specificity of such antibodies. These principles are expected to apply to antibodies in general. Therefore, further detailed epitope mapping of peptide antibodies and characterization of motif-specific antibodies is valuable, especially in relation to the understanding of antibody affinity and cross-reactivity and in relation to theories of molecular mimicry.

Relevant databases and programs are expanding and improving (*see* **Notes 13** and **14**) and should be further refined with data on individual contributions of side chains and backbone peptide bonds to specificity and affinity. Molecular modeling methods, including new artificial intelligence algorithms, must be further improved and may eventually allow de novo construction of (peptide) antibodies of predetermined specificity and affinity. Similarly, designed libraries may become useful for (de novo) production of peptide antibodies in the future, but classical methods of production and characterization remain the cornerstone techniques for peptide antibodies at present, however, in combination with recombinant technologies.

Therapeutic peptide antibodies and peptide-based vaccines remain an active research area, which may help to solve important medical problems or contribute to treatment and prevention of autoimmune and infectious diseases in the future.

8 Notes

1. *See* Chapters 2 and 13.
2. *See* Chapter 5.
3. *See* Chapter 9 and [49].
4. *See* Chapter 3.
5. *See* Chapters 7 and 8.
6. *See* Chapters 5 and 6. Preactivation of carrier proteins with iodoacetic acid N-hydroxysuccinimide ester and conjugation through the SH group of Cys allow determination of conjugation ratio by quantitation of carboxymethyl-cysteine (cmCys) relative to other amino acids of the carrier.
7. *See* Chapters 10 and 11.
8. Currently, most antibody libraries rely on immunization or natural immunity at some point during development [20, 50–52].
9. The techniques are listed in order of ability to determine the structure of whole antibody molecules. NMR spectroscopy can determine the structure of antibody domains in solution. X-ray crystallography requires suitable crystals, which is difficult with whole antibody molecules. XL-MS is feasible with whole IgGs. Cryo-EM is in principle applicable to all antibody molecules.
10. Antibodies reacting with defined, continuous (linear) parts of proteins can also be regarded as peptide antibodies, and studies of such antibodies have shown that they show the same characteristics regarding antibody-epitope interactions, that is, dependency on a few side chain interactions in combination with a number of backbone peptide bond interactions [41, 53–61].
11. *See* Chapter 14.
12. *See* Chapters 19 and 20.
13. Relevant databases and associated programs:

    Immune epitope database www.iedb.org

    Immune epitope database analysis resource http://tools.iedb.org

    Cancer epitope database analysis resource https://cedar.iedb.org

    Protein structure database www.pdb.org

    Protein sequence database (Uniprot/Swissprot) www.expasy.org

    Protein predicted structure database (AlphaFold) https://alphafold.ebi.ac.uk

    Antibody structure database https://opig.stats.ox.ac.uk
14. *See* Chapter 2 and [10].

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Chapter 2

B-Cell Epitope Prediction for Antipeptide Paratopes with the HAPTIC2/HEPTAD User Toolkit (HUT)

Salvador Eugenio C. Caoili

Abstract

B-cell epitope prediction is key to developing peptide-based vaccines and immunodiagnostics along with antibodies for prophylactic, therapeutic and/or diagnostic use. This entails estimating paratope binding affinity for variable-length peptidic sequences subject to constraints on both paratope accessibility and antigen conformational flexibility, as described herein for the HAPTIC2/HEPTAD User Toolkit (HUT). HUT comprises the Heuristic Affinity Prediction Tool for Immune Complexes 2 (HAPTIC2), the HAPTIC2-like Epitope Prediction Tool for Antigen with Disulfide (HEPTAD) and the HAPTIC2/HEPTAD Input Preprocessor (HIP). HIP enables tagging of residues (e.g., in hydrophobic blobs, ordered regions and glycosylation motifs) for exclusion from downstream analyses by HAPTIC2 and HEPTAD. HAPTIC2 estimates paratope binding affinity for disulfide-free disordered peptidic antigens (by analogy between flexible-ligand docking and protein folding), from terms attributed to compaction (in view of sequence length, charge and temperature-dependent polyproline-II helical propensity), collapse (disfavored by residue bulkiness) and contact (with glycine and proline regarded as polar residues that hydrogen bond with paratopes). HEPTAD analyzes antigen sequences that each contain two cysteine residues for which the impact of disulfide pairing is estimated as a correction to the free-energy penalty of compaction. All of HUT is freely accessible online (https://freeshell.de/~badong/hut.htm).

Key words B-cell epitopes, B-cell epitope prediction, proteins, peptides, antibodies, binding free-energy changes, conformational disorder, polymer collapse, polyproline II helix, disulfide bonding

1 Introduction

1.1 B-Cell Epitope Prediction (BCEP)

A B-cell epitope (BCE) is a structural feature (e.g., part of a molecule or of a supramolecular complex) recognized by a paratope (i.e., antigen-binding site of an immunoglobulin) via thermodynamically favorable binding in some context (e.g., immunization in vivo or immunoassay in vitro) [1]. As such, a BCE can be defined only in relation to a paratope and vice versa [2]. BCEs and paratopes are thus relational entities, characterized by many-to-many relationships with each BCE recognized by structurally distinct paratopes that each recognize structurally distinct BCEs [3]. Yet,

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BCE recognition is a matter of degree rather than an all-or-none phenomenon; and it may be quantified in terms of paratope-BCE binding affinity, which itself depends on underlying structural factors (e.g., paratope accessibility and conformational state) [4].

Accordingly, BCE prediction (BCEP) is the computational identification of putative BCEs among antigen structures. In essence, this entails partitioning such structures into plausible candidate BCEs and subsequently estimating paratope-BCE binding affinity (e.g., as an equilibrium binding constant or some measure of biological function) to identify those candidate BCEs that are likely to be immunodominant (i.e., tending to elicit the production of antibodies more than other BCEs) [4]. The overall process is described herein for the production of antipeptide antibodies (i.e., peptide antibodies) in particular, with a view to supporting the development of related practical applications as exemplified by peptide-based vaccines.

1.2 Antipeptide Antibody Responses

Antipeptide antibodies are typically obtained via immunization using peptide-based immunogens, such as synthetic peptides conjugated to suitable immunogenic carriers (e.g., proteins and aggregates thereof) [5]. Insofar as the immunizing peptides thus used are sufficiently short, they may be conveniently regarded as comprising only BCEs that are themselves peptidic sequences, each being a continuous BCE (as opposed to a discontinuous BCE, which contains at least two residues that are separated in the primary structure by one or more extraneous amino-acid residues) [6]. These BCEs tend to be conformationally flexible, as the peptides are so short that they fail to assume folded conformations. However, the peptides are often identical in sequence to segments of proteins that may be at least partly folded (i.e., conformationally rigid), such that the BCE sequences may themselves be at least partly folded in the proteins. Consequently, antipeptide antibodies may fail to cross-react with their cognate BCE sequences where the latter form parts of proteins (e.g., as the paratopes fail to recognize said sequences in folded conformations), which is problematic for applications such as peptide-based vaccines, therapeutic antibodies and even antibody-based immunodiagnostics [4].

Some previous work on BCEP was thus directed toward establishing an integrative structure-based framework for predicting biological effects mediated by antipeptide antibodies, by way of paratope-BCE binding affinity estimates using protein structural energetics, initially based on anticipated changes in solvent-accessible surface area (SASA) due to immune-complex formation [7]; but this considered only candidate BCEs of uniform length (e.g., hexapeptide sequences), which is arguably unphysical in view of known structures of BCEs bound by antipeptide paratopes [6]. More recently, a more physicochemically realistic alternative approach has been developed for variable-length BCEs, by analogy

between antipeptide paratope-BCE binding and protein folding modeled as polymer collapse, with implementation as a computer program, namely the Heuristic Affinity Prediction Tool for Immune Complexes (HAPTIC) [8]. To greatly simplify BCEP, HAPTIC completely dispenses with the use of atomic coordinates and even SASA values under the assumption that all input data pertain to only conformationally flexible and thus entirely paratope-accessible peptidic antigens, though this fails to account for folded antigen structure (e.g., among native proteins) and also other barriers to paratope binding (e.g., biomembranes). Moreover, HAPTIC neglects the temperature dependence of peptidic-chain flexibility, which tends to increase with temperature. Hence, the work presented herein provides a more comprehensive toolkit that comprises means to facilitate preprocessing of input antigen sequence data (e.g., to mask residues that are folded and/or paratope-inaccessible) plus an improved version of HAPTIC named HAPTIC2 as well as an additional program derived from HAPTIC2 to handle antigens containing a disulfide-bonded cysteine-residue pair.

2 Theory and Methods

2.1 Extended HAPTIC Framework

A more comprehensive and physicochemically plausible analytical framework for BCEP was developed by modifying the original approach embodied by HAPTIC [8], to subsume peptidic antigens in general (e.g., including proteins that comprise collapsed, folded, and/or posttranslationally modified structures), while more closely aligning the estimation of paratope-BCE binding affinity with available data on conformational preferences and binding modes among said antigens. The definition of candidate BCEs as nested sequences within said antigens was retained, as was the expression of paratope-BCE binding affinity in terms of the standard free-energy change of binding $\Delta G_{\mathrm{b}}^{\circ}$, viz.:

$$\Delta G_{\mathrm{b}}^{\circ} = -RT\ln(C^{\circ}K_{\mathrm{A}}) \tag{1}$$

$$K_{\mathrm{A}} = 1/K_{\mathrm{D}} \tag{2}$$

where R is the gas constant, T the absolute temperature, C° the standard concentration (set to 1M by convention), K_{A} the association constant, and K_{D} the dissociation constant [9]; but $\Delta G_{\mathrm{b}}^{\circ}$ was partitioned differently, as:

$$\Delta G_{\mathrm{b}}^{\circ} = \Delta G_0 + \Delta G_1 + \Delta G_2 + \Delta G_3 \tag{3}$$

where the right-hand terms are contributions due to processes of establishing unrestricted freedom to bind (0), compaction (1), collapse (2), and contact (3), as discussed below.

2.1.1 Unrestricted Freedom to Bind

Unrestricted freedom to bind is a generalization of accessible disorder, viz., the state of a BCE when it is both paratope-accessible and conformationally flexible (i.e., dynamically disordered), such that paratope-BCE binding can occur via induced fit and/or conformational selection [4]. In particular, said generalization also subsumes the special case of polyproline sequences adopting an essentially rigid polyproline-II (PPII) helical conformation [10], wherein the rigidity arises from steric interactions between consecutive residues (i.e., nearest neighbors) rather than folding in the sense of constraints on conformation that include interactions between nonconsecutive residues. Unrestricted freedom to bind thus pertains to individual residues and sequences thereof (e.g., BCEs) whose potential for binding to paratopes is restricted by neither folding nor any barriers to physical contact with paratopes. This is the default state assumed for all residues of peptidic antigen sequences provided as input to HAPTIC, which is appropriate for sufficiently short, hydrophilic, and extracellularly located peptides, but exceptions abound. Most notably, residues may be folded, which can preclude cross-reactive binding of antipeptide antibodies to native antigenic targets [6]. Furthermore, residues may be sequestered within paratope-inaccessible (e.g., intramolecular and/or intracellular) locations due to processes of biomolecular organization such as folding (e.g., of natively folded proteins), coil-to-globule collapse (e.g., of intrinsically disordered proteins), and supramolecular assembly (e.g., of biomembranes and membraneless biomolecular condensates) [11–14].

Accordingly, a simple and convenient way of defining ΔG_0 (in Eq. 3) for a candidate BCE is as follows:

$$\Delta G_0 = \begin{cases} 0, & N_u = N \\ \infty, & N_u < N \end{cases} \tag{4}$$

where N_u is the number of residues in a state of unrestricted freedom to bind and N is the total number of residues (i.e., the sequence length). This computationally expedient approach is based on the notion that ΔG_0 is either negligible (e.g., for a nonfolded extracellular antigen in solution, as assumed by HAPTIC) or a penalty (e.g., to account for the energetic cost of antigen unfolding and/or antibody transport across cell membranes) [7, 8], with the caveat that potentially useful BCEs might be excluded from further consideration because said penalty is deemed too severe.

In order to use Eq. 4, putative folded and/or paratope-inaccessible residues can be identified by various means. For instance, structural data (e.g., from crystallographic and other experimental studies) and structure prediction tools (e.g., employing ab initio calculations and/or homology modeling) [15–17] may be used to identify residues that are likely to be folded, noting

that this can be accomplished indirectly by way of identifying residues that are likely to be nonfolded (e.g., using disorder prediction tools) [18, 19]. Moreover, paratope accessibility can be inferred using computational tools for predicting biological localization (e.g., within transmembrane regions and intracellular compartments) [20–22]. However, a much simpler (albeit less accurate) alternative to using multiple specialized tools is tagging of sequence segments that consist of hydrophobic residues and/or order-promoting residues. Hydrophobic sequence segments can form hydrophobic blobs that cluster together, thereby shielding their constituent residues from the aqueous environment and paratopes therein, possibly with consequent folding and/or sequestration within paratope-inaccessible (e.g., transmembrane) locations. Likewise, sequence segments consisting of order-promoting residues, which are mostly hydrophobic and/or aromatic, tend to form folded structures. Still, paratope access to polar disordered sequences may also be limited, notably where such sequences form collapsed globules or other condensed albeit nonfolded structures due to intramolecular hydrogen bonding among polar tracts (i.e., sequences enriched for neutral polar residues such as histidine, glutamine, asparagine, threonine, serine, and glycine) [23, 24] and/or electrostatic binding interactions between sequences of opposite net charge [23, 25].

From a practical standpoint, N_u can be more narrowly defined to also exclude residues that are likely to be chemically altered via posttranslational modification (e.g., glycosylation, lipidation, phosphorylation, and various forms of oxidation). This may be regarded as equivalent to imposing a penalty for the energetic cost of reversing the covalent modification, which is thus viewed as yet another barrier to paratope-BCE binding, noting that paratopes can discriminate between BCEs that differ only in one chemical group [26]. Such an approach also provides the opportunity to tag residues that are potentially problematic because they are chemically labile (e.g., by virtue of their susceptibility to natural oxidation, as in the case of cysteine and methionine, or to unintended artificial modification by reagents used for peptide-based immunogen preparation, as in the case of lysine where glutaraldehyde is used as an amine-reactive crosslinking agent for covalent coupling of peptides to immunogenic carrier proteins [27]).

2.1.2 Compaction and Collapse

Compaction is the fitting of a candidate-BCE backbone within an enclosing sphere whose radius is that of an idealized circular-paratope footprint, whereas collapse is the transition of a candidate BCE from the compacted (i.e., postcompaction) state to an ensemble of conformations suitable for paratope binding [8]. From a polymer-theoretic perspective of protein folding as occurring in successive stages [28], compaction and collapse are, respectively,

analogous to the initial stage that is dominated by local elastic effects arising from residue backbone conformational propensities and the subsequent stage when nonlocal excluded-volume effects become dominant.

With regard to compaction, the approach to estimating ΔG_1 was modified for more general applicability, as:

$$\Delta G_1 = \begin{cases} \Delta G_e, & h_{Pro}^{max} \leq W \\ \infty, & h_{Pro}^{max} > W \end{cases} \tag{5}$$

$$W = \lfloor 2R_f / L_r \rfloor \tag{6}$$

where h_{Pro}^{max} is the maximum number of consecutive proline residues; W is the maximum number of residues in an inherently compact sequence (i.e., which fits within an enclosing sphere of radius R_f when adopting an entirely PPII helical conformation); $R_f = 10$ Å is the paratope-footprint radius [29]; $L_r = 3.1$ Å is the rise per residue in a PPII helix [10]; and ΔG_e is a finite free-energy change for compaction based on the sequence, defined herein as:

$$\Delta G_e = \begin{cases} 0, & N \leq W \\ \Delta G_d, & N > W \end{cases} \tag{7}$$

$$\Delta G_d = \begin{cases} 0, & R_f \geq R_d \\ \Delta G_y(R_f) - \Delta G_y(R_d), & R_f < R_d \end{cases} \tag{8}$$

$$\Delta G_y(R_y) = \begin{cases} 0, & R_y \geq L_c/2 \\ \max(0, y(R_y)), & R_y < L_c/2 \end{cases} \tag{9}$$

$$y(R_y) = RT\left(\frac{0.56 L_c L_p}{R_y^2} - \frac{1.1 L_p}{R_y} + \frac{3}{2}\ln\left(\frac{L_c L_p}{R_y^2}\right) + 0.44\right) \tag{10}$$

$$L_c = N L_s \tag{11}$$

where R_d is the estimated radius of the minimal enclosing sphere for the sequence when disulfide bonding is considered; L_c is the contour length; L_p is the persistence length; and $L_s = 3.8$ Å is the sequence-segment length (i.e., between consecutive CA [Cα] atoms), such that Eq. 10 is in its corrected form [30] with R used in place of the Boltzmann constant k_B for consistency with Eqs. 1 and 17. This differs from the approach previously described for HAPTIC, which neglects the possibility of disulfide-bond formation and thus implicitly assumes that $R_d = L_c/2$ (i.e., the minimal enclosing sphere must be large enough to accommodate the sequence adopting a maximally extended conformation) in all cases. On a related note, the definition of L_c in Eq. 11 is identical to that in previous work on which Eq. 12 is based [31], whereas HAPTIC assumes $L_c = (N-1)L_s$ (i.e., only structure between the N- and C-terminal CA atoms is considered by HAPTIC).

Using Eqs. 5 and 6 with their given parameter values, all sequences with $N \leq 6$ are candidate BCEs having $\Delta G_1 = 0$ (as they are inherently compact), while all sequences with $h_{\mathrm{Pro}}^{\max} > 6$ have $\Delta G_1 = \infty$ (as they are essentially inflexible), as is also the case with HAPTIC. All other sequences are candidate BCEs having $\Delta G_1 \geq 0$, such that $\Delta G_1 > 0$ only if $\Delta G_e > 0$ (i.e., compaction is thermodynamically unfavorable) from Eqs. 7 through 11, for which L_p is estimated herein as:

$$L_p = \left(R_g^2 N/(2L_s)\right) \Big/ \sum_{k=1}^{N} (1 - k/N) k^{2\nu} \tag{12}$$

$$R_g = \rho R_h \tag{13}$$

$$R_h = 2.16 N^{0.503 - 0.11 \ln(1 - f_{\mathrm{PPII}})} + 0.26|Q| - 0.29 N^{0.5} \tag{14}$$

$$f_{\mathrm{PPII}} = (1/N) \sum_{i=1}^{N} P_{\mathrm{PPII},i} \tag{15}$$

$$P_{\mathrm{PPII},i} = 1/(1 + K_{\mathrm{PPII},i}) \tag{16}$$

$$K_{\mathrm{PPII},i} = K_{\mathrm{PPII},i}^{\dagger} \exp\left((\Delta H_{\mathrm{PPII}}/R)(1/T^{\dagger} - 1/T)\right) \tag{17}$$

where R_g is the radius of gyration; $\nu = 0.6$ is the Flory scaling exponent for unfolded peptides [31]; $\rho = 1.06$ is the ratio of R_g to R_h for unfolded peptides [32]; R_h is the hydrodynamic radius; Q is the net charge (summed from signed formal-charge counts per sequence); f_{PPII} is the mean PPII helical propensity of all residues in the sequence; $P_{\mathrm{PPII},i}$ is the PPII helical propensity of the i^{th} residue; $K_{\mathrm{PPII},i}$ is the equilibrium constant for the transition of the i^{th} residue from PPII to non-PPII conformational states at temperature T; $K_{\mathrm{PPII},i}^{\dagger}$ is the value of $K_{\mathrm{PPII},i}$ at the standard reference temperature $T^{\dagger} = 298.15$ K (i.e., 25 °C); and $\Delta H_{\mathrm{PPII}} = 10$ kcal/mol is the enthalpy change for said transition [33]. This is consistent with more recently published work [34] in that f_{PPII} is based on the entire sequence (whereas HAPTIC neglects the N- and C-terminal residues), a newer version of Eq. 14 is used (cf. the one used for HAPTIC [35]), and the temperature dependence of $P_{\mathrm{PPII},i}$ is explicitly captured (cf. HAPTIC, which uses representative fixed values of $P_{\mathrm{PPII},i}$ including additional ad hoc ones for non-glycine residues immediately preceding proline in the sequence, though in retrospect this is redundant in view of said temperature dependence [33, 34]).

As for R_d, this can be estimated for a candidate BCE that forms all or part of a peptidic sequence comprising exactly one pair of cysteine residues linked by an intramolecular disulfide bond, which thus form the ends of a disulfide loop, as follows:

$$R_d = \begin{cases} L_c/2, & N_o \leq N_a/2 \\ (N_a/2 + N_t) L_s/2, & N_o > N_a/2 \end{cases} \tag{18}$$

$$N_t = \max(N_v, N_w) \tag{19}$$

where N_o is the number of BCE residues that form part of the loop; N_a is the number of loop residues; N_t is the number of BCE residues in the longest contiguous segment of nonloop residues; and N_v and N_w are the numbers of nonloop BCE residues located N-terminal and C-terminal to the loop, respectively.

With regard to collapse, the original approach of HAPTIC to estimating ΔG_2 was retained, viz.:

$$\Delta G_2 = \begin{cases} \Delta G_x, & N \le M \\ \infty, & N > M \end{cases} \tag{20}$$

$$M = \lfloor \pi R_f^2 N \Big/ \sum_{i=1}^{N} A_i \rfloor \tag{21}$$

$$A_i = (3\sqrt{\pi} V_i/4)^{2/3} \tag{22}$$

$$\Delta G_x = \begin{cases} 0, & N \le W \\ RT \ln\left((M - W + 1)^{(N-W)} (M - N)!/(M - W)!\right), & N > W \end{cases} \tag{23}$$

where M is the sequence-dependent estimated maximum number of BCE residues that can fit on the paratope; A_i is the assumed effective cross-sectional area of the i^{th} residue; and V_i is the van der Waals volume of the i^{th} residue [36].

2.1.3 Contact

Contact is the binding of a collapsed candidate BCE by a paratope [8]. For this, the approach to estimating ΔG_3 was modified on the basis of a simpler yet more accurate scoring system [37], as (in units of kcal/mol):

$$\begin{aligned} \Delta G_3 = {} & 0.097 \sum_{i=1}^{N} \Delta X_{ap,i} - 0.43 \sum_{i=1}^{N} X_{sb,i} \\ & - 0.78 \sum_{i=1}^{N} X_{hb,i} - 1.4 \end{aligned} \tag{24}$$

where $\Delta X_{ap,i}$ is the change in the number of solvent-exposed apolar non-H (i.e., C and S) atoms upon paratope-BCE binding while $X_{sb,i}$ and $X_{hb,i}$ are the numbers of salt bridges and hydrogen bonds, respectively, formed between BCE and paratope for the i^{th} residue. This differs from the original approach of HAPTIC, as more weight is assigned for $\Delta X_{ap,i}$ (0.097 vs. 0.088) and $X_{hb,i}$ (0.78 vs. 0.76) while less weight is assigned for $X_{sb,i}$ (0.43 vs. 0.58); but the most important difference is due to elimination of term ΔX_{tor} representing the change in the number of torsions (accounting for restricted conformational freedom upon paratope-BCE binding), which is explicitly considered by HAPTIC for all BCE residues with use of special ad hoc ΔX_{tor} values for non-proline N-terminal residues and also for non-glycine residues immediately N-terminal to proline in the sequence (in view of

backbone dihedral angle ϕ [having its central bond between N and CA atoms], which is missing at the N-terminus, fixed in proline and severely constrained in said non-glycine residues) [8].

Concomitant to adopting Eq. 24, $\Delta X_{\mathrm{ap},i}$ was decreased for all residues, while $X_{\mathrm{hb},i}$ was increased for glycine and proline. This was based in part on observed tendencies of particular BCE residue atoms to contact paratope atoms at paratope-BCE interfaces [38] and also on a related dichotomous classification of all residues as either polar or apolar, notably with both glycine and proline classed as polar. The basic premise was that backbone and side-chain atoms of BCEs contact paratope atoms, but only a subset of BCE atoms can do so simultaneously; and when three non-H atoms are all covalently bonded to a trigonal planar BCE atom (i.e., a carbonyl or aromatic C atom), it is unlikely to contact paratope atoms [38].

Although glycine and proline are often classed as apolar because their side chains lack polar atoms, both can, as BCE residues, form hydrogen bonds with paratopes. Hence, both were classed as polar and each assigned a $X_{\mathrm{hb},i}$ value of 1 (vs. 0 under HAPTIC) at internal sequence positions (though both were each assigned $X_{\mathrm{hb},i}$ values of 1 and 2 at N- and C-terminal positions, respectively, as under HAPTIC), noting that peptide BCEs form around one hydrogen bond per residue with their cognate antipeptide paratopes on average [39]. Furthermore, each was assigned a $\Delta X_{\mathrm{ap},i}$ value equal to the number of its non-carbonyl C atoms (i.e., 1 for glycine and 4 for proline, vs. 2 and 5, respectively, under HAPTIC). Other residues classed as polar were all those having side chains that each comprise at least one N or O atom and all those at either N- or C-terminal positions, such that the only residues classed as apolar were at internal sequence positions with side chains devoid of N and O atoms. For all non-glycine and non-proline residues, their assigned $X_{\mathrm{hb},i}$ values were identical to those under HAPTIC; but their assigned $\Delta X_{\mathrm{ap},i}$ values were decreased to exclude all CA atoms, carbonyl C atoms (i.e., in peptide, other amide, and carboxyl groups), and aromatic C atoms that were each covalently linked to three other non-H atoms (i.e., in histidine [CG], phenylalanine [CG], tyrosine [CG and CZ] and tryptophan [CG, CD2, and CE2]). As for $X_{\mathrm{sb},i}$, all assigned values were identical to those under HAPTIC.

At internal sequence positions, HAPTIC2 residue contributions to BCE-paratope contact energetics thus define a conceptually simple BCE residue hierarchy, that is, R > E > D > H > Q > N > K > W > Y > P > T > S, G > F > M, L, I > V > C > A. This is based on a hierarchy of three criteria: $X_{\mathrm{hb},i}$, $X_{\mathrm{sb},i}$, and $\Delta X_{\mathrm{ap},i}$ (i.e., parameters of Eq. 24 with values listed in Table 1), in order of decreasing priority. Hence, a BCE residue is ranked higher on the basis of its greater hydrogen-bonding atom count quantified as $X_{\mathrm{hb},i}$ (e.g., for R > E), its greater formal-charge count quantified as $X_{\mathrm{sb},i}$ (e.g., for D > H) or its more negative

Table 1
BCE residue (X) default parameter values at internal (i.e., nonterminal) sequence positions (cf. Table 2)

	Equation Numbers and Parameters						
	14	17	21	24			
	q_i	$K^{\dagger}_{\mathrm{PPII},i}$	A_i	$\Delta X_{\mathrm{ap},i}$	$X_{\mathrm{sb},i}$	$X_{\mathrm{hb},i}$	$\Delta G_{3,i}$
			(Å2)				(kcal
X							/mol)
A	0	1.703	19.9	−1	0	0	−0.097
C	0	3.000	23.6	−2	0	0	−0.194
V	0	1.564	26.9	−3	0	0	−0.291
I	0	1.564	30.1	−4	0	0	−0.388
L	0	3.167	30.1	−4	0	0	−0.388
M	0	1.778	30.1	−4	0	0	−0.388
F	0	4.882	31.8	−6	0	0	−0.582
G	0	6.692	16.0	−1	0	1	−0.877
S	0	3.167	21.1	−1	0	1	−0.877
T	0	2.125	24.8	−2	0	1	−0.974
P	0	0.000	24.3	−4	0	1	−1.168
Y	0	3.000	32.8	−5	0	1	−1.265
W	0	3.000	36.1	−6	0	1	−1.362
K	+1	0.786	31.8	−4	1	1	−1.598
N	0	2.704	25.3	−1	0	2	−1.657
Q	0	0.887	28.4	−2	0	2	−1.754
H	0	4.000	29.1	−3	0	2	−1.851
D	−1	2.333	24.5	−1	1	2	−2.087
E	−1	1.381	27.6	−2	1	2	−2.184
R	+1	1.632	33.8	−3	1	3	−3.061

[*] q_i: net charge ($Q = \sum q_i$ in Equation 14); $K^{\dagger}_{\mathrm{PPII},i}$: equilibrium constant of PPII-to-non-PPII transition at 25∘C; A_i: equivalent-sphere cross-sectional area (from Equation 22); $\Delta X_{\mathrm{ap},i}$: change in solvent-exposed apolar non-H (i.e., C and S) atom count; $X_{\mathrm{sb},i}$: salt-bridge count ($= |q_i|$); $X_{\mathrm{hb},i}$: hydrogen-bond count; $\Delta G_{3,i}$: contribution of i^{th} residue to ΔG_3 (in Equation 24)

Table 2
BCE residue (X) parameter value adjustments relative to Table 1 at N- and C-terminal sequence positions (*)

*	Affected Parameters (and Value Adjustments); Structural Basis	Change in Contribution to ΔG_3 (kcal/mol)
N	q_i (+1), $X_{sb,i}$ (+1),	− 0.43 (X=G ∨ X=P) or
	$X_{hb,i}$ (0 if X=G ∨ X=P,	− 1.21 (X≠G ∧ X≠P)
	+1 if X≠G ∧ X≠P);	
	positive formal charge due to protonation of backbone N atom	
C	q_i (−1), $X_{sb,i}$ (+1),	− 1.21 (X=G ∨ X=P) or
	$X_{hb,i}$ (+1 if X=G ∨ X=P,	− 1.99 (X≠G ∧ X≠P)
	+2 if X≠G ∧ X≠P);	
	negative formal charge due to deprotonation of backbone with two carboxylate O atoms	

value of $\Delta X_{ap,i}$ (e.g., for Q > N); so all polar residues outrank all apolar residues, with larger apolar residues outranking smaller ones and alanine thus ranking lowest (though all N- and C-terminal residues are classed as polar as per Table 2).

2.2 Tool Development and Deployment

HAPTIC was revised to obtain HAPTIC2, by retaining the general features of the original prediction algorithm (i.e., to identify the predicted most immunodominant BCE as the candidate BCE with the highest estimated paratope binding affinity for each input sequence) in line with Eqs. 1 and 2, while modifying procedures for selecting candidate BCEs and estimating their paratope binding affinity in accordance with Eqs. 3 through 17 and 20 through 24, in conjunction with the assigned parameter values including those for individual BCE residues as presented in Tables 1 and 2. HAPTIC2 thus selects candidate BCEs for estimating paratope binding affinity only where the values of ΔG_0, ΔG_1 and ΔG_2 are all finite (i.e., $< \infty$) according to Eqs. 4, 5, and 20. Like HAPTIC, HAPTIC2 accepts input sequence data in FASTA format; but unlike HAPTIC, it distinguishes between upper- and lower-case residue codes in said data, such that unrestricted freedom to bind is affirmed only for residues represented by upper-case codes (i.e., $\Delta G_0 < \infty$ only for candidate BCEs represented entirely by upper-case codes). With regard to cysteine residues, HAPTIC2 (like HAPTIC) assumes that they all remain in their reduced form (i.e., without forming any disulfide bonds).

HAPTIC2 was forked (i.e., copied and modified) to obtain the HAPTIC2-like Epitope Prediction Tool for Antigen with Disulfide (HEPTAD), which inherits most features of HAPTIC2 (as regards

case-sensitive processing of FASTA-format input sequence data, selection of candidate BCEs, and other prediction algorithm details) but proceeds with full sequence analysis only where each provided input sequence contains exactly two cysteine residues. HEPTAD assumes that said residues are covalently linked by an intramolecular disulfide bond and then predicts the most immunodominant BCEs according to Eqs. 1 through 24 (i.e., including 18 and 19). This provides an alternative to HAPTIC2 especially for relatively short sequences (e.g., segments of proteins) that comprise a single disulfide loop, without complicating HAPTIC2 or attempting to explore more complex cases (e.g., sequences that each comprise multiple cysteine residues with consequent ambiguity of disulfide-bond topology, noting that unrestricted freedom to bind is also less plausible where two or more disulfide bonds occur within a single BCE).

In order to facilitate tagging of individual residues among input sequences for exclusion of candidate BCEs, the HAPTIC2/HEPTAD Input Preprocessor (HIP) was also developed. HIP accepts FASTA-format sequence input and can tag residues in the sequence data by rendering residue letter codes in lower case in the output thus generated (also in FASTA format and suitable as input for both HAPTIC2 and HEPTAD). HIP enables tagging of contiguous sequence segments of apolar residues (which tend to form hydrophobic blobs) and of order-promoting residues (which tend to occur in folded structures), with user-defined minimum segment lengths (e.g., four residues, which may be regarded as the minimum size of a hydrophobic blob [40] and also the minimum length of an α-helix as a representative ordered structure). For this purpose, apolar residues are those for which $X_{\mathrm{hb},i} = 0$ in Table 1 (i.e., A, C, V, I, L, M and F at internal rather than N- or C-terminal sequence positions), while order-promoting residues each have a side chain that is larger than a methyl group as well as devoid of N and O atoms and/or uncharged with a trigonal planar CG atom (i.e., C, V, I, L, M, F, Y, W, N, and H) [41]. HAPTIC also enables tagging of residues by letter codes (e.g., C and M, to exclude the most oxidation-prone candidate BCEs from further consideration) and of putative N-glycosylation sites within canonical sequence motifs (i.e., NXS and NXT where X $\neq$ P).

Like HAPTIC, HIP, HAPTIC2, and HEPTAD were all developed as common gateway interface (CGI) programs written in the Python programming language, to be freely accessed online via web interface user input forms or used as command-line programs (e.g., on Linux and other UNIX-type systems, either locally or remotely) in non-CGI mode. HIP, HAPTIC2, and HEPTAD were packaged together as the HAPTIC2/HEPTAD User Toolkit (HUT) and made free for online use (https://freeshell.de/~badong/hut.htm). HUT was used to analyze previously curated data (sourced from published literature) that had been analyzed using HAPTIC

[8], in particular to compare the output of HAPTIC2 with that of HAPTIC. Additional data were also obtained via the Immune Epitope Database (https://www.iedb.org) [42] and publications cited therein on peptide immunogen sequences that each comprise exactly two cysteine residues, in order to compare the output of HEPTAD with that of HAPTIC2 as regards the predicted most immunodominant BCEs.

3 Example Results

3.1 HAPTIC2 vs. HAPTIC

Results obtained using HAPTIC2 for immunogenic peptide sequences and data thereon previously analyzed using HAPTIC [8] are depicted in Figures 1 through 4. Using HAPTIC2 instead of HAPTIC, each predicted most immunodominant BCE (Fig. 1) either remained the same (in most cases) or lengthened to comprise additional N- or C-terminal residues (for sequences E, F, H, L, M, N, O, and S), in all cases being devoid of HIP-tagged putative hydrophobic blobs and ordered regions. The observed lengthening of some predicted BCEs is due to decreased PPII helical propensity at the immunization temperatures (versus the lower reference temperature of 25∘ C) that favors compaction (according to Eqs. 5 through 17), as well as more favorable contributions from hydrogen-bond formation upon contact without explicit penalization of restricted torsions (according to Eq. 24 and Table 1).

Using data presented in Fig. 1, HAPTIC2 thus consistently yields higher estimates of antipeptide-antibody binding affinities for immunoassays than does HAPTIC, due to more favorable contributions from hydrogen-bond formation upon contact in all cases and also decreased PPII helical propensity in the case of peptide P (for which the immunoassay temperature exceeds the reference temperature of 25∘ C). Hence, HAPTIC2 consistently overestimates said affinities (Fig. 3), whereas these are underestimated by HAPTIC for peptides I, Q, R, and V (Figure 4 of [8]). With regard to the predicted in-vivo antibody affinity ceiling of 4.75×1011 $M-1$ [43], affinity estimates exceed this for peptides B, D, K, T, and U using HAPTIC2 (but only for peptides D and U using HAPTIC) in the context of immunoassay. However, affinity estimates in the context of immunization (versus immunoassay) exceed said ceiling only for peptides D, K, and U using HAPTIC2 (Fig. 4) and only for peptide U using HAPTIC [8]. In view of these observations, HAPTIC2 yields more plausible predictions than HAPTIC in that HAPTIC2 better avoids underestimating antibody affinity while also still avoiding overestimation above the predicted in-vivo ceiling in most cases, with said overestimation being mitigated by larger compaction free-energy penalties at immunoassay temperatures that are lower than the corresponding immunization temperatures (Fig. 2), noting that compaction free-energy penalties are temperature-independent for HAPTIC.

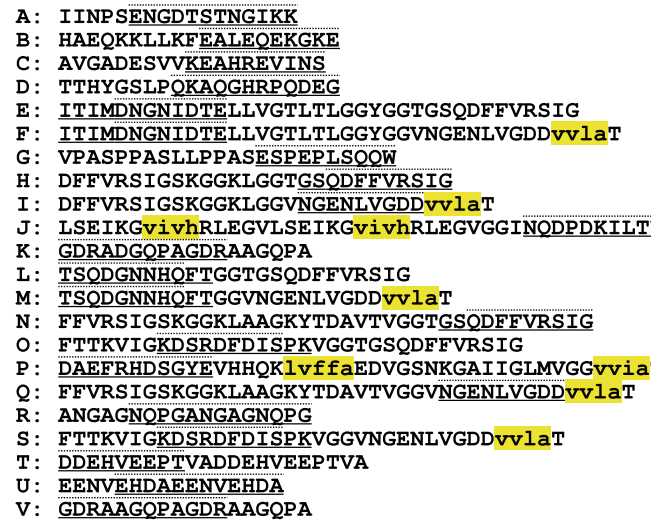

Fig. 1 HAPTIC2- and HAPTIC-predicted most immunodominant BCEs (indicated by solid underlines and dotted overlines, respectively) among immunogenic peptide sequences (labeled A through V) that are all devoid of cysteine residues. HIP-tagged sequence segments comprising putative hydrophobic blobs (e.g., VVLA in F, I, M, Q, and S) and ordered regions (e.g., VIVH in J) are rendered in lower case with yellow highlighting. Immunization temperature was either 39.2∘C (in rabbits, for B, D, G and P) or 37∘C (in mice, for all other sequences). Immunoassay temperature was 4∘C (for B, D, and J), 25∘C (for A and C), 37∘C (for P) or 20∘C (for all other sequences)

3.2 HAPTIC2 vs. HEPTAD

Results obtained using HAPTIC2 and HEPTAD for example peptidic immunogen sequences are depicted in Figs. 5 through 7. Although HAPTIC2 and HEPTAD may yield identical predicted most immunodominant BCEs for an input sequence (e.g., when the disulfide loop is sufficiently long, with $N_o \leq N_a/2$ according to Eq. 18), divergence between HAPTIC2- and HEPTAD-predicted most immunodominant BCEs is observed for each of the example sequences (Fig. 5). Said divergence is due to higher HEPTAD-predicted antibody binding affinity for BCEs that each comprise all or part of a disulfide loop (Fig. 6) relative to HAPTIC2-predicted antibody binding affinity for the same BCEs wherein the cysteine residues are in reduced form (i.e., without any disulfide bonds formed). This affinity difference is in turn due to correspondingly lower HEPTAD-predicted (vs. HAPTIC2-predicted) compaction free-energy penalties (Fig. 7) according to Eqs. 5 through 19 and values of $K^{\dagger}_{\mathrm{PPII},\,i}$ in Table 1. A HAPTIC2-predicted most immunodominant BCE may thus be a subsequence of its HEPTAD-predicted counterpart (e.g., as for sequences b, e, f, i, and j) or vice versa (e.g., as for sequence c). Alternatively, the two predicted BCEs may either overlap nonetheless (e.g., as for

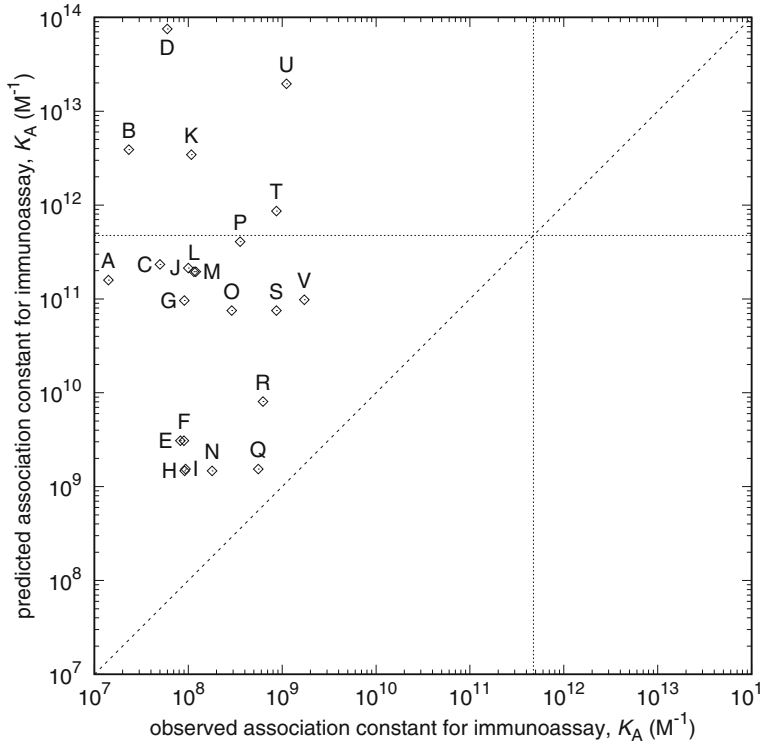

Fig. 2 HAPTIC2-predicted versus observed antipeptide-antibody binding affinities for peptides at corresponding immunoassay temperatures specified in Fig. 1. Dotted crosshair lines are at 4.75×10^{-11} M^{-1} (i.e., predicted in-vivo antibody affinity ceiling). Identity line (for perfect agreement between predicted and observed binding affinities) is dashed

sequences a, d, g, h, l, and m) or be nonoverlapping (e.g., as for sequences k, n, and o, each of which comprises a disulfide loop that is nested within the HEPTAD-predicted most immunodominant BCE).

HEPTAD-predicted most immunodominant BCEs thus tend to comprise (or at least occur near) cysteine residues, more so than the corresponding HAPTIC2-predicted most immunodominant BCEs, insofar as HEPTAD attenuates the compaction free-energy penalty for candidate BCEs that comprise or overlap disulfide loops. Because cysteine is classed as both apolar and order-promoting, HEPTAD-predicted most immunodominant BCEs may contain residues that are tagged by HIP as forming parts of putative hydrophobic blobs and/or ordered regions (e.g., as for sequences a, k, and o). Hence, such BCEs may be excluded from further consideration if HIP output is provided as input for HEPTAD. However, this approach is arguably excessive, in view of the possibility that BCEs could comprise HIP-tagged residues. As a case in point, the immunogenic peptide SS-Aβ 42 (a mutated

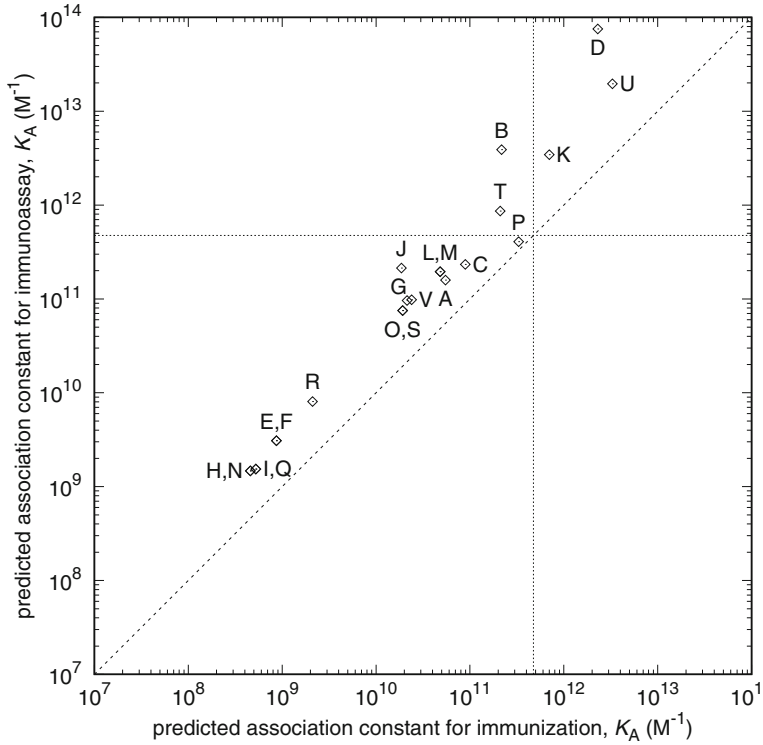

Fig. 3 HAPTIC2-predicted antipeptide-antibody binding affinities for immunoassay and immunization with peptides at corresponding temperatures specified in Fig. 1. Values for immunoassay, crosshair lines and identity line are as in Fig. 2

version of human Aβ42 amyloid peptide) comprises the internal sequence QKCVFFAEDVGSNCGA wherein the two cysteine residues form a disulfide-bonded pair; and immunization of mice with SS-Aβ42 has yielded the monoclonal antibody TxCo-1 for which the peptide subsequence QKCVFFAE consists of paratope-contacting BCE residues [44], noting that the nested tetrapeptide subsequence CVFF might be tagged by HIP as both a hydrophobic blob and an ordered region.

4 How to Use HUT

4.1 General Workflow

1. Prepare user data as peptidic sequence/s in FASTA format (*see* **Note 1**).
2. Point web browser to HUT (https://freeshell.de/~badong/hut.htm).
3. Click link to web interface for HIP, HAPTIC2, or HEPTAD (*see* **Note 2**).

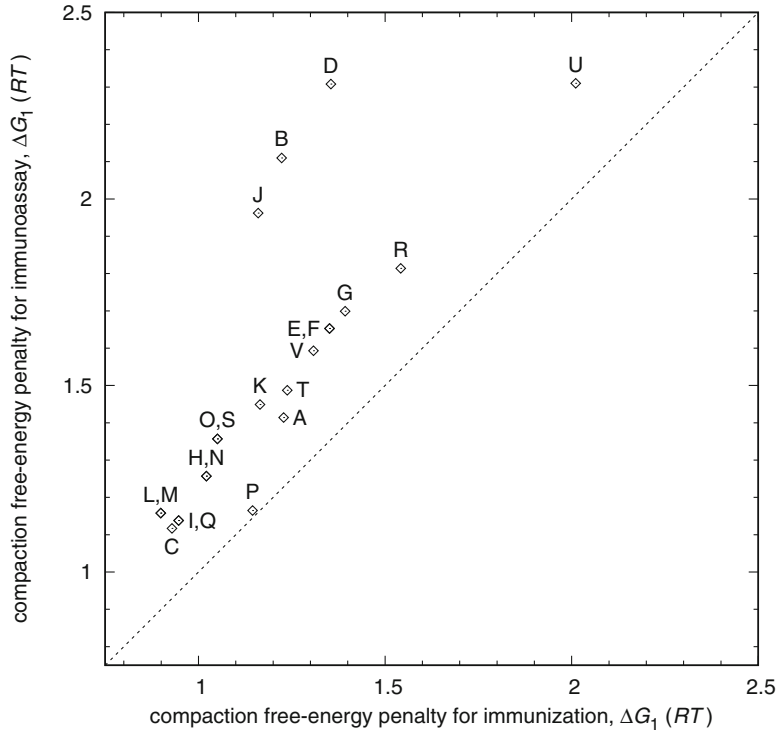

Fig. 4 HAPTIC2-predicted compaction free-energy penalties for immunoassay and immunization with peptides at corresponding temperatures specified in Fig. 1. Identity line is dashed

```
a: EclllSKYCMPS        a: 130474    i: 31566
b: CPSKKPYEEVTC        b: 79528     j: 1855240
c: SPLLGCIGSTCAE       c: 60116     k: 1378185
d: CNNPHRILDGINC       d: 6713      l: 37377
e: HRKTTCTRCPATSP      e: 1965990   m: 97505
f: RRSHPCRTCTTHTP      f: 1970295   n: 39336
g: NDYCLRECKQQGYKG     g: 43545     o: 123164
h: NNFLSKEECERKCGG     h: 952290
i: KKICKMEKCSSVFNV
j: YQAGSTPCNGVEGFNCY
k: CNGVEGfncyfPLQSYGFQP
l: LLGYKKGEGNTCVENNNPTC
m: MSLLTEVETPTRNEWECRCSDSSD
n: LSESSVTVTPPDGTSALVECECGGT
o: FEPTGFQNMLSGLYNPIVFSASGAnLTDAhlfcllacDRD
```

Fig. 5 HAPTIC2- and HEPTAD-predicted most immunodominant BCEs (indicated by solid underlines and dotted overlines, respectively) at an immunization temperature of 37◦ C among peptidic immunogen sequences (labeled a through o) that each contain two cysteine residues, which are assumed to form a disulfide-bonded pair only for HEPTAD predictions. HIP-tagged sequence segments comprising putative hydrophobic blobs (e.g., CLLL in a), ordered regions (e.g., FNCYF in k), and glycosylation site (i.e., N of NLT in o) are rendered in lower case with yellow highlighting. ID numbers are from the Immune Epitope Database (IEDB; https:/www.iedb.org), for which each corresponding record can be accessed via a URL of the form https:/iedb.org/epitope/ < X > where < X > is the ID number

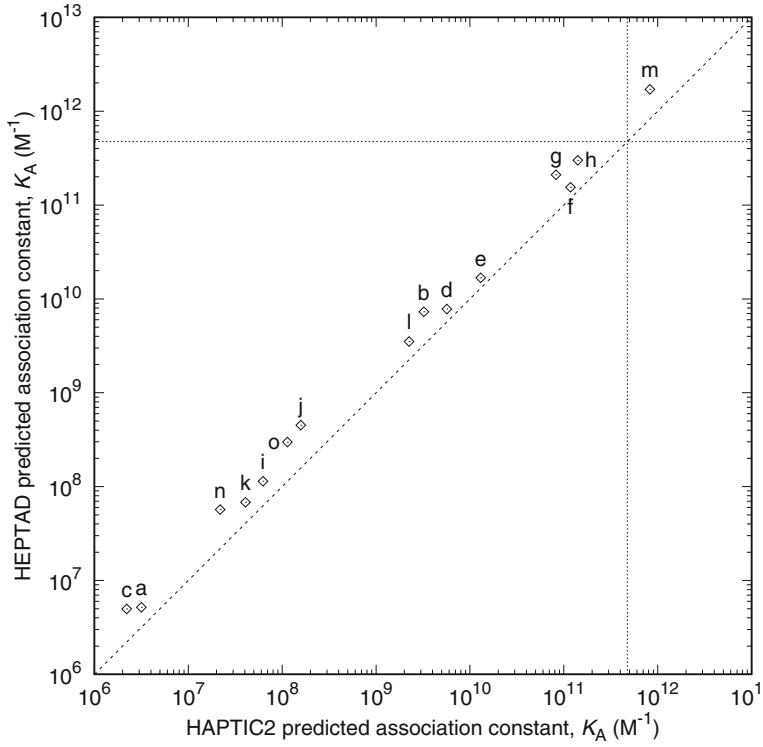

Fig. 6 HEPTAD- versus HAPTIC2-predicted antipeptide-antibody binding affinities for sequences and immunization temperature specified in Fig. 5. Crosshair lines and identity line are as in Fig. 3

4. Change any tool setting/s where appropriate (see details per tool below).
5. Replace default input peptidic sequence data with user data (*see* **Note 3**).
6. Click "send" button to submit user data with specified tool settings.
7. Capture plain-text output generated by tool as in Fig. 8 (*see* **Note 4**).

4.2 HIP Settings

1. Set minimum hydrophobic residue run length (*see* **Note 5**).
2. Set minimum excluded order-promoting residue run length (*see* **Note 6**).
3. Select excluded amino-acid residue/s using checkbox/es (*see* **Note 7**).
4. Select excluded amino-acid residues in sequence motifs (*see* **Note 8**).

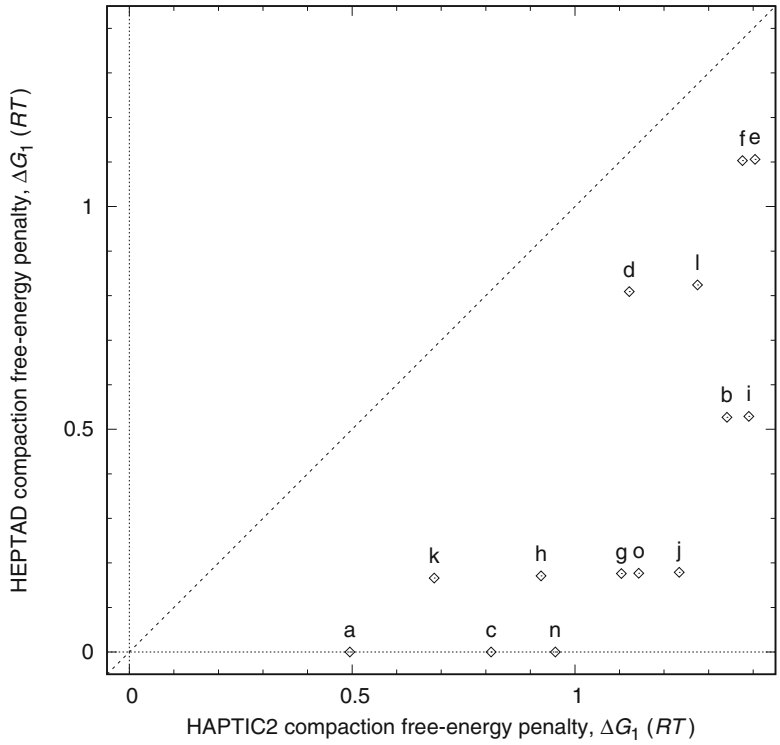

Fig. 7 HEPTAD- versus HAPTIC2-predicted compaction free-energy penalties for sequences and immunization temperature specified in Fig. 5. Identity line is dashed

4.3 HAPTIC2 and HEPTAD Settings

1. Set paratope-footprint radius in Å (*see* **Note 9**).
2. Set temperature of immunization in K (*see* **Note 10**).
3. Set temperature of immunoassay in K (*see* **Note 11**).

5 Notes

1. User data should be rendered in plain text consisting of at least one valid sequence entry in FASTA format. Each sequence entry should thus consist of a header line of the form "> *" where " > " is the greater-than character and "*" is a string comprising zero or more printable characters (e.g., representing sequence metadata) followed by at least one line of sequence data. Only single-letter codes for 20 standard proteinogenic amino-acid residues (ACDEFGHIKLMNPQRSTVWY) should be used for representing sequence data. Both upper- and lower-case codes are accepted, but only upper-case codes are processed (i.e., lower-case codes are masked). Residues can be selectively masked manually (e.g., based on user knowledge) and/or automatically (e.g., using HIP).

```
# Paratope-footprint radius   (Å):  10.0
# Temperature of immunization (K): 310.15
# Temperature of immunoassay  (K): 298.15
#
# Output headers for epitope predictions:
# A:        Antigen-chain list serial no.
# NAA:      N-terminal sequence position
# CAA:      C-terminal sequence position
# L:        Location: N-/C-/Di-terminal or Internal
# Q:        Net charge (based on formal-charge counts)
# N:        Number of AAs in sequence (AA count)
# M:        Upper bound on N (sequence-dependent)
# SPHER:    Spherical-volume compaction free-energy penalty (kT)
# CIRCUL:   Circular-surface collapse free-energy penalty (kT)
# KD_IMMUN: Dissociation constant for immunization (mol/L)
# KD_ASSAY: Dissociation constant for immunoassay (mol/L)
# SEQUENCE: Epitope sequence
# ANTIGEN:  Antigen sequence
#
# Predicted most immunodominant epitope/s in query antigen/s:
# A NAA CAA L   Q  N  M SPHER CIRCUL KD_IMMUN KD_ASSAY SEQUENCE/ANTIGEN
  1   5  12 C   0  8 11 0.461  0.588 2.02e-07 1.19e-07 LSKYCMPS/ECLLLSKYCMPS
  2   3  12 C  -1 10 11 1.053  2.380 2.58e-10 1.12e-10 SKKPYEEVTC/CPSKKPYEEVTC
  3   1  13 D  -1 13 13 1.409  6.031 2.03e-07 9.55e-08 SPLLGCIGSTCAE/SPLLGCIGSTCAE
  4   1  10 N   1 10 11 0.947  2.380 1.63e-10 6.62e-11 CNNPHRILDG/CNNPHRILDGINC
  5   1   8 N   4  8 11 0.943  0.588 7.68e-11 3.23e-11 HRKTTCTR/HRKTTCTRCPATSP
  6   1   8 N   4  8 11 1.020  0.588 6.60e-12 2.48e-12 RRSHPCRT/RRSHPCRTCTTHTP
  7   6  15 C   1 10 11 0.949  2.380 8.36e-12 3.15e-12 RECKQQGYKG/NDYCLRECKQQGYKG
  8   1  11 N   0 11 11 0.930  4.171 5.04e-12 1.73e-12 NNFLSKEECER/NNFLSKEECERKCGG
  9   1   8 N   4  8 10 1.043  0.734 1.60e-08 8.74e-09 KKICKMEK/KKICKMEKCSSVFNV
 10   9  17 C  -2  9 12 0.794  1.050 4.55e-09 2.18e-09 NGVEGFNCY/YQAGSTPCNGVEGFNCY
 11  12  20 C  -1  9 11 0.926  1.281 1.55e-08 7.86e-09 PLQSYGFQP/CNGVEGFNCYFPLQSYGFQP
 12   7  18 I  -2 12 13 1.415  3.951 3.22e-10 1.27e-10 GEGNTCVENNNP/LLGYKKGEGNTCVENNNPTC
 13   8  18 I  -1 11 11 1.180  4.171 8.84e-13 2.83e-13 ETPTRNEWECR/MSLLTEVETPTRNEWECRCSDSSD
 14   1  13 N  -1 13 13 1.542  6.031 1.84e-08 8.00e-09 LSESSVTVTPPDG/LSESSVTVTPPDGTSALVECECGGT
 15   1   8 N   0  8 11 0.449  0.588 5.25e-09 2.64e-09 FEPTGFQN/FEPTGFQNMLSGLYNPIVFSASGANLTDAHLFCLLACDRD
# Candidate epitope/s in query antigen/s:
# A NAA CAA L   Q  N  M SPHER CIRCUL KD_IMMUN KD_ASSAY SEQUENCE
  1   1   3 N   0  3 11 0.000  0.000 1.63e-04 1.15e-04 ECL
  1   1   4 N   0  4 11 0.000  0.000 8.67e-05 5.95e-05 ECLL
  1   1   5 N   0  5 11 0.000  0.000 4.62e-05 3.09e-05 ECLLL
  1   1   6 N   0  6 11 0.000  0.000 1.11e-05 7.04e-06 ECLLLS
  1   1   7 N   1  7 11 0.283  0.182 1.33e-06 8.50e-07 ECLLLSK
  1   1   8 N   1  8 11 0.495  0.588 3.16e-07 1.88e-07 ECLLLSKY
  1   1   9 N   1  9 11 0.687  1.281 5.59e-07 3.32e-07 ECLLLSKYC
  1   1  10 N   1 10 11 0.874  2.380 1.08e-06 6.33e-07 ECLLLSKYCM
  1   1  11 N   1 11 11 1.146  4.171 1.28e-06 7.04e-07 ECLLLSKYCMP
  1   2   4 I   0  3 11 0.000  0.000 2.14e-02 1.83e-02 CLL
  1   2   5 I   0  4 11 0.000  0.000 1.14e-02 9.51e-03 CLLL
  1   2   6 I   0  5 11 0.000  0.000 2.74e-03 2.16e-03 CLLLS
  1   2   7 I   1  6 11 0.000  0.000 2.05e-04 1.46e-04 CLLLSK
  1   2   8 I   1  7 11 0.267  0.182 4.13e-05 3.01e-05 CLLLSKY
  1   2   9 I   1  8 11 0.478  0.588 5.59e-05 4.06e-05 CLLLSKYC
  1   2  10 I   1  9 11 0.681  1.281 7.29e-05 5.26e-05 CLLLSKYCM
  1   2  11 I   1 10 11 0.968  2.380 4.38e-05 2.96e-05 CLLLSKYCMP
  1   2  12 C   0 11 11 0.990  4.171 2.57e-06 1.46e-06 CLLLSKYCMPS
  1   3   5 I   0  3 10 0.000  0.000 1.56e-02 1.32e-02 LLL
  1   3   6 I   0  4 11 0.000  0.000 3.76e-03 3.00e-03 LLLS
  1   3   7 I   1  5 10 0.000  0.000 2.81e-04 2.02e-04 LLLSK
  1   3   8 I   1  6 10 0.000  0.000 3.61e-05 2.39e-05 LLLSKY
  1   3   9 I   1  7 11 0.267  0.182 4.13e-05 3.01e-05 LLLSKYC
  1   3  10 I   1  8 10 0.488  0.734 4.77e-05 3.45e-05 LLLSKYCM
  1   3  11 I   1  9 11 0.795  1.281 1.68e-05 1.14e-05 LLLSKYCMP
  1   3  12 C   0 10 11 0.826  2.380 4.97e-07 2.83e-07 LLLSKYCMPS
  1   4   6 I   0  3 11 0.000  0.000 7.06e-03 5.78e-03 LLS
  1   4   7 I   1  4 11 0.000  0.000 5.28e-04 3.90e-04 LLSK
  1   4   8 I   1  5 10 0.000  0.000 6.78e-05 4.61e-05 LLSKY
  1   4   9 I   1  6 11 0.000  0.000 4.95e-05 3.32e-05 LLSKYC
  1   4  10 I   1  7 11 0.278  0.182 4.18e-05 3.07e-05 LLSKYCM
  1   4  11 I   1  8 11 0.608  0.588 1.31e-05 9.03e-06 LLSKYCMP
  1   4  12 C   0  9 11 0.651  1.281 2.61e-07 1.51e-07 LLSKYCMPS
  1   5   7 I   1  3 11 0.000  0.000 9.91e-04 7.50e-04 LSK
  1   5   8 I   1  4 10 0.000  0.000 1.27e-04 8.87e-05 LSKY
  1   5   9 I   1  5 11 0.000  0.000 9.29e-05 6.39e-05 LSKYC
  1   5  10 I   1  6 11 0.000  0.000 4.95e-05 3.32e-05 LSKYCM
  1   5  11 I   1  7 11 0.403  0.182 1.33e-05 9.35e-06 LSKYCMP
  1   5  12 C   0  8 11 0.461  0.588 2.02e-07 1.19e-07 LSKYCMPS
  1   6   8 I   1  3 10 0.000  0.000 2.39e-04 1.71e-04 SKY
  1   6   9 I   1  4 11 0.000  0.000 1.74e-04 1.23e-04 SKYC
  1   6  10 I   1  5 11 0.000  0.000 9.29e-05 6.39e-05 SKYCM
  1   6  11 I   1  6 11 0.000  0.000 1.40e-05 8.90e-06 SKYCMP
  1   6  12 C   0  7 11 0.252  0.182 2.06e-07 1.23e-07 SKYCMPS
```

Fig. 8 Topmost plain-text lines of HAPTIC2 output for sample peptidic-antigen sequence input data in FASTA format with default settings

2. HIP, HAPTIC2, and HEPTAD can be installed and run locally as command-line tools, but this may require some system-dependent modification of the underlying Python code (e.g., to set the path for the local Python interpreter). Said code is available upon request from the author.
3. Default input peptidic sequence data are provided in the input text field of the web interface for each tool so that users can easily test the tool simply by clicking the "send" button. After any initial testing, said data should be deleted by setting focus to (i.e., selectively activating) said text field (e.g., by clicking within it or repeatedly pressing "Tab") and then using the "Select all" and "Cut" functions in succession (e.g., by right-clicking and selecting said functions, or by pressing "Ctrl+A" followed by "Ctrl+X"). User data should then be copied (e.g., from a text-editor window) and pasted into said text field as plain text.
4. Plain-text output can be transferred to another application by copying and pasting (e.g., into a text-editor window).
5. Minimum hydrophobic residue run length is the minimum size of a hydrophobic blob, which consists of only hydrophobic residues (ACFILMV) and is deemed inaccessible for binding by paratopes. Only nonnegative integers are accepted. Specifying zero (0) prevents masking of hydrophobic blobs.
6. Minimum excluded order-promoting residue run length is the minimum size of an order-promoting region, which consists of only order-promoting residues (CFHILMNVWY) and is deemed inaccessible for binding by paratopes. Only nonnegative integers are accepted. Specifying zero (0) prevents masking of order-promoting regions.
7. Excluded residue/s may be deemed problematic in view of chemical reactivity (e.g., susceptibility to oxidative damage, as in the case of methionine [M], or to covalent modification by aldehyde crosslinking agents, as in the case of lysine [K]).
8. Excluded residues of sequence motifs comprise asparagine (N) residues in canonical eukaryotic N-glycosylation motifs of the form NXS or NXT where X is any residue except proline (P).
9. Paratope-footprint radius is the idealized circular-paratope radius (with default value of 10.0 Å), which determines maximum length of PPII helical segment in a candidate BCE according to Eq. 6.

10. Temperature of immunization is assumed (e.g., normal) body temperature for immunized animal species (e.g., 310.15 K = 37°C for human/mouse [default] or 312.35 K = 39.2°C for rabbit).
11. Temperature of immunoassay is for experiment to measure paratope-BCE binding affinity, with default value assumed for ambient/room temperature of 298.15K = 25°C.

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Chapter 3

Fmoc Solid-Phase Peptide Synthesis

Paul Robert Hansen and Alberto Oddo

Abstract

Synthetic peptides are important as drugs and in research. Currently, the method of choice for producing these compounds is solid-phase peptide synthesis. Here, we describe the scope and limitations of Fmoc solid-phase peptide synthesis. Furthermore, we provide a detailed protocol for Fmoc peptide synthesis.

Key words Fmoc solid-phase synthesis, Resins, Linkers, Coupling reagents

1 Introduction[1]

Synthetic peptides are important as therapeutics [1, 2] and for a number of research purposes including cancer diagnosis and treatment [3], antibiotic drug development [4], epitope mapping [5], production of antibodies [6], and vaccine design [7]. Chemical synthesis of biologically active peptides may provide enough material for additional study, structure-activity relationships, or result in new analogues with improved properties.

Large peptides and small proteins of more than 50 residues may be produced by recombinant methods [8]. However, most researchers prefer the solid-phase method (SPPS) for the chemical synthesis of peptides less than 50 residues. Using the SPPS approach, peptides may be synthesized in virtually any scale, synthesis may be carried out by a fully automated peptide synthesizer and non-proteinogenic amino acids, or posttranslational modifications may be introduced during synthesis.

In solid-phase peptide synthesis (Scheme 1), the C-terminal amino acid residue, masked with a temporary protecting group on the α-amino group and a semipermanent protecting group on the side-chain, is coupled through its C^{α}-carboxylic acid group to a resin. The resin is a polymer that swells in organic solvents and expands as the peptide grows. Furthermore, the resin is equipped

Gunnar Houen (ed.), *Peptide Antibodies: Methods and Protocols*, Methods in Molecular Biology, vol. 2821,
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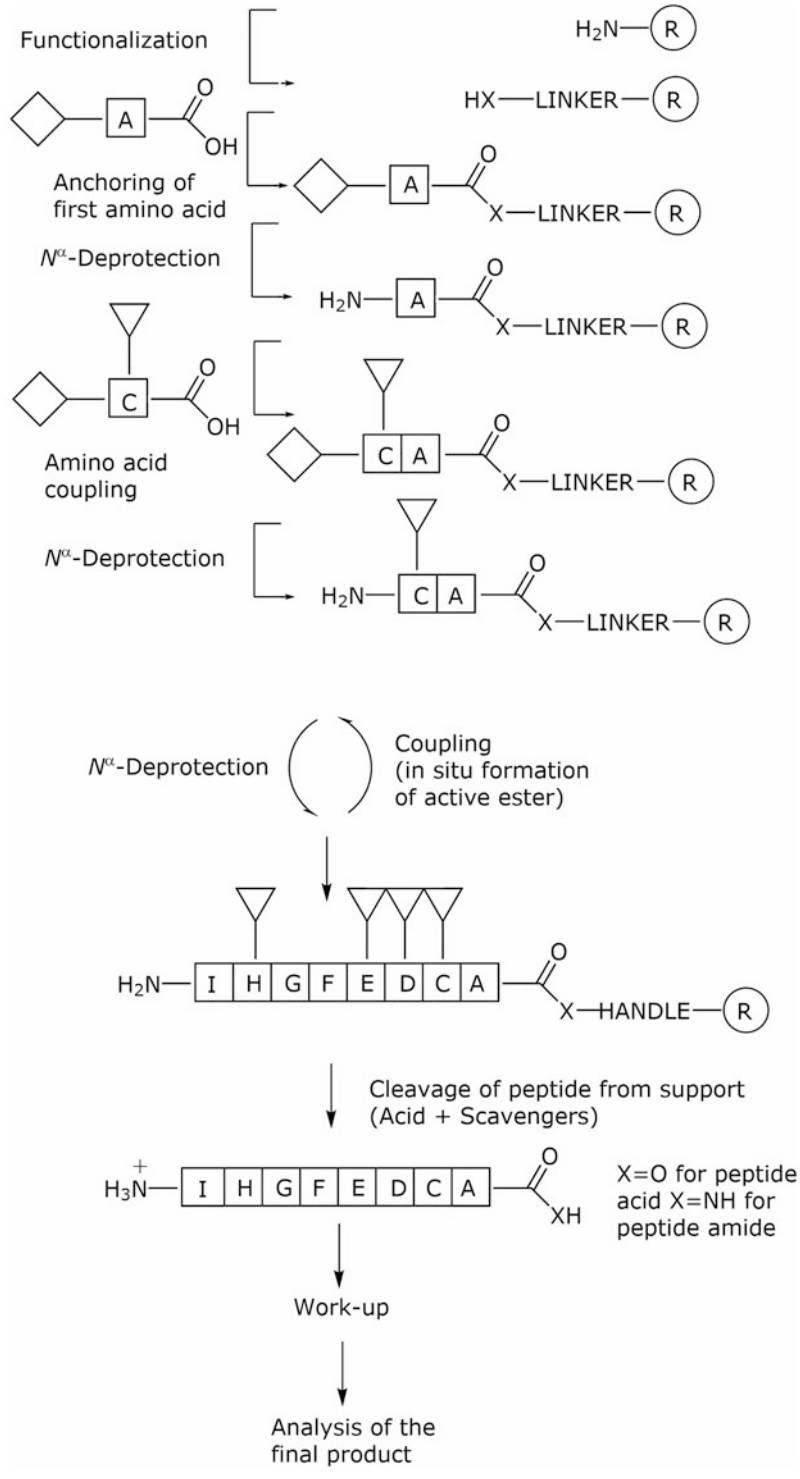

Scheme 1 Overview of solid-phase peptide synthesis

with a bifunctional linker, which allows for the cleavage of the final product following synthesis. The linker most often provides either a peptide acid or peptide amide as the end product.

Following coupling of the first amino acid to the resin, the temporary protecting group masking the α-amino group is removed. The synthesis cycle is continued from the C-terminus toward the N-terminus until the desired protected peptide is obtained. By convention, the resin is placed to the right in Scheme 1, which is very important for correct interpretation of sequence and stereochemistry. The product is then cleaved from the resin concurrently with the semipermanent side-chain protecting groups, isolated and characterized.

The advantages of SPPS over solution synthesis are: (i) All reactions are carried out in a single vessel; (ii) excess amino acid and reagents are used to drive reactions to completion and can subsequently be filtered from the system, eliminating the need for purification of intermediates after each step; and (iii) the solid-phase approach consists of many repetitive steps making automation possible. The only drawback of the method is that removal of by-products during synthesis is impossible.

Two strategies for the solid-phase synthesis of peptides exist, the Boc/benzyl and the Fmoc/tBu strategy.

In the Boc/benzyl strategy, which was introduced by R.B. Merrifield in 1963 [9], the α-amino group is protected by the *tert*-butyloxycarbonyl (Boc) group (**1**, Fig. 1) and the side-chain functional groups by benzyl-based protecting groups. The Boc group is removed by trifluoroacetic acid (TFA) in dichloromethane (DCM), while the semipermanent side-chain protecting groups require hydrogen fluoride (HF) for removal. For a review on Boc SPPS, *see* [10].

In the Fmoc/tBu strategy, which was developed by Atherton and Sheppard [11], the α-amino group is protected by the base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) group (**2**) and side-chain functional groups by the acid-labile tert-butyl (**3**) or trityl-based protecting groups (**4**). The Fmoc group is removed by 20% piperidine in dimethylformamide (DMF) and the side-chain protecting groups by TFA. This is a truly orthogonal protecting scheme as opposed to the Boc/Benzyl strategy, which relies on gradual acid lability.

Since the Boc/Benzyl strategy requires the use of HF for final deprotection, the Fmoc/tBu strategy is the most widely used. In this review, we will focus on the scope and limitations of the Fmoc SPPS strategy [12–14].

1.1 Resins

Currently, resins may be divided into three different groups: (i) poly-styrene-based, (ii) poly(ethylene glycol) (PEG)-grafted polystyrene, and (iii) PEG resins without polystyrene.

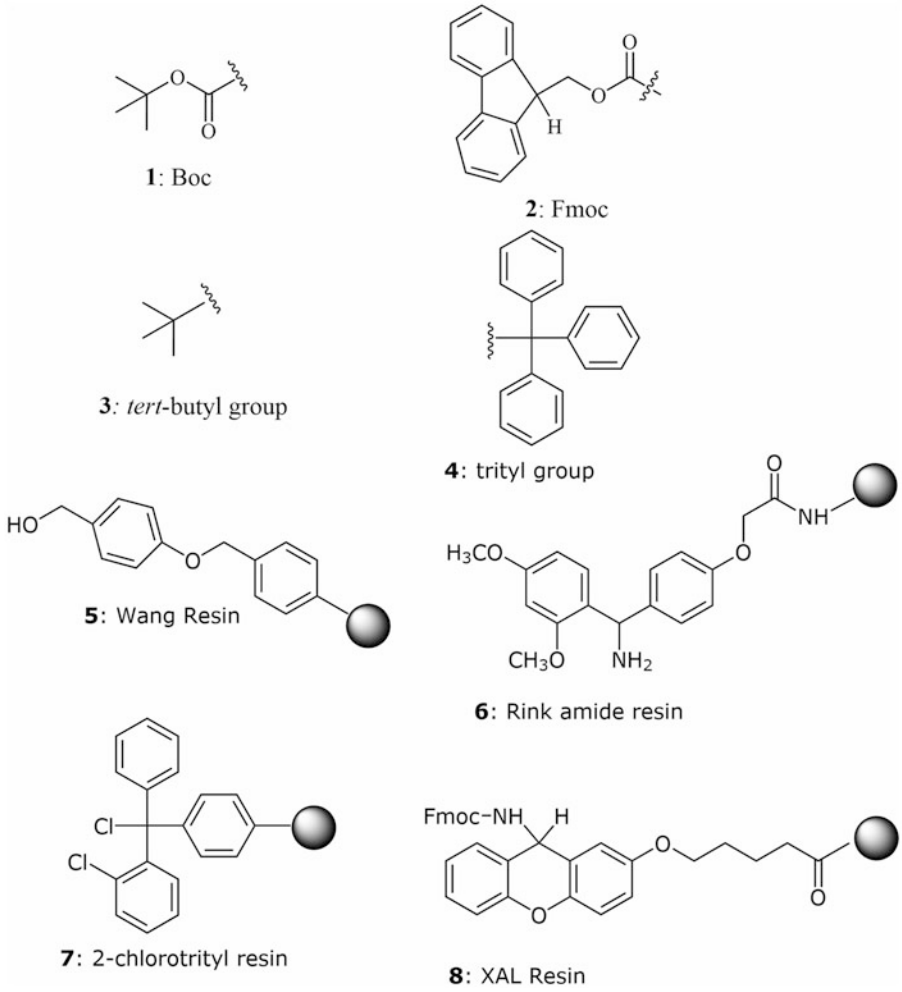

Fig. 1 α-Amino-protecting groups and commonly used linker-functionalized resins in Fmoc SPPS

The classical polystyrene resin is a resin cross-linked with 1% of divinylbenzene (DVB), which swells well in DMF and DCM but not water. However, this type of resin is not well suited for Fmoc SPPS of longer peptides.

The most widely used resin in Fmoc SPPS is the TentaGel® resin developed by Rapp and coworkers [15]. TentaGel® resins are grafted copolymers consisting of a low cross-linked polystyrene matrix on which approximately 50–70% PEG (w/w) is grafted. The PEG-graft copolymer swells in almost all relevant solvent systems, including H_2O. The end of the PEG chain is functionalized with either a hydroxyl group or an amino group. A number of these resin are commercially available.

ChemMatrix® is a resin developed by Albericio and coworkers, which is made exclusively from PEG [16]. The authors demonstrated that the highly complex β-amyloid (1–42) peptide could be

synthesized in a crude purity of 91% using this resin. Finally, Meldal developed PEGA, which is a beaded polyethylene glycol dimethylacrylamide copolymer resin [17] that allows enzymes to penetrate [18] for on-resin assays.

1.2 Linkers

A number of different linkers are available, and most of them release the peptide as a peptide acid or peptide amide when treated by with TFA. For a review of linkers, *see* [19] and linkers for protected peptides [20]. The most popular peptide acid and amide linkers are the Wang (**5**) [21] and Rink (**6**) [22], respectively.

The 2-chloro trityl linker (**7**), which most often comes attached to a polystyrene resin, is well suited for the synthesis of peptide acids [23]. Furthermore, protected peptide acids may be cleaved from the resin using 1% TFA or 10–20% acetic acid, but Trt-groups are not very stable in these conditions. This strategy is treated in other aspects of peptide synthesis. For protected peptide amides, the Xanthenylamide (XAL) linker (**8**) may be used [24].

It should be noticed that in common practice resins are often identified by their linker. For example, the expression "Wang resin" refers to a polystyrene-based resin functionalized with the Wang linker. Other linkers are available, which are cleaved by hydroxide ions [25], fluoride ions [26], 2% hydrazine [27], or photolysis [28]. Finally, the Backbone Amide Linker (BAL) should be mentioned. In this approach, the growing peptide is anchored through a backbone nitrogen, allowing the synthesis of peptide acids and of a number of modifications at the C-terminus [29]. The linker determines the way in which the first residue is anchored to the solid support: for the Rink Amide linker (RAM), often used in combination with TentaGel® resins, this operation is straightforward as it is performed in the same way as a standard amino acid coupling; for the Wang and the 2-chlotrityl linkers, another protocol has to be followed, which ultimately makes it more convenient to buy a preloaded resin bearing the desired residue.

Manufacturers usually indicate a loading value (mmol/g) for their resins, which refers to the amount of functional groups (mmol) per gram of resin. This value is used to calculate the amount of reagents to use for each coupling. Low-loading resins offer a loading $\leq$0.25 mmol/g, while high-loading resins offer a functionalization $\geq$0.9 mmol/g. The latter are more cost-effective and ensure a better ratio between the amount of resin and the coupling solution, but they also favor peptide aggregation and other undesired intermolecular reactions. A loading extent of 0.4–0.7 mmol/g is very versatile and suitable to most applications. In case no loading has been indicated, it is possible to determine it spectrophotometrically after the first Fmoc-deprotection step [13].

Bead size is also often indicated either as particle diameter (μm) or mesh number. Most commercial resins are labeled either as 100–200 mesh (particle size = 74–149 μm) or 200–400 mesh

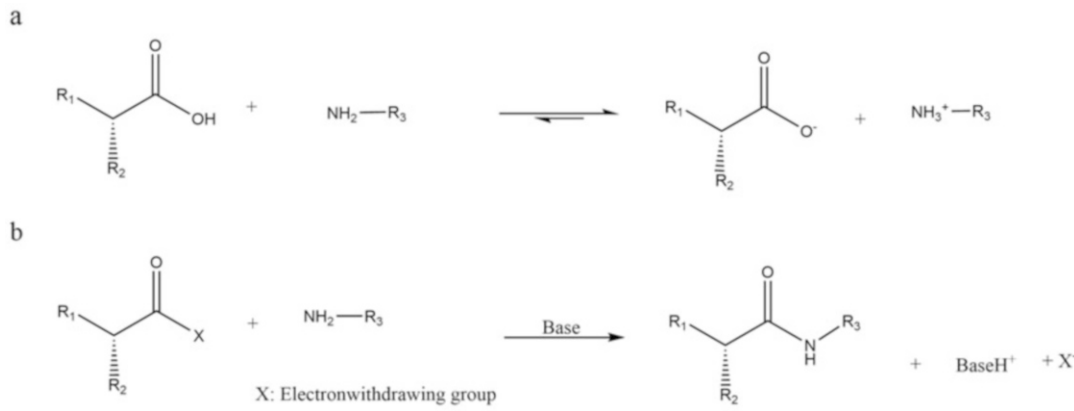

Fig. 2 Proton transfer versus activation of amino acids

(particle size = 37–74 μm). In our lab, we prefer to use the 100–200 mesh size as bigger beads have a lower tendency to aggregate; this phenomenon can create pockets of resin that may be difficult to reach during the wash and deprotection procedures, leading to less pure products.

1.3 Peptide Bond Formation

Peptide synthesis is based on the formation of an amide (peptide) bond in a controlled, quantitative, and sequential fashion. If a carboxylic acid and an amine are mixed in solution, their acidic/basic properties predominate and only a proton transfer occurs (Fig. 2a). For this reason, it is necessary to activate the carboxylic acid with a coupling reagent—and often, an auxiliary nucleophile, too (Fig. 2b). Most of them result in a benzotriazol ester (**9**, Fig. 3) that reacts with the growing peptide chain on the resin, providing a nonacidic, efficient leaving group that ultimately allows the formation of the amide bond. This strategy typically results in a >99% coupling efficiency.

Since the shelf-stability of this type of compounds is very poor, the benzotriazol ester is prepared in situ. A number of coupling reagents exist, and they have been reviewed extensively [30].

Coupling protocols with DIC (*N,N*-diisopropylcarbodiimide) (**10**) and HOBt (1-hydroxybenzotriazole) (**11**) have been used for many years and work well for the synthesis of most short peptides (<15 amino acids) without clusters of branched or hydrophobic amino acids. The advantages of this approach are that the reagents are cheap and no base is used, eliminating the risk of epimerization at the α-carbon. In recent years, coupling protocols involving DIC and Oxyma (ethyl 2-cyano-2-(hydroxyimino)acetate) (**12**) [31] have gained increasingly popularity. More difficult peptides require phosphonium or aminium type of coupling reagents such as PyBOP [32] (1-benzotriazolyloxy-tris-pyrrolidinophosphonium hexafluorophosphate) (**13**), HBTU [33] (N-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-N-methylmethanaminium hexafluorophosphate

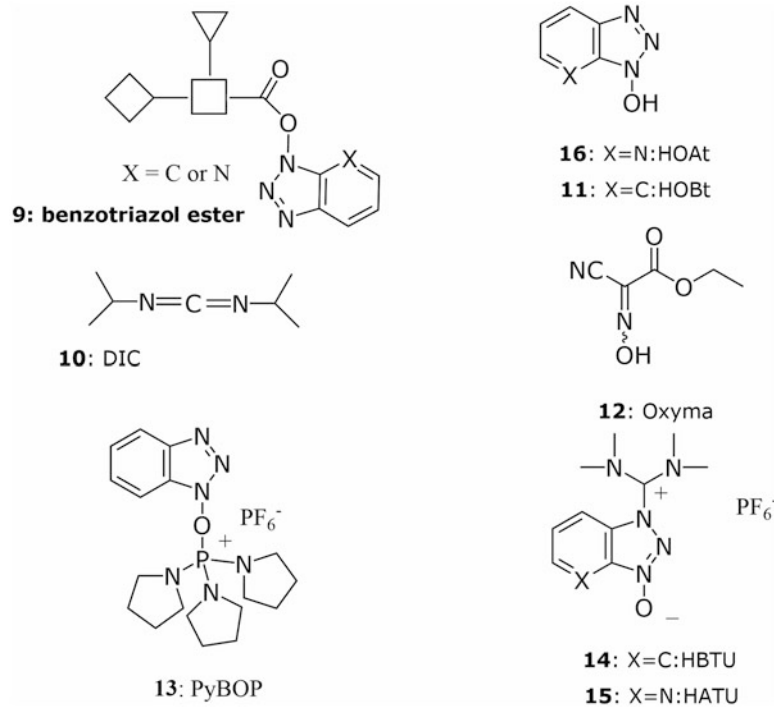

Fig. 3 Active ester generated in situ and coupling reagents

N-oxide)(**14**), or HATU [34] ((N-[(dimethylamino)-1H-1,2,3-triazole[4,5-b]pyridine-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide)) (**15**). These coupling reagents are typically used with HOBt or HOAt (7-aza-1-hydroxybenzotriazole) (**16**) and two equivalents (relative to the coupling reagent) of a tertiary amine such as diisopropylethylamine (DIPEA). Generally, HOAt is a more effective additive than HOBt since it contains a nitrogen atom in the aromatic ring [34]. The coupling reaction is carried out in DMF or the less toxic NMP. The former is preferred in most labs since Fmoc-protected amino acids and triazole-based reagents are more soluble in this solvent.

In our lab, we prefer to use three equivalents of HATU and HOAt and six equivalents of DIPEA relative to the resin loading (mmol/g resin) in DMF for 1.5 h. For couplings after sterically hindered amino acids, such as Arg, Ile, Val, and Leu, we do a 1 h recoupling.

Following synthesis, the peptide resin is then washed thoroughly with DMF and ethanol and lyophilized. This step is important since residual DMF may add to the *N*-terminus of your peptide. Next, the peptide is cleaved from the resin concurrently with the protecting groups.

1.4 Protecting Groups

When the carboxylic acid is activated toward substitution, it can react with a variety of nucleophile species. Besides the terminal α-amino group of the growing chain, nucleophiles can be present in the side chain of other residues in the sequence. From a synthetic perspective, this would lead to an unacceptable amount of impurities. Hence, there is a need for protecting groups.

The acid-labile protecting group tert-Bu is used to protect the side-chain functional groups of Ser, Thr, Tyr, Glu, and Asp [35]. The trityl (Trt) group [36] is employed for Cys, Asn, Gln, and His. For Arg, 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) [37] is the most commonly used. Finally, the Boc group is preferred for Lys and Trp [38]. A number of protecting groups are also available for special purposes as described in the section on other aspects of SPPS (Table 1).

It is usually straightforward to remove the acid-labile protecting groups while cleaving the peptide from the solid support using a cocktail based on ~95% TFA for 2 h. However, if a fully protected peptide is desired, particular attention has to be paid in order to ensure that the protecting groups can survive the cleavage conditions. Particularly, labile groups like Trt are never stable in acidic conditions, even when no TFA is used.

1.5 Cleavage Cocktails

An alphabet soup of cleavage cocktails is available, which all contain TFA as the main component and scavengers that react with carbocations generated during cleavage. In our lab, we use Reagent B´ TFA/H_2O/Triisopropylsilane (95:2.5:2.5) (v/v), which works well for most peptides. We recommend that you do a test cleavage using approximately 5 mg of resin before choosing your cleavage cocktail.

Other popular cleavage cocktail include the following:

Reagent K: TFA/thioanisole/H_2O/phenol/EDT (82.5:5:5:5:2.5) [39].

Reagent L: TFA/TIS/dithiothreitol/H_2O (88:2:5:5) [40].

Table 1
Characteristics of protecting groups for solid-phase peptide synthesis

Protecting group	Resistant to	Labile to	Removed with
Fmoc	Acids	Bases	10–20% Piperidine, ≥10′
tBu	Bases	Acids	≥50% TFA in DCM, 30′
Boc	Bases	Acids	≥50% TFA in DCM, 30′
Trt	Bases	Acids	≥2% TFA, 30′
Pbf	Bases	Acids	~95% TFA, 2 h

Reagent R: TFA/thioanisole/ethandithiol/anisole (90:5:3:2) [41].

Reagent B: TFA/phenol/H_2O/triisopropylsilane (88:5:5:2) [42].

For protected peptides: acetic acid/TFE/dichloromethane (20:20:60) [43].

Following cleavage for 2 h, the solution is collected, and TFA is evaporated by a stream of nitrogen down to a few hundred microliters. The peptide is precipitated in cold ether and centrifuged; the supernatant is then discarded, and the washing procedure is repeated two times washed three times. Short and/or very lipophilic peptides can be considerably soluble in diethyl ether; in such case, they can be precipitated from fully apolar solvents such as hexane or heptane. For a successful wash, it is important to obtain a fine peptide suspension; a short immersion in an ultrasound bath can help disintegrating undesired peptide aggregates. After drying, the peptide is dissolved in 10% acetic acid (we actually use 10–20% MeCN and 0.1% TFA; solutions of acetic acid in water tend to defrost quickly in the freeze dryer; for very insoluble peptides, 100% AcOH can be used—after considering compatibility with possible protecting groups) and lyophilized to give a fluffy white powder.

1.6 Analysis

Following synthesis, the product should be analyzed by LC-MS or analytical reverse-phase HPLC and MALDI-time-of-flight mass spectrometry. Analytical HPLC and preparative HPLC are most often done on a C-18 column using 0.1% aqueous TFA as buffer A and CH_3CN/H_2O (9:1) as buffer B. For MALDI-TOF-MS, α-cyano-4-hydroxycinnamic acid is the most popular matrix. Alternatively, the HPLC UV and mass spectra may be generated by a liquid chromatography-MS (LC-MS) instrument.

1.7 Other Aspects of SPPS

1.7.1 Modifications

A number of peptide modifications are possible (Fig. 4) [44 review]. Some of the modifications include (I) modification of N-terminus and Lys side-chain: acetylation [45], lipidation [46], pegylation [47], fluorescent probe [48] radionuclei radiolabeled peptides for cancer diagnosis and treatment, for example, Ga^{68} [49], biotinylation [50], and *N*-locked peptides [51]; (II) cyclization: C->N termini cyclization [52], Asp->Lys lactamization [53], side chain to side chain [54], disulfide bond formation [55], macrocyclization [56], (III) amide [41], aldehyde [57], ester [58], thioester [59], peptide hydrazine [60], lipidation [61], or *C*-locked modification of the C-terminus [62]; (IV) backbone modifications: aza [63], retroinverso [64], endothiopeptides [65], depsipeptides [66], *N*-methylated [67], and peptoid residues for proteolytic stability [68]; introduction of non-proteinogenic amino acids such as D-amino acids [69], citrulline for studies of autoimmune diseases [70], photoactivable amino acids for peptide-

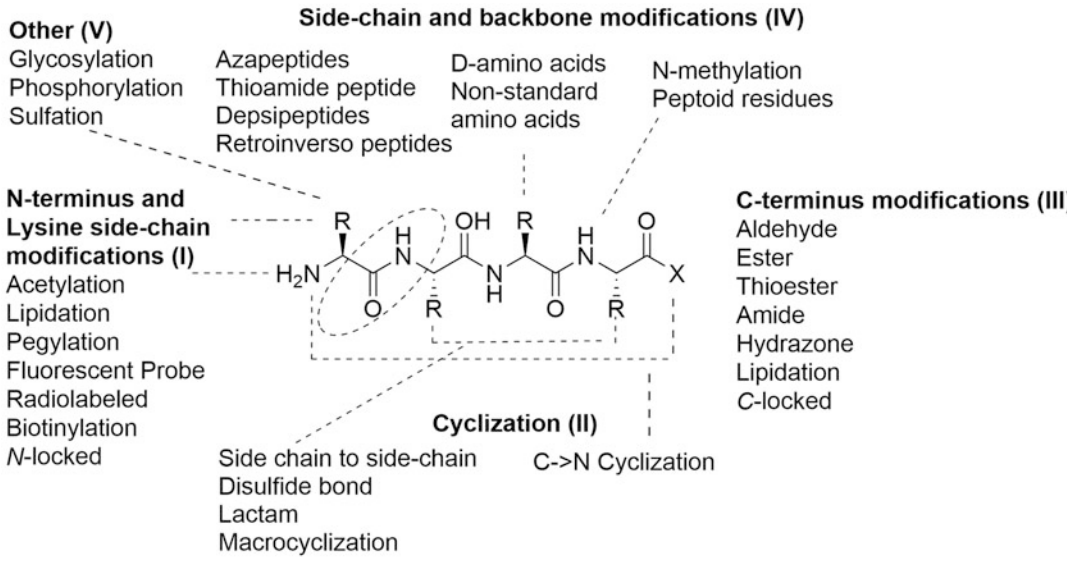

Fig. 4 Peptide modifications

protein conjugation [71]; and other: (V) glycosylation [72], phosphorylation [73], or sulfation [74] of serine, threonine, or tyrosine, branched lysine peptide constructs for antibody generation [75]. For these modifications, special protecting groups are needed.

1.7.2 Special Protecting Groups

Lysine may be protected with a number protecting groups (PGs) that can be cleaved in the presence of other PGs, while the peptide is still attached to the resin. This may be useful for selective introduction of modifications to the ε-amino group, for example, biotin. The PGs include ivDde (2% hydrazine) [76]; Mtt and Mmt (more acid-sensitive analogues of Trt, removed with ≤1.8% TFA) [77]; and Aloc (Pd + nucleophile) [78] or photolysis [79]. Similarly, C^{α}, Asp, and Glu may be protected with allyl ($PhSiH_3$ + $Pd(PPh_3)_4$) [80] or Dmab (2% hydrazine) [81] for the synthesis of C-terminal modified peptides. Strategies involving unusual protecting groups and anchoring of the side chain of Asp and Glu [82], Arg [83], Lys [84], or Cys [85] to the resin have been described. The latter is especially useful since Cys attached to the resin via an ester bond, but not amide bond, is prone to epimerization [86].

The acetamido group (Acm) [87] is often used for Cys when synthesizing peptides with two or more disulfide bonds. The Acm group is not cleaved by TFA but requires I_2 [88] or $Tl(Tfa)_3$ [89] for cleavage. Furthermore, the disulfide bonds can be formed on resin or in solution following cleavage [90].

For an excellent review on amino acid-protecting groups, *see* Isidro-Llobet et al. [91].

1.7.3 Microwave Heating

Some peptides may form β−sheet type structures during synthesis or involve many difficult couplings with β-branched amino acids. For such sequences, microwave heating may lead to significant reductions in synthesis times and an increase in the crude peptide purity. However, conditions often need to be optimized for peptides containing Asp, Cys, and His. This is also true for phosphopeptides, glycopeptides, and N-methylated peptides since epimerization, aspartimide formation, and β-elimination may occur. For an excellent review on microwave heating in SPPS, *see* [92].

In the Fmoc strategy, the Fmoc group is cleaved by 20% piperidine in DMF. However, the Fmoc group may also be cleaved by the non-nucleophilic reagent DBU (1,8 diazobicyclo(5.4.0)-undec-7-ene) [93] for phosphorylated or glycosylated residues, which are prone to β-elimination. Typically, a 2% solution of DBU in DMF is used.

1.7.4 Synthesis of Long Peptides and Proteins

Synthesis of long peptides and proteins is most commonly carried out using segment condensation strategies [20] or native chemical ligation [94].

In the segment strategy, protected peptide fragments are synthesized on a 2-chlorotrityl resin, cleaved by 1% TFA, purified, and coupled to another resin-bound peptide. The HIV fusion inhibitor Fuzeon is synthesized using this strategy [95].

Native chemical ligation was introduced by Dawson and coworkers [94]. This strategy involves chemoselective reaction of two unprotected peptide segments, one having a thioester at the C-terminal end and the other a Cys residue at the N-terminus. Reaction gives an initial thioester-linked species. Spontaneous rearrangement gives a full-length product with a native peptide bond at the ligation site. Native chemical ligation has been used to make hundreds of proteins ranging in size up to more than 200 amino acids. For a recent review, *see* [96]. Since thioesters are base labile, they are more difficult to synthesize by Fmoc chemistry than Boc chemistry [97].

1.7.5 Side Reactions in SPPS

Many side reactions in Fmoc SPPS have been described, including racemization (epimerization) [98], diketopiperazine [99], aspartimide [100], and lactam formation [101]. However, these are not a problem as long as a standard protocol is followed, such as the one described in Subheading 2. For a review of side reactions in SPPS, *see* Yang [102].

1.7.6 Greening Solid-Phase Peptide Synthesis

In recent years, much attention has been directed toward identifying more sustainable solvents and reagents in SPPS, since most of them are classified as environmentally problematic substances by

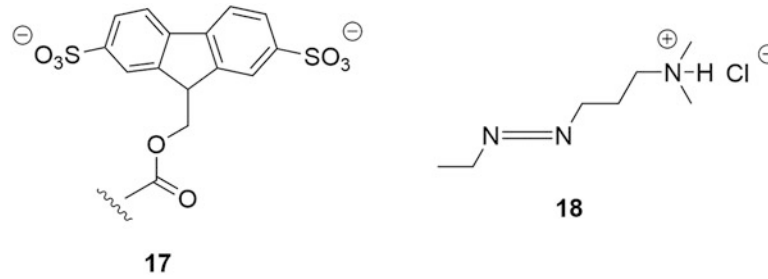

Fig. 5 Water-soluble coupling reagent Smoc-protecting group **17** and EDC **18**

the European Chemicals Agency (ECHA) under the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) regulation [103].

One of the most promising approaches is solid-phase peptide synthesis in aqueous media (ASPPS), in which conventional SPPS has been modified to the presence of water, recently reviewed by Jaradat [104]. For example, Knauer and co-workers synthesized a number of small peptides in good yield and purity using ChemMatrix H-Rink amide resin and water-compatible Smoc-(N^{α}-2,7-disulfo-9-fluorenylmethoxycarbonyl) amino acids (Fig. 5) and EDC-HCl/Oxyma EDC = 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in the presence of $NaHCO_3$ as coupling reagents [105].

Another approach has been to explore different binary solvent mixtures, which show comparable polarity and viscosity properties to DMF but are less toxic. Researchers from Novo-Nordisk and Bachem synthesized the peptide therapeutic Bivalirudin (H-fPRPGGGGNGDFEEIPEEYL-OH) in different binary solvents. The authors found that coupling reactions went well in N-formyl morpholine/1,3-dioxolane and Fmoc deprotection is efficient in DMSO in combination with 1,3-dioxolane, 2-Me-THF, or ethyl acetate [106].

1.8 Concluding Remarks

Solid-phase peptide synthesis is a mature methodology that allows most peptides up to approximately 50 amino acids to be synthesized in good yield and purity. Furthermore, non-proteinogenic amino acids or other modifications may be incorporated, including cyclization, glycosylation, phosphorylation, fluorescent labeling, biotinylation, or disulfide bond formation. However, it is important to realize that certain peptide sequences are difficult to prepare even for experienced peptide chemists. In these cases, careful attention should be paid to the peptide sequence, protecting groups, coupling reagents, heating protocol, and cleavage conditions as outlined in this chapter and the literature cited.

2 Materials and Preparations

General procedure for peptide synthesis in disposable syringes.

2.1 Choosing the Resin

This mostly depends on two variables:

(a) Is the goal to obtain a peptide acid or a peptide amide?

(b) Should the side-chain protecting groups be removed when the peptide is released from the solid support?

If a free peptide is desired (a), it is usual to employ a resin that releases the peptide after treatment with a 95% TFA cocktail. A preloaded Wang resin would be the choice for peptide acids, a non-preloaded TentaGel/HypoGel resin functionalized with a RAM linker that is very useful for the synthesis of peptide amides. If the peptide should be obtained without removing the side-chain protecting groups (b), a 2-chlorotrityl or Sieber resin are good choices for peptide acids and amides, respectively.

2.2 Synthesis Scale

The amount of resin to be used depends on how much peptide is desired. This can be calculated using the following formula:

$$\text{g of Resin} = \frac{\text{mmol of Peptide (desired)}}{\text{Resin loading}}$$

As this is a purely theoretical value (i.e., not factoring impurities and other losses), it is convenient to multiply it by 1.5 or 2.

2.3 Reactors

The reactor is the vessel where reactions take place. For peptide synthesis, it is important that the reactor has one upper and one lower opening and one filter (usually made of PTFE, polytetrafluoroethylene) to allow solvents to flow while retaining the resin (*see* **Note 2**). This protocol is based on the use of 5-mL syringes equipped with a PTFE filter as reactors (Fa. Gerhardt, Wolfhagen, Germany) for 50–200 mg of resin and assumes the availability of a suction device (*see* **Note 3**). The suction device is however not strictly necessary. The use of pipette tips of 200-μL capacity is advisable, as these provide a disposable interface between the syringe and the solvents, reagents, and suction plate. It is often necessary to trim the wide end of such tips to achieve a good fit on the syringes.

2.4 HOAt and HATU Solutions

Three equivalents of HOAt and HATU are needed for each coupling, based on the synthesis scale calculated before (*see* **Note 4**).

It is convenient to calculate the total number of couplings and prepare a 0.4-M stock solution of both in dimethylformamide (DMF):

$$\text{mg HOAt} = 135.12 * 3 * [\text{synthesis scale (mmol)}] * [\text{number of couplings}]$$

$$\text{mg HATU} = 380.23 * 3 * [\text{synthesis scale (mmol)}] * [\text{number of couplings}]$$

The respective total amounts are then to be dissolved in DMF up to a volume calculated as follows:

$$\text{V Solution (mL)} = \frac{3 * [\text{synthesis scale (mmol)}] * [\text{number of couplings}]}{0.4\ \text{M}}$$

The HATU solution should be divided in as many individual tubes as the total number of couplings. The HOAt solution shall be used to dissolve the amino acid samples (*see* next point). It is advisable to prepare a standard template using Excel to perform these calculations quickly and automatically.

2.5 Amino Acid Solutions and N,N-diisopropylethylamine (DIEA)

In our lab, we prepare 0.4-M solutions of our amino acids by dissolving them in an appropriate volume of the HOAt stock solution prepared in Subheading 2.4. One individual sample tube containing three equivalents of amino acid and HOAt should be prepared for each coupling.

The required amount of amino acid for each coupling should be weighed out in an individual tube.

$$\text{mg Amino acid} = \text{Molecular weight of Amino acid} * 3 * [\text{synthesis scale (mmol)}]$$

Equally divide the HOAt stock solution among the amino acid samples.

By doing so, a tube containing the amino acid and HOAt solution and a tube containing the HATU solution have been prepared for each coupling. For overnight or medium-term storage, place the tubes in a −20 °C freezer. Let them warm up again to room temperature for at least 20 min before use.

During coupling of each amino acid, DIEA is added to the solution. The amount for each coupling is calculated as follows:

$$\mu\text{L DIEA} = [129.24\ \text{g/mol} * 6 * [\text{synthesis scale (mmol)}]]/0.742\ \text{g/mL}$$

2.6 Piperidine Solution

This solution is needed to remove the Fmoc-group between one coupling and the following. To prepare a 20% v/v solution of piperidine in DMF, simply measure both volumes (e.g., 40 and 160 mL, respectively) separately in a graduated cylinder and mix them together in the final container. The final container should be a brown bottle with a screw cap.

3 Methods

3.1 Initiating the Synthesis

1. Weigh out the resin directly inside the reactor (*see* **Note 5**), reinsert the piston, and push it all the way down.
2. Transfer 3 mL of DMF into a beaker and draw it in the syringe.
3. Close the bottom end with a pressure cap and let it swell in DMF for at least 30 min (preferably overnight).

3.2 Quick Wash

1. Remove the piston and place the syringe on the suction plate.
2. Wash down with DMF any resin residue on the piston head.
3. Wash three times with DMF.

3.3 Amino Acid Coupling (See Note 6)

1. Add the HATU solution to the HOAt/amino acid solution.
2. Add DIEA (Sigma-Aldrich) and shake briefly: the solution should turn to intense yellow.
3. Draw the coupling solution into the syringe avoiding to introduce too much air.
4. Cover the syringe with tin foil and leave on a shaker for 1:30 h, and then discharge the coupling solution.
5. If a double coupling is desired, do a *quick wash* (Subheading 3.2) and then repeat Subheading 3.3, **steps 1–4**.

3.4 Full Wash

1. Remove the piston and place the syringe on a suction plate.
2. Wash down with DMF any resin residue on the piston head.
3. Wash with DMF (three times), DCM (Sigma-Aldrich) (three times), and DMF again (five times); fill the reactor all the way up at least a couple of times in order to wash the walls.

3.5 Fmoc-Group Removal

1. Transfer 2–3 mL of piperidine solution into the syringe and leave standing.
2. After 4 min, drain the solution and do a *quick wash* (Subheading 3.2).
3. Repeat Subheading 3.5, **steps 1** and **2** twice (thus a total of three deprotection cycles; for peptides longer than ten residues, extend the time of the last two deprotection cycles to 7 min each).
4. Put the piston back in place and change the syringe tip.

3.6 Full Wash (as in Subheading 3.4)

3.7 Reiteration

1. Subheadings 3.3, 3.4, 3.5, and 3.6 are repeated for each subsequent amino acid until the sequence is completed.

3.8 Final Fmoc-Group Removal

1. Do as in Subheading 3.5, and then wash the resin five times with ethanol (rinse the whole reactor and the piston, too).
2. Insert the piston and push it half-way down, and then discard the tip and leave the reactor in a lyophilizer overnight or at least for 2 h.

3.9 Cleavage

1. Freshly prepare at least 6 mL of TFA/H_2O/Triisopropylsilane (95:2.5:2.5) (v/v) cleavage cocktail (*see* **Note 7**) for each peptide.
2. Push the piston all the way down.
3. Transfer 3.5 mL of cleavage cocktail in a beaker and draw it into the syringe.
4. Carefully cap the lower end of the syringe with a pressure cap.
5. Place on a shaker for at least 2 h.
6. Using the piston, push the cleavage solution into a 5-mL cryotube without touching the resin with the piston head.
7. Remove the piston.
8. Wash the resin twice pouring ~1 mL of cleavage cocktail from above collecting the eluate in the cryotube.
9. Push the piston all the way down to collect the last drops of solution into the cryotube.
10. Evaporate the solution with a gentle stream on N_2.
11. When 300 μL or less are left, add 4 mL of cold (−20 °C) diethyl ether (*see* **Note 8**). Put the cap on and shake gently.
12. Centrifuge at 2000 rpm for 6 min.
13. Using a pipette, carefully remove and discard the supernatant.
14. Resuspend the solid into another 3–4 mL of cold ether.
15. Repeat Subheadings 3.9, **steps 12–14** for a total of three washes. The last centrifuge run should be at 4000 rpm.
16. Leave the cryotube standing open overnight to let the residual ether evaporate.
17. Dissolve the crude product in 90% water and 10% acetonitrile and freeze-dry to obtain fluffy white crystals (*see* **Note 9**).

4 Notes

1. This chapter is an updated version of P. R. Hansen and A. Oddo (2015) Fmoc Solid-Phase Peptide Synthesis In: Methods in Molecular Biology: Peptide Antibodies, edited by G. Houen pp 33–50.

2. Standard polypropylene 5-mL syringes can be fitted with a PTFE filter and used for this purpose. In this case, pressure caps from Sigma-Aldrich (Cat# Z120979) to close the narrow end are useful. The syringe piston head must not have a rubber O-ring, as this could be unstable in the reaction and cleavage conditions. It is also possible to buy 5-mL polypropylene reactors equipped with a PTFE filter, a bottom and a top cap from Thermo Scientific (Cat# 29922). The main difference between the two approaches is that, in the case of syringes, the bottom opening serves both as inlet and outlet and the piston can be used to drive the flow; with other reactors, the inlet is the top opening, and the outlet is the bottom one, and a suction device is needed to drive the flow. If no suction device is available, syringes must be used: washing solvents and the piperidine solution must be transferred in a beaker and then drawn into (and ejected from) the syringe using the piston.
3. This is basically an in-house made Teflon® plate featuring five holes with a diameter of ~4 mm. This size allows to accommodate syringes, 200-μL pipette tips, and the Thermo Scientific reactors. The plate and the vacuum pump are connected to a 2.5-L glass reservoir using Omnifit® fittings (Fig. 6). We drill a hole in a cork stopper to let the drain pipe pass through, and then we insert it in a rubber ring adapter (the sort used for filtration) to ensure a tight fit with the reservoir neck. A three-way flow switch is needed to prevent the piperidine solution from being drained from the reactors during deprotection.
4. It is usual to acylate one amino-functionality at a time, but when acylations are taking place at multiple positions, this should be accounted for. For example, when lysine-based

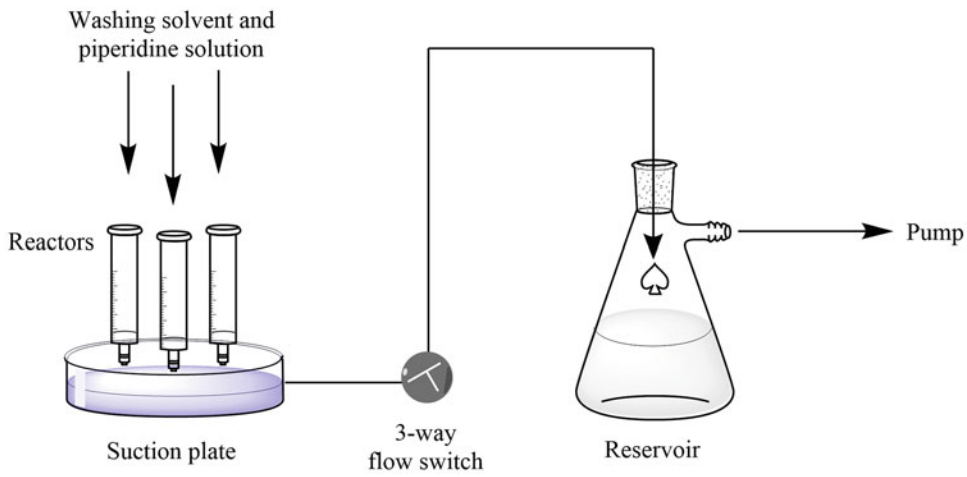

Fig. 6 Peptide synthesis setup

branched peptides are synthesized by coupling on both amino groups simultaneously, this has the same effect as doubling the resin loading as far stoichiometry is concerned.

5. When synthesizing multiple peptides at once, it is very convenient to label the reactors with tape of different colors.
6. If the amino functionality on the resin (preloaded or not) is Fmoc-protected, **steps 5** and **6** need to be completed before the first coupling can take place. Please note that, except for 2-chlorotrityl resins, most other resins (preloaded or not) are Fmoc-protected.
7. Cleavage cocktails based on TFA and other caustic substances are highly corrosive and need to be handled with care. Remember that acids are always added to water and never the other way around. Always use glass containers and glass Pasteur pipettes to prepare and transfer the cocktail. These cocktails cannot be stored and have to be freshly prepared before use. Triisopropylsilane dissolves slowly in TFA and persists as oily spots on the solvent surface; make sure that dissolution is complete before exposing the resin to the cocktail.
8. Short ($\leq$6 AA) and lipophilic peptides can be considerably soluble in diethyl ether; in this case, use n-hexane instead. If in doubt, the supernatant can be saved and kept at −20 °C overnight. In the morning, check for the presence of precipitate.
9. Note that the peptide will be obtained as a TFA-salt.

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Chapter 4

Peptide-Carrier Conjugation

Paul Robert Hansen

Abstract

To produce antibodies against synthetic peptides, it is necessary to couple them to a protein carrier. This chapter provides a nonspecialist overview of peptide-carrier conjugation. Furthermore, a protocol for coupling cysteine-containing peptides to bovine serum albumin is outlined.

Key words Peptide-carrier conjugation, Bovine serum albumin, m-maleimidobenzoyl-N-hydroxy-succinimidyl ester (MBS)

1 Introduction

In order to produce antibodies against synthetic peptides with a molecular weight less than 1500 Da, it is necessary to couple them to a protein carrier [1]. This is because both a T-cell epitope and B-cell epitope are required for a strong immune response and small synthetic peptides cannot provide both. For excellent reviews on peptide-carrier conjugation, see Muller [2] and Hermanson [3].

The choice of carrier molecule is determined by the number of functional groups available for conjugation, immunogenicity of the carrier protein, cost, and most importantly whether the produced peptide-protein conjugate is water soluble. Commonly used carriers are [3], BSA (MW 67,000), KLH (MW 4.5×10^5 to 1.3×10^7) thyroglobulin (MW 660,000), and ovalbumin (MW 43,000).

It is not well established how many peptide molecules per carrier protein is necessary to generate a good antibody response. However, Hodges et al. have shown that two peptides per BSA molecule are enough to generate antibodies against peptides related to herpex simplex virus [4]. The peptide-carrier conjugation ratio may be determined by amino acid analysis [5], spectrophotometry [6], or mass spectrometry [7].

The most widely used strategy for preparation of peptide-carrier conjugates is solution conjugation.

Gunnar Houen (ed.), *Peptide Antibodies: Methods and Protocols*, Methods in Molecular Biology, vol. 2821,
https://doi.org/10.1007/978-1-0716-3914-6_4,

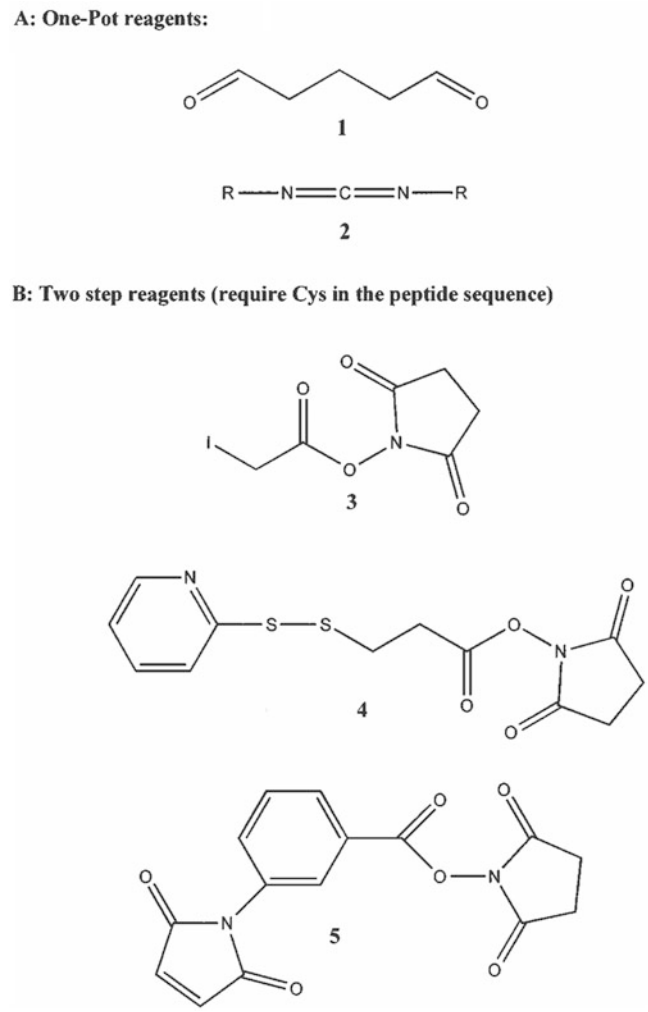

Fig. 1 Peptide-protein coupling reagents. (**a**) One-pot reagents. (**b**) Two-step reagents (require Cys in the peptide sequence)

The solution strategy may be divided into an (i) one-pot or (ii) two-step conjugation method. The majority of them reacts with the ε-amino group of lysine and/or the thiol group of cysteine (Figs. 1, 2, and 3).

In the one-pot method, the cross-linking reagent and peptide are added to the carrier protein followed by dialysis to remove unwanted compounds.

In the two-step method, two separate reactions are used to form the covalent bond between carrier protein and peptide. In the first reaction, the cross-linking reagent is attached to the carrier protein via an amide bond. Gel filtration or dialysis are often used to remove excess of the coupling reagent, followed by conjugation of the peptide.

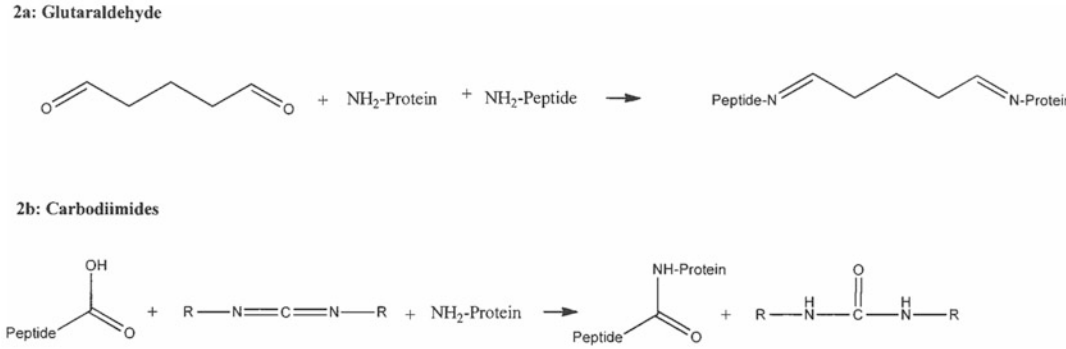

Fig. 2 One-pot peptide-protein coupling reagents

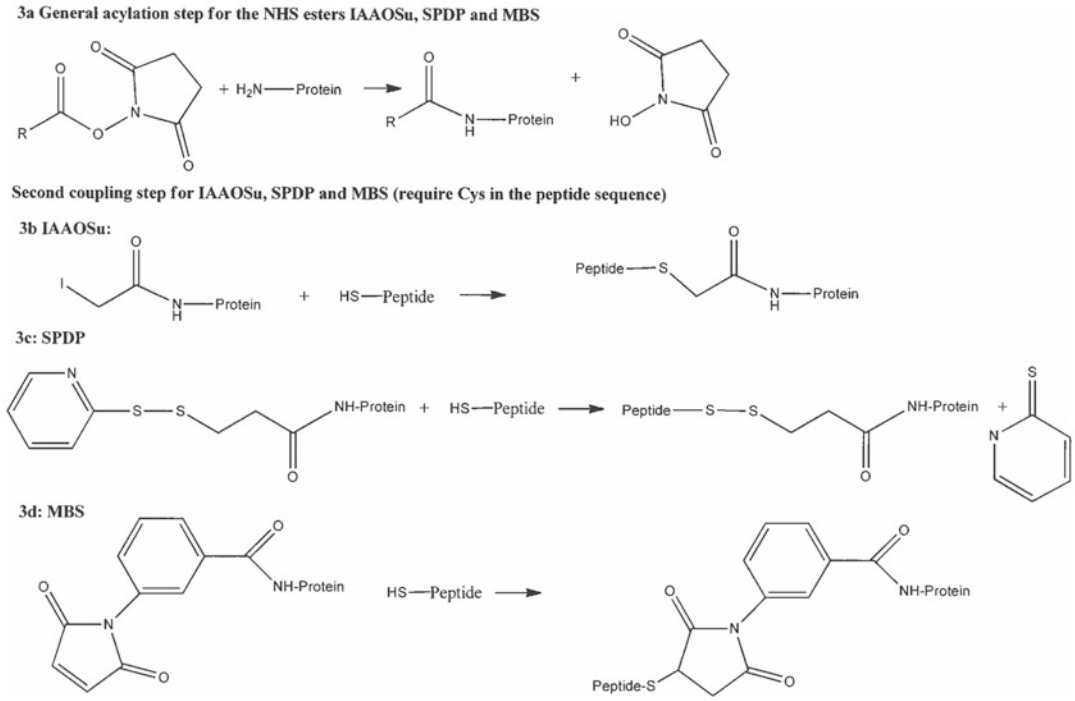

Fig. 3 Peptide-protein coupling reagents

1.1 One-Pot Method

One of the oldest one-step cross-linking reagents is glutaraldehyde **1**, which reacts with amino groups on the carrier protein and peptide to form Schiff bases (Fig. 2a). However, the reagent is very nonspecific and known also to form peptide and protein aggregates [8].

The most popular one-step reagents for peptide-protein conjugation are the carbodiimides **2**, which was first used to generate antibodies against bradykinin and angiotensin [9]. As shown in

Fig. 2b, this class of reagents activates a C-terminal carboxylic acid group in the peptide or protein, which in turn reacts with an amino group in the protein or peptide to form an amide bond.

1.2 Two-Step Method

The most widely used two-step reagents are iodoacetic acid *N*-hydroxysuccinimide ester (IAAOSu) **3** [10], *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) **4** [6], and m-maleimidobenzoyl-*N*-hydroxysuccinimidyl ester (MBS) **5** [11]. These three coupling reagents all require that a Cys residue is present in the peptide sequence. If this is not the case, it may be added to the *N*-terminus during solid-phase synthesis. The described two-step reagents all react in the same way in the initial step (Fig. 3a). First, they form an amide with amino groups on the carrier protein via its NHS-ester. The cysteine residue of the peptide then reacts with either the alkyl iodide (Fig. 3b), the 2-pyridyldisulfide group (Fig. 3c), or m-maleimidobenzoyl group (Fig. 3d), resulting in a covalent bond between carrier and peptide.

IAAOSu: Rector et al. introduced iodoacetic acid N-hydroxysuccinimide ester (**3**) to produce well-defined protein-protein conjugates using ovalbumin and IgG as test proteins [10]. Houen and coworkers demonstrated that IAAOSu also works well for peptide-carrier conjugation. The authors conjugated several peptides, including CGHEKEGFMEAEQC and glutathione to ovalbumin, which gave high antibody titers [12].

SPDP: N-succinimidyl 3-(2-pyridyldithio)-propionate **4** was first described by Carlsson and coworkers in 1978. Since pyridine-2-thione is released during the second step of the conjugation reaction, the peptide-protein carrier ratio may be estimated spectrometrically [6].

MBS: m-maleimidobenzoyl-*N*-hydroxysuccinimidyl ester **5** was first reported by Kitagawa and Aikawa who conjugated insulin to β-D-galactosidase [11]. Since then, MBS has been used to conjugate a number of peptides to proteins including peptides of two regions of the surface protein VP1 of foot-and-mouth disease virus to KLH [13]. When using MBS as coupling reagent, the carrier protein is typically dissolved in phosphate-buffered saline (PBS) followed by the addition of MBS in dimethylformamide (DMF). After 1 h, the MBS-protein conjugate is passed through a PD-10 desalting column. The peptide is then dissolved in DMF and mixed with MBS-conjugated carrier protein. Following overnight reaction, 0.1-M bicarbonate is added and the peptide-carrier conjugate lyophilized.

Lateef and coworkers successfully raised antipeptide antibodies using the above described procedure [7]. Two test peptides, EMVAQLRNSSEPAKKC and RNTKGKRKGQGRPSPLAPC, were conjugated to BSA and the products analyzed by MALDI-TOF MS. The authors found that between 1 and 13 peptides were conjugated to BSA. A detailed peptide-protein conjugation protocol using MBS is provided in the Subheading 3.

Most published peptide-protein conjugation procedures work quite well. However, there are pitfalls. In an excellent study by Briand et al., nine peptides, three carrier proteins, and four coupling reagents were used to prepare a number of peptide-protein conjugates [5]. The authors examined some of the problems that may be encountered when peptide-carrier conjugates are prepared for immunochemical assay such as (i) generation of specific antibodies that react with unrelated carrier proteins treated with the same coupling agent, (ii) instability of the conjugate, and (iii) alteration of the antigenic properties of the peptide moiety. Finally, some guidelines as to which control experiments are suitable are given.

1.3 Other Approaches

Multiple antigenic peptides: An alternative to traditional peptide-carrier conjugates is multiple antigenic peptides (MAPs), which were introduced by Tam [14] and have been used extensively in diagnostics and vaccine research [15, 16]. MAPs are a branched lysine core matrix carrying four copies of the antigenic peptide, which is synthesized entirely by solid-phase synthesis. The advantage of this approach is that single chemically defined peptide antigens are produced, which yields good immune responses. The drawback is that MAP constructs containing hydrophobic peptide antigens may be difficult to synthesize and analyze.

2 Materials

1. Beaker 50 mL.
2. Bovine serum albumin (BSA).
3. Cysteine-containing peptide (*see* **Note 1**).
4. Dialysis tubing average flat width 43 mm, molecular weight cutoff 12,000 Da with dialysis tubing closures 50 mm.
5. Dimethylformamide (DMF).
6. Eppendorf tubes (1 and 5 mL).
7. m-maleimidobenzoyl-*N*-hydroxysuccinimidyl ester (MBS) (*see* **Note 2**).
8. Phosphate-buffered saline (0.01 M), PBS, pH 7.4 (*see* **Note 3**).
9. Sodium azide.

3 Methods

3.1 Procedure for Coupling a Peptide to BSA Using MBS [2, 7]

1. Dissolve BSA (5 mg) in 0.5 mL of 0.01-M phosphate buffer (pH 7).
2. Prepare a solution of 10 mg/mL MBS in DMF.

3. Add 100 μL of the MBS solution drop-wise and stir for 30 min.
4. To remove excess of MBS, transfer the conjugation solution to a dialysis tubing and close with dialysis tubing closures.
5. Put the dialysis tubing in a 50-mL beaker with 30 mL of 0.01-M phosphate buffer (pH 7.4) for 2 h in a fridge. Repeat twice and leave the last dialysis overnight.
6. Dissolve the cysteine-containing peptide (5 mg) in 100-μL DMF.
7. Transfer the solution in the dialysis tubing to a 5-mL Eppendorf tube and add the peptide solution.
8. Following overnight reaction at RT, dialyze the conjugation mixture as described in **steps 4** and **5**.
9. Store aliquots in a freezer or in a fridge with 0.02% sodium azide (*see* **Note 4**).

4 Notes

1. The peptide may be produced as described by Hansen and Oddo (Chapter 10) or obtained commercially. A number of companies offer custom peptide synthesis. It is recommended that you order a purity of >90%.
2. MBS conjugation kits are commercially available from several suppliers.
3. To prepare a 0.01-M PBS solution, mix 8.064-g NaCl and 0.201-g KCl in a 1-L volumetric flask and H_2O to the calibration mark.
4. Ready to use PBS with azide buffer is commercially available from several suppliers.

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Chapter 5

Solid Phase Peptide Carrier Conjugation

Gunnar Houen and Dorthe T. Olsen

Abstract

Conjugation to carrier proteins is necessary for peptides to be able to induce antibody formation when injected into animals together with a suitable adjuvant. This is usually performed by conjugation in solution followed by mixing with the adjuvant. Alternatively, the carrier may be adsorbed onto a solid support followed by activation and conjugation with the peptide by solid-phase chemistry. Different reagents can be used for conjugation through peptide functional groups (-SH, -NH2, -COOH), and various carrier proteins may be used depending on the peptides and the intended use of the antibodies. The solid phase may be an ion exchange matrix, from which the conjugate can subsequently be eluted and mixed with adjuvant. Alternatively, the adjuvant aluminum hydroxide may be used as the solid-phase matrix, whereupon the carrier is immobilized and conjugated with peptide. The resulting adjuvant-carrier-peptide complexes may then be used directly for immunization.

Key words Peptide, Carrier, Conjugation, Solid phase, Antibodies

1 Introduction

Peptides of smaller size do not elicit production of antibodies (Abs) due to a lack of T-cell epitopes and an inherently low immunogenicity, and in order to induce specific Abs, they need to be conjugated covalently to a larger carrier protein and to be injected together with a suitable adjuvant [1–3]. Conjugation can be done in solution by one- or two-step procedures with a number of reagents [3–6]. Alternatively, conjugation may be performed by solid-phase chemistry using principles developed for peptide synthesis [7, 8]. Here, convenient procedures for conjugation to different solid phase-immobilized carrier proteins are described (Fig. 1). The solid phase can be an ion exchange matrix, from which the resulting conjugates can be eluted and mixed with adjuvant (Fig. 1a) [8]. Alternatively, the adjuvant aluminum hydroxide can be used as the solid phase, whereupon the carrier is immobilized (Fig. 1b) [7]. In this case, the resulting adjuvant-carrier-

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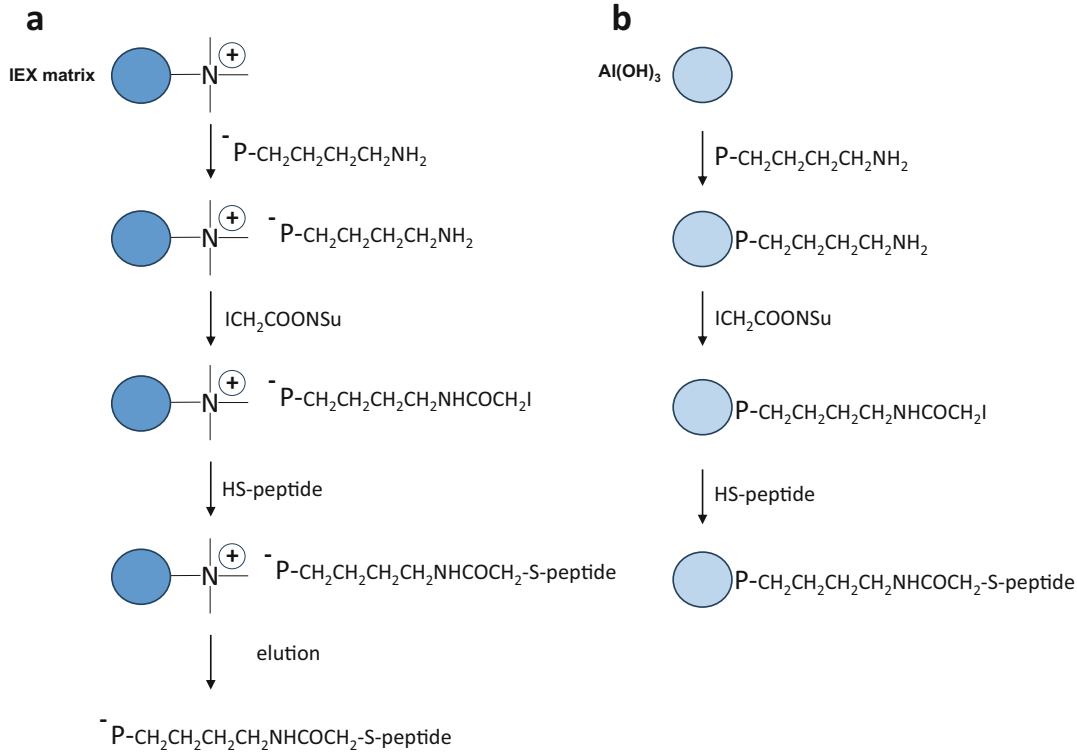

Fig. 1 (**a**) Conjugation to ion exchange matrix (IEX)-adsorbed carrier protein. (**b**) Conjugation to aluminum hydroxide ($Al(OH)_3$) adjuvant-adsorbed carrier protein. Conjugation takes place on lysine side chain amino groups

peptide complexes may be used directly for immunization. The advantages of these methods include improved control of conjugation chemistry and peptide orientation and ease of the different steps involved.

2 Materials

1. Ion exchange matrix (strong quaternary cation exchanger) (*see* **Notes 1** and **2**).
2. Aluminum hydroxide, aqueous suspension, for example, 2% by dry matter (determined as Al_2O_3), pH approximately 8.
3. Carrier proteins (e.g., ovalbumin, bovine serum albumin, keyhole limpet hemocyanin).
4. Sodium phosphate buffer (PB), 10 mM, pH 9.0 (*see* **Note 3**).
5. NaOH, 1-M stock, dilute to 0.1, 0.01, 0.001 M with water.
6. Dimethylformamide (DMF).
7. Mercaptoethanol.

8. Iodoacetic acid *N*-hydroxysuccinimide ester (IAAOSu), for example, synthesized as described by Hampton et al. [9].
9. Synthetic peptide(s) with cysteine added at the N- or C-terminus as appropriate for conjugation (*see* **Note 4**).
10. Amino acid analysis (AAA) apparatus or access to this service, for example, as described by Barkholt and Jensen [10].
11. Chromatographic equipment.
12. End-over-end rotator.
13. Centrifuge.

3 Methods

3.1 Conjugation to Ion Exchange Matrix-Adsorbed Protein

3.1.1 Adsorption of Carrier Protein

1. The required amount of ion exchange matrix (here 10-mL settled matrix) is washed with 10-mM PB, pH 9.0.
2. The carrier (10 mL, 1–2 mg/mL in PB) (e.g., ovalbumin) is mixed with the prewashed ion exchange matrix and incubated with gentle end-over-end agitation for 1 h or overnight at 5 °C. The matrix is settled by centrifugation (2000 g), and the amount of carrier protein bound is estimated by determining the UV absorption of the first supernatant (*see* **Note 5**).
3. The matrix is washed three times with 10-mM PB (centrifugation in between at 2000 g).

3.1.2 Activation

1. The settled matrix with bound carrier is resuspended with 10-mL 10-mM PB.
2. 1-mL iodoacetic acid N-hydroxysuccinimide ester in DMF (10 mg/mL) is added and incubated at 5 °C for 1 h.
3. The activated carrier-matrix is washed three times with 10-mM PB as above.

3.1.3 Conjugation

1. Resuspend the activated matrix in 10-mL 10-mM PB and add the cysteine-containing peptide (*see* **Note 4**) dissolved in 1-mL PB or DMF as required (*see* **Note 6**).
2. Incubate overnight at 5 °C with gentle end-over-end agitation.
3. Wash three times with PB (centrifugation in between at 2000 g). The amount of conjugated peptide may be determined by AAA (*see* **Note 7**).
4. At this stage, the peptide-carrier-matrix may be used directly for immunization with or without added adjuvant. Alternatively, the peptide-carrier complexes may be eluted, characterized, and then used for immunization.

3.1.4 Elution

1. The matrix with peptide-conjugated carrier is packed in a small column and eluted with a gradient of increasing NaCl concentration (0–1 M) in 10-mM PB. The absorbance at 280 nm is recorded. Conjugates elute according to their isoelectric points (pIs), which are determined by the composition and number of conjugated peptides together with the composition of the carrier.
2. The eluted conjugates may be dialyzed or used directly for immunization. The conjugation ratios may be determined by AAA as above.

3.1.5 Mixing with Adjuvant

1. Mix conjugate 1:1 with 2% aluminum hydroxide (recommended) or another adjuvant.

3.1.6 Immunization

1. Mice are injected subcutaneously with 0.1 mL and rabbits with 1 mL, for example, every 2 weeks until a desired titer is obtained (as determined by ELISA or other method) (*see* **Notes 8** and **9**).

3.2 Peptide Conjugation to Aluminum Hydroxide-Adsorbed Carrier Protein

3.2.1 Adsorption of Carrier Protein to Aluminum Hydroxide

1. Ten mL 2% aluminum hydroxide in water is mixed with 10-mg carrier protein (e.g., ovalbumin) in 1-mL water (*see* **Note 10**) and incubated with end-over-end rotation overnight at 5 °C.
2. The adjuvant-carrier matrix is washed three times with 10-mL dilute base (0.001–0.01 M NaOH) (it is important to control the pH, which must remain above 7 at all times).

3.2.2 Activation

1. Add 1-mL iodoacetic acid N-hydroxysuccinimide ester (10 mg/mL) in DMF and incubate overnight at 5 °C with end-over-end agitation.
2. The activated carrier-adjuvant matrix is collected by centrifugation (2000 g).
3. Wash three times with dilute base (0.001–0.01 M NaOH) (centrifugation at 2000 g in between).
4. Resuspend in 10-mL dilute base (0.001–0.01 M NaOH).

3.2.3 Conjugation

1. Add peptide containing a cysteine (*see* **Note 4**) dissolved in 1-mL water, dilute base (0.001–0.01 M NaOH), DMF, or mixtures of these as required (*see* **Note 6**).
2. Incubate overnight at 5 °C with end-over-end agitation. The pH may be checked in between to assure it is high enough for successful conjugation (*see* **Note 11**).
3. Excess of reactive groups may be blocked by addition of 5-μL mercaptoethanol, but this is not mandatory.

4. Wash three times with water or dilute base (0.001–0.01 M NaOH) (*see* **Note 12**).
5. The amount of conjugated peptide may be determined at this stage by AAA (*see* **Note 7**).
6. The adjuvant-carrier-peptide conjugates are now ready for immunization.

3.2.4 Immunization

1. Mice are injected subcutaneously with 0.1 mL and rabbits with 1 mL, for example, every 2 weeks until a desired titer is obtained (as determined by ELISA or other method) (*see* **Notes 8** and **9**).

4 Notes

1. A strong anion exchange matrix (quaternary amine) is chosen for carrier proteins with a low isoelectric point and a strong cation exchange matrix for carrier proteins with a high pI.
2. The nature of the matrix restricts the types of conjugation chemistry that may be used.
3. A 0.1-M stock can be made and diluted afterwards. Make 0.1-M stocks of NaH_2PO_4 and Na_2HPO_4 and mix to obtain the desired pH.
4. The cysteine is added preferably at the N-terminus (for C-terminal peptide(s) or C-terminus (for N-terminal peptide (s)), but any position may in principle be used, for example, naturally occurring cysteine residues.
5. Use Lambert-Beer's equation: $A_{280} = \varepsilon \cdot l \cdot c$.
6. 0.1–1 mg/mL depending on peptide availability and solubility.
7. If the conjugation is successful, carboxymethyl-cysteine will be formed by the reaction of the cysteine side chain with the iodoacetic acid. Furthermore, the ratios of the individual amino acids can be used to calculate a conjugation number, provided that the amino acid compositions of the carrier end the peptide are known.
8. For mAb production, mice are injected intraperitoneally the first and last time or intravenously the last time (only eluted conjugates without adjuvant for intravenous injection).
9. Animals are followed daily for adverse reactions. If required, animals may have to be treated or sacrificed according to ethical guidelines.
10. Depending on the availability and the degree of saturation wanted, smaller or larger amounts of carrier protein may be used.

11. The reactivity of the cysteine side chain SH group is optimal at pH 8–9.
12. Washing with DMF or mixtures of DMF and water or dilute base may be required to remove excess of very hydrophobic peptides. However, the final washes must be without organic solvent.

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Chapter 6

Analysis of Polypeptides by Amino Acid Analysis

Peter Højrup

Abstract

Amino acid analysis is an accurate method for the composition and quantitation of polypeptides and among these synthetic peptides. Combined with mass spectrometry, it yields a reliable control of peptide quality and quantity prior to conjugation and immunization.

Initially peptides are hydrolyzed, preferably in the gas phase, with 6-M HCl at 110 °C for 20–24 h and the resulting amino acids analyzed by chromatography, where the most reliable form is ion exchange chromatography with post-column ninhydrin derivatization. Depending on the hydrolysis conditions, tryptophan is destroyed, and likewise cysteine, unless derivatized, and the amides, glutamine, and asparagine are deamidated to glutamic acid and aspartic acid, respectively. Three different ways of calculating results are suggested, and taking the above limitations into account, a quantitation better than 5% can usually be obtained.

Key words Peptides, Amino acid analysis

1 Introduction

Since the conception of the automatic amino acid analyzer in the 1950s by Moore and Stein [1], amino acid analysis (AAA) has transformed analytical biochemistry, enabling the quantitative analysis of proteins, peptides, and free amino acids.

The result of an amino acid analysis is the absolute amount of each amino acid in the sample. These data can be used for determining the composition of the sample and/or the total amount of sample present. If your purpose is only quantitation (typically when analyzing complex samples like used for proteomics), you have a multitude of choices (e.g., Bradford, BCA, Lowry); however, most of these methods are based on derivatization of amines with a chromophore followed by comparison to a standard. Although simple and fast, the main problem of these types of analyses is that you are measuring amino groups, not just amino acids. Another common method, which has the advantage of being nondestruc-

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tive, is to measure the concentration by UV absorbance. Although this method is quite fast and precise, it is mainly recommended for routine analysis of identical samples, as the results can easily be disturbed by UV-absorbing substances.

Particularly, the last method can be quite accurate if used in a controlled environment; that is, you always have the same buffer, concentration range, type of protein, etc., but if the conditions are not under complete control, quantitation can be wrong by a significant amount.

On the other hand, when using amino acid analysis, you measure on the level of the individual amino acid, and most of the problems with the colorimetric methods mentioned above can readily be recognized and compensated for. However, the price to pay is an increase in cost and a longer analysis time. Amino acid analysis usually takes place by chromatographic separation of the amino acids, followed by determination of the exact quantity of each residue. As the amino acids do not have any absorbance in themselves, except for a rather low absorbance in the 250–280 nm UV range for the aromatic amino acids, they must be derivatized prior to quantitation. Derivatization of amino acids can take place either prior to or following separation, also known as pre- and post-column derivatization. A multitude of methods are available (e.g., [2, 3]), with the pre-column derivatization clearly showing higher sensitivity than post-column. However, two things speak in favor of post-column derivatization, one is stability, as the ion-exchange column used for separation is extremely stable, and the second is that the derivatization step in pre-column analysis is much more sensitive toward contaminations, both regarding the derivatization itself and UV-absorbing substances showing up in the following HPLC separation. This can be particularly important in a core lab where you do not have control of the samples you receive, and thus, the following description will be based on a post-column derivatization system with separation taking place by cation exchange chromatography.

Prior to AAA, polypeptides must be hydrolyzed to obtain the free amino acids for the analysis. Since the early inception of AAA, acid hydrolysis using 6-N HCl at 110 °C for 20–24 h has been the method of choice, mainly because hydrochloric acid is easily evaporated and produces few artifacts. The volatility of hydrochloric acid also enables gas-phase hydrolysis, thus further decreasing contaminations. A negative aspect of the acid hydrolysis method is that tryptophan is destroyed, and cysteine also, unless derivatized, and the amides, glutamine, and asparagine are deamidated to glutamic acid and aspartic acid, respectively (*see* **Note 1**). However, taking these limitations into account, the accuracy obtained by AAA is generally unmatched by other methods.

2 Materials

Hydrolysis solution: Amino acid analysis-grade 6-N HCl containing 0.1% phenol and 0.1% 2-thioglycolic acid.

Mini-inert valve (VICI PS-614163) (*see* **Note 2**).

40-mL screw-cap bottle (e.g., number ND24, EPA, Mikrolab Aarhus A/S, Århus, Denmark).

Large-gauge syringe needle.

Stock solution of amino acids (e.g., Pierce amino acid standard) appropriately diluted.

Norleucine and/or sarcosine to be used as internal standards. Ninhydrin and chromatographic running buffers.

Unless otherwise noted, chemicals must be analytical grade.

3 Methods

3.1 Sample Preparation

Sample preparation is essential for correct amino acid analysis, as you are analyzing the entire content of your sample. In many other forms of peptide analysis, you either do not see minor contaminants, or like in mass spectrometry, you can perform a micro purification prior to analysis to get rid of low-molecular weight contaminants.

If you are mainly interested in the composition, you should transfer your sample to a salt-free buffer, using solid-phase extraction, dialysis, gel filtration, or similar. However, in most cases, you are also interested in the quantity and often have a limited amount of sample. In these cases, you have to consider contaminants, particularly in the last preparation steps.

3.1.1 Contaminations

Three kinds of contaminants are common in amino acid analysis:

Salts are to be avoided, as they may inhibit hydrolysis. If you have a large amount of salts, you may have to forsake gas-phase hydrolysis and add a drop of HCl to the sample. Salts may also disturb the chromophore derivatization and the final chromatographic separation. Note here that both the ion exchange and the post-column derivatization is much less affected than pre-column derivatization. A special case is amine-containing buffers, in particular urea and guanidinium chloride. For pre-column derivatization, they exhaust the chromophore leading to under-derivatization (and large chromatographic peaks), and for post-column methods, they may obscure the arginine peak. Again, ion exchange with post-column derivatization is much less affected.

Amino acids can often be contaminants. Glycine is often used as a buffer, and a drop of spilled buffer may dry, become airborne, and contaminate other samples. However, this can be observed in the chromatogram and compensated for.

Finally, contaminating *proteins* will skew the results. Keratin is a typical contaminant arising from hair and skin and gives rise to increased serine and glycine levels. If the purpose of the analysis is quantitation, a 5% contamination may be in the same range as the accuracy of the method and thus be compensated for, but a similar contamination may lead to erroneous conclusions of the composition if analyzing a supposedly "pure" protein.

3.1.2 Amounts

When considering the amount to submit for amino acid analysis, two things must be taken into consideration: the sensitivity of the analyzer and the sensitivity of the hydrolysis. Pre-column derivatization, particularly when carried out with fluorescent detection, can be made extremely sensitive (about 1 pmol/aa), while post-column detection using ninhydrin is difficult to drive below 100 pmol/aa—using fluorescent detection you can thus give you more than ten times higher sensitivity. However, when preparing samples routinely, and particularly when running samples in a core-like facility, it is quite difficult to ensure total cleanliness in the preparation of samples. This typically results in contaminations, particularly of the small amino acids, Gly, Ala, Ser, and Thr, at a level of 10–30 pmol. This means that, in practice, it is difficult to obtain reliable results of less than 1 μg of the starting material. As people almost always overestimate the amount they are working with, you should always ask for 2-μg samples. If you have a high-sensitivity system, you are then able to run multiple technical replicates.

3.2 Gas-Phase Hydrolysis

The classical way of performing hydrolysis calls for pre-treated glass tubes, but for most practical purposes, 500-μL polypropylene tubes (Eppendorf tubes) can be used (*see* **Note 3**).

The samples are transferred to 500-μL polypropylene tubes, two to three holes are made in the lid with a wide-gauge needle, and the samples are subsequently dried in a vacuum centrifuge. Place 200–300 μL hydrolysis solution in the bottom of a 40-mL screw-cap bottle, followed by three to five polypropylene tubes with samples. Be careful not to stack them directly on top of each other, as condensing HCl may drip into the tubes below.

The glass vial is flushed with argon (alternatively nitrogen) before being closed with a mini-inert valve (*see* **Note 4**). The glass vial is then evacuated to less than 10 Torr and placed at 110 °C for 20–24 h (*see* **Note 5**).

After hydrolysis, the mini-inert valve is opened in a fume hood, pointing away from you (acid fumes!). The valve is then removed, and the sample tubes withdrawn, dried on the outside, and then

dried in a vacuum centrifuge. Be careful to have a −80 °C trap between the centrifuge and the vacuum pump to catch residual HCl vapor.

3.3 Amino Acid Analysis

After removal of all traces of HCl, the samples are dissolved in loading buffer (sodium citrate pH 2.20) spiked with an internal standard (*see* below). The autosampler of the BioChrom 30 amino acid analyzer is unable to load and inject the entire sample, but setting the autosampler to "microliter-pickup," you can inject 60 of 70 μL if you use vials with tapered inserts.

The analysis is most easily done using BioChrom standard program and solvents, and by using the accelerated analysis buffers, the cycle time per analysis is about 50–60 min.

In order to verify the running of the system and to calibrate for loss of ninhydrin efficiency, standard samples are run at regular intervals (in our case 1400 pmol amino acid standard but should be adjusted to your standard running conditions).

3.4 Data Interpretation

After each analysis, the data are automatically integrated according to the current calibration and saved to a single data file containing the results in text format. This can be read by Excel or a dedicated program (e.g., MyAAA, *see* **Note 6**).

3.4.1 Calibration and Standards

Typical results from an analysis are shown in Fig. 1. Standards can be included in several positions to improve accuracy.

Initially, you must make certain that the system is calibrated for each residue, using at least a three-point calibration curve. On our ninhydrin-based system, this is carried out every third month and whenever changes are made to the system.

To improve the results, you may include a standard during the hydrolysis. A typical standard is norleucine, which is stable to acid hydrolysis, and usually separates nicely from the other amino acids during the chromatography. The amount recovered can thus be used to compensate for handling losses during the hydrolysis and loading.

Secondly, a standard (norleucine, sarcosine) may be included in the loading buffer to compensate for injection errors. Standards also help to identify errors when you suddenly find yourself with a blank run: Is this caused by no sample or an error in the machine? In our lab, every seventh sample is a standard, which is used for checking the chromatographic separation and, when integrated, to compensate for the loss of quality of ninhydrin over time (*see* **Note 7**). By analyzing several pure standard proteins, a hydrolysis compensation factor may be calculated for each amino acid (*see* **Note 8**).

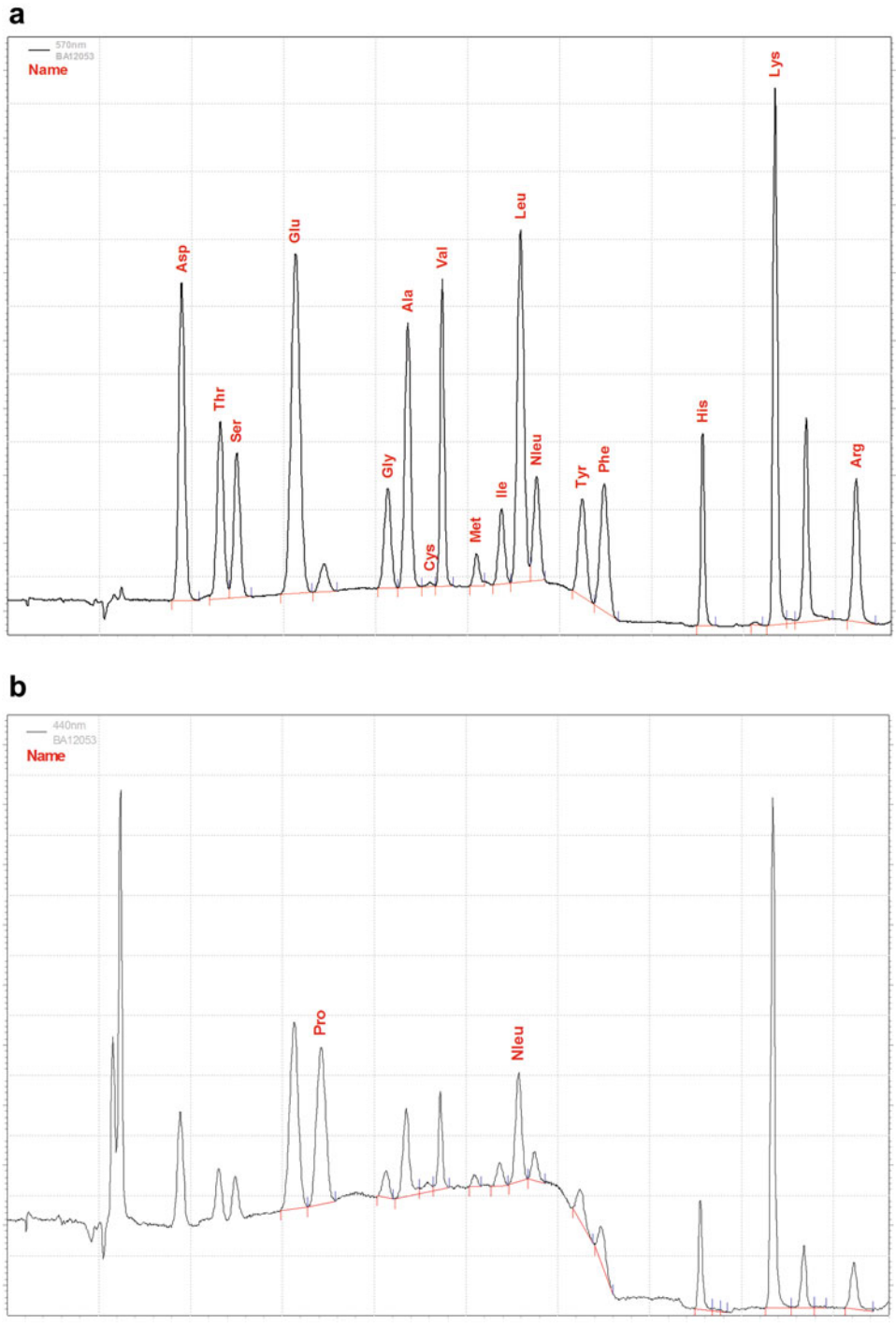

Fig. 1 Amino acid analysis chromatogram of hydrolyzed bovine serum albumin. The amino acids were separated by cation exchange chromatography and detection was after post-column derivatization with ninhydrin. *Top* chromatogram shows 560-nm and *below* 440-nm trace. Only proline is calculated from the 440-nm chromatogram

3.4.2 Quantitation

After compensating the measured amounts according to the outline above, the total amount of protein can be calculated as follows:

Quantitation: the fast and easy way. Add the amount of each residue together. Remember to omit the internal standard (if included) and to include Pro, which for ninhydrin systems is calculated at 440 nm.

The summed value is multiplied by 110 (the average mass of a residue), and the result is the total amount of protein in picogram. Divide by 10^6 to get μg if the results of the analysis are reported in picomoles.

This method usually agrees within 5% with methods 2 and 3 below if the protein composition is "average." If the protein composition is skewed (deviating strongly from the composition in Table 1, e.g., collagen contains approximately 1/3 Gly), this method is unsuitable.

Quantitation: the slightly more complicated way. The amount of each residue is multiplied by the mass of each residue (remember to multiply by the residue mass, not the amino acid mass—e.g., Gly is calculated as 57 Da, not 75 Da), as the amino acids are originally part of a protein, not free. You then sum the resulting values and get a result in picogram as above. For both methods 1 and 2, you will underestimate slightly, as Trp and Cys are not included in the calculations. If you are analyzing mixtures of proteins, you can usually get a more accurate estimate by adding 3.1% mass due to the average content of Cys and Trp (Table 1).

Quantitation: *the best, but most complicated, way.* This method only applies to the analysis of pure proteins with a known sequence. Start by calculating the composition of the given protein.

Remember that Asn and Gln are deamidated during acid hydrolysis and are to be calculated as Asp and Glu, respectively. Then divide each measured amount with the given residue composition. This calculates amount in pmol per residue. In an ideal world, this value would be identical for each residue; however, due to losses (mainly hydrolytic), there will be some variation. If a value is obviously wrong (too high—contamination; too low—losses), you can omit it before taking the average of the rest.

This average value represents the amount of one residue in the sequence and thus the total *amount* of protein analyzed. Multiply it with the molecular mass of the protein (based on the sequence or the mass determined by, e.g., mass spectrometry), and you have the total mass of the protein. This has the advantage that you can compensate for contaminants, and you can include "missing" residues (Trp, Cys) and posttranslational modifications like glycosylation in the mass.

Table 1
Composition in percent of the 20 common amino acids in the UniProt protein database

Ala	(A)	9.00	Gln	(Q)	3.80	Leu	(L)	9.84	Ser	(S)	6.85
Arg	(R)	5.84	Glu	(E)	6.25	Lys	(K)	4.94	Thr	(T)	5.55
Asn	(N)	3.80	Gly	(G)	7.25	Met	(M)	2.23	Trp	(W)	1.30
Asp	(D)	5.47	His	(H)	2.22	Phe	(F)	3.88	Tyr	(Y)	2.87
Cys	(C)	1.30	Ile	(I)	5.52	Pro	(P)	5.01	Val	(V)	6.86

Data are from https://www.ebi.ac.uk/uniprot/TrEMBLstats

Example 1 Table 2 shows the calculations related to the amino acid analysis chromatogram shown in Fig. 1. The first column shows the name of the amino acid in three-letter code. Note that Asx represents Asp and Asn like Glx represents Glu and Gln due to deamidation of the side-chain amide during hydrolysis. The next column shows the raw data as integrated by the amino acid analyzer. The third column has a compensation of 1.4% added due to a decrease in ninhydrin efficiency, calculated from the standards run intermixed with the samples. The fourth column shows the residue mass of each amino acid, which is used to generate column five, which then shows the total mass of each residue. Finally, column 6 lists the number of each residue in BSA, and the last column (number 7) shows the values in column 5 divided with column 6. The values in the last column should theoretically all be identical, but Ser and Met are lower than expected due to hydrolytic loss, while Glu is slightly higher due to a contamination. Taking the average residue amount (129.9 pmol/res) and multiplying with the mass of BSA (66,652 Da) yield a total mass of 8.65 μg.

Comparing the calculated total mass shows that the initial 7.83 μg is increased to 7.94 μg by compensating for decreased ninhydrin sensitivity. This is again increased to 8.29 μg in column 5, mainly due to BSA having a lower-than-average content of small residues (e.g., Gly is only 2.7% compared to an average of 7.25%, Table 1).

3.4.3 Calculating Protein/Peptide Composition

Finally, when calculated based on the average pmol/residue, the total mass is calculated as 8.65 μg. The reason for this large increase in total mass is that BSA has two tryptophan residues and a large content of cysteine (6%). For the average protein, the combined content of Trp and Cys is only 2.35% (Table 1).

Calculating the composition of an unknown protein or peptide can be a little tricky. For this, you need first to estimate the lowest common denominator. You start by summing the amount of all residues. As each residue should contribute equally to the composition, you will get the lowest common denominator by dividing

Table 2
Amino acid composition of BSA as determined by amino acid analysis after acid gas-phase hydrolysis

pmol		pmol	g/mol	pg	Residues	pmol/res
Asp	7087.6	7186.8	115.03	826,701	54	133.1
Thr	4188.0	4246.7	101.05	429,126	33	128.7
Ser	3257.0	3302.6	87.03	287,423	28	117.9
Glu	11,461.5	11,622.0	129.04	1,499,702	79	147.1
Gly	2142.9	2172.9	57.02	123,898	16	135.8
Ala	6045.0	6129.6	71.04	435,445	47	130.4
Val	4596.7	4661.0	99.07	461,770	36	129.5
Met	458.9	465.4	131.04	60,981	4	116.3
Ile	1654.3	1677.5	113.08	189,689	14	119.8
Leu	7769.6	7878.4	113.08	890,890	61	129.2
Tyr	2579.9	2616.0	163.06	426,560	20	130.8
Phe	3670.5	3721.8	147.07	547,370	27	137.8
His	2179.2	2209.7	137.06	302,864	17	130.0
Lys	7431.0	7535.1	128.09	965,168	59	127.7
Arg	3156.2	3200.4	156.1	499,577	23	139.1
Pro	3470.3	3518.9	97.05	341,510	28	125.7
Sum	71,148.62	72,144.70		8,288,675	546	2079.0
					Average	129.9
Amount	7.83 µg	7.94 µg		8.29 µg	Av.[a] 66,652	8.65 µg

[a]Average mass of intact BSA

the sum of all residue-amounts with the number of residues. The number of residues can be estimated by dividing the mass of the protein by 110 (the mass of an average residue) considering the presence of Cys and Trp, which are not part of the amino acid analysis. If you do not know the mass of the protein, you will have to estimate the lowest common denominator. This is most easily done based on stable residues present in low amounts (e.g., His, Arg, Phe, Met).

Example 2 The example in Table 3 shows the composition of bovine ACBP. The column labeled pmol shows the amount of each residue with the sum below. The protein weighs 10 kDa and contains 86 residues of which two are known to be tryptophan and thus not seen in the analysis, leaving 84 residues. Dividing the sum

Table 3
Amino acid composition of ACBP as determined by amino acid analysis after acid gas-phase hydrolysis

pmol		Residues	Integer
Asp	7588.8	9.8	10
Thr	3313.4	4.3	4
Ser	1643.4	2.1	2
Glu	10,613.0	13.7	14
Gly	3153.8	4.1	4
Ala	7137.9	9.2	9
Val	2487.3	3.2	3
Met	2386.8	3.1	3
Ile	3009.7	3.9	4
Leu	4095.7	5.3	5
Tyr	2988.3	3.9	4
Phe	2256.6	2.9	3
His	1564.0	2.0	2
Lys	9864.0	12.7	13
Arg	798.3	1.0	1
Pro	2169.1	2.8	3
Sum	65,070.0		
Residues	84		Total 84
Amount/res	774.64		

of 65,070 pmol with 84 residues yields 774.6 pmol/residue. Dividing this value into each amount gives the number of residues in column 3, which when rounded to integer values are shown in column 4.

If you had no knowledge of the mass of the protein, you could start the search for the lowest common denominator by noting that the lowest value was Arg as 798 pmol, while His and Ser were double that at 1564 and 1643 pmol. Furthermore, at triple values around 2400, you find Val, Met, and Phe. Taking the average of these residues, you would end up with 795 pmol/residue, which could then be used as starting point for the remainder of the amino acids. By making a least square fit to the data, you can obtain a quite accurate value.

4 Notes

1. Although acid hydrolysis using HCl is the standard method, a few methods have been developed for analysis of sensitive residues. Tryptophan can be recovered after hydrolysis in 2.5 M mercaptoethanesulfonic acid (170–185 °C for 12.5 min) or mixtures of HCl, mercaptoethanol, and phenol [4]. Cysteine can be determined after oxidation with performic acid [5] or sodium azide or by chemical derivatization before hydrolysis. Several not-so-stable residues can be recovered after basic hydrolysis [6].

   As these compounds are not volatile, you need to neutralize or purify the sample prior to analysis, thus increasing complexity and decreasing sensitivity.

2. The mini-inert valve is equipped with a rubber cylinder that enables the insertion of a needle into the vial. Before being taken into use, this rubber cylinder must be pushed out, cut in the middle to allow airflow, and reinserted to close the hole. Be careful that the flow through the valve is not blocked.

3. For the classical hydrolysis you use 6 × 50 mm glass tubes. These are then pyrolyzed overnight at 400 °C and kept free of contaminants. For hydrolysis, the sample is dried at the bottom of the tube, and five to six vials can be placed in a 40-mL screw-cap vial for gas-phase hydrolysis. These are likely to contribute less to contamination when attempting high-sensitivity analysis; however, in our hands using >1 μg samples, the advantage is minimal compared to polypropylene tubes, as contaminations are more likely to originate from the sample and sample handling.

4. Always check that the mini-inert valve has an unrestricted airflow when the green button is depressed and is closed when the red button is depressed, as the Teflon bar may rotate during use.

5. As the stability of amino acids toward acid hydrolysis varies (*see* **Note 8**), you can increase accuracy by making a time-course hydrolysis. If you hydrolyze for 24, 48, and 72 h, you can extrapolate the quantity of the difficult-to-hydrolyze residues (Val, Ile) toward infinity, while the unstable ones (Ser, Thr, Tyr, Met) can be extrapolated to 0 h.

   Alternatively, if time is at a premium, you can increase the temperature and shorten the hydrolysis time. You can effectively halve the time for every 10 °C increase (i.e., 6 h at 130 °C). The results are not quite as accurate but are usually acceptable. Note that some polypropylene tubes are not stable at high temperatures; please check beforehand.

6. MyAAA is an in-house-developed program for the analysis of data from a BioChrom 30 amino acid analyzer. Please contact the author for more information.
7. We typically observe a loss of 2–3% ninhydrin intensity/week. This is compensated as a general factor.
8. Please observe that the yield of a given residue varies with the sequence, and absolute compensation factors for hydrolytic loss cannot be calculated reliably. The yield of beta-branched residues (Ile and Val) is thus reduced greatly when they are linked to each other (i.e., Ile-Val, Val-Val, Ile-Ile) due to steric hindrance. A yield lower than normal due to hydrolytic loss can also be expected for Ser (up to 10%), Thr (up to 5%), Tyr (dependent on phenol in the hydrolysis buffer), and Met (oxidation). Cysteine (unless derivatized) and tryptophan are completely lost during acid hydrolysis.

Acknowledgments

The Carlsberg Foundation is gratefully acknowledged for financial support.

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Chapter 7

Characterization of Synthetic Peptides by Mass Spectrometry

Victor G. Chrone, Andrea Lorentzen, and Peter Højrup

Abstract

In the quality control of synthetic peptides, mass spectroscopy (MS) serves as an optimal method for evaluating authenticity and integrity. Typically, the sequence of a synthetic peptide is already established, thereby directing the focus of analysis towards validating its identity and purity. This chapter outlines straightforward methodologies for conducting MS analyses specifically tailored for synthetic peptides.

Key words Synthetic peptide, Mass spectrometry, MALDI-TOF-MS, LC-MS

1 Introduction

While synthetic peptides today are generally of a high quality [1, 2], errors in the form of chemical modifications still occur, mainly not only during synthesis but also during purification and storage [3]. Prior to initiating resource-intensive immunization and testing protocols, it is therefore imperative to ensure quality control for both the chemical identity and purity, as impurities found in commercial synthetic peptides have the potential to generate false-positive results, thus underscoring the critical importance of high-quality input peptides [4, 5]. MS analysis is the method of choice due to its high precision, sensitivity, dynamic range, and wide mass range. Typically, a mass spectrum obtained through matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry furnishes adequate information to proceed with peptide studies for immunological purposes. However, in case of impurities, you will often have the need for analyzing the synthetic peptide in detail, which requires the use of MS/MS or MS/MS/MS instrumentation [6].

MALDI emerges as the favored MS technique for several reasons. Notably, MALDI exhibits considerable tolerance toward salts and solvents while boasting high sensitivity, robustness, and

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high-throughput capacity and detecting even sub-millimolar concentrations [7, 8]. Additionally, peptides analyzed via MALDI-TOF MS predominantly yield singly charged ions, facilitating easier interpretation compared to alternate techniques like LC-MS. The methodology of MALDI-TOF-MS involves co-crystallization of the peptide and matrix on a metal plate, typically utilizing a compound like α-cyano-4-hydroxycinnamic acid for normal peptides, while some modified peptides (e.g., glycosylated peptides) need sinapinic acid or 2,5-dihydroxybenzoic acid. Upon activation by a UV laser, desorption of the peptide-matrix mixture transfers the sample to the gas phase for ionization and analysis [7]. Although lacking the resolving power of LC-MS/MS, MALDI-TOF MS has a significant edge in sample analysis time.

As an alternative, liquid chromatography coupled with mass spectrometric detection (LC-MS or tandem MS, LC-MS/MS) has gained widespread application in peptide analysis. LC-MS instruments exhibit an exceptional sensitivity, often achieving detection limits in the picomole per liter (pmol/L) range [9]. A typical LC-MS/MS setup encompasses components such as atmospheric pressure ionization source, typically electrospray ionization (ESI), which is coupled to an ion-inlet and focusing systems, mass-filtering devices, collision chambers, and ion detectors [10]. Various mass spectrometer types like quadrupole, ion-trap, LTQ-Orbitrap, and time-of-flight analyzers exist, employing diverse ionization, or atmospheric pressure photoionization techniques [11]. A disadvantage of LC-MS is the slow turnaround time, unless you use a sample loading system like Evosep [12]. Another disadvantage is that the data files are so large that you need a dedicated search program (e.g., Mascot, X!Tandem, proprietary programs etc.). This may result in finding only the modifications you search for unless you perform an open search (using, e.g., MSFragger/FragPipe, PEAKS). Finally, peptides that are small and/or contain no or multiple positively charge residues (e.g., Fig. 1) will be quite difficult to analyze by LC-MS.

As you can use almost any mass spectrometer with sufficient mass range, this chapter outlines simple procedures for sample preparations for MALDI-TOF MS and LC-MS, whereas comprehensive techniques and interpretation of MS/MS spectra can be found in the cited literature and subsequent chapters.

2 Methods

2.1 MALDI-TOF-MS

1. Dissolve the peptide in milli-Q water or 0.1% trifluoroacetic acid (TFA). The use of ultrapure water (18.2 MΩ) is essential in all solutions involved in MS.

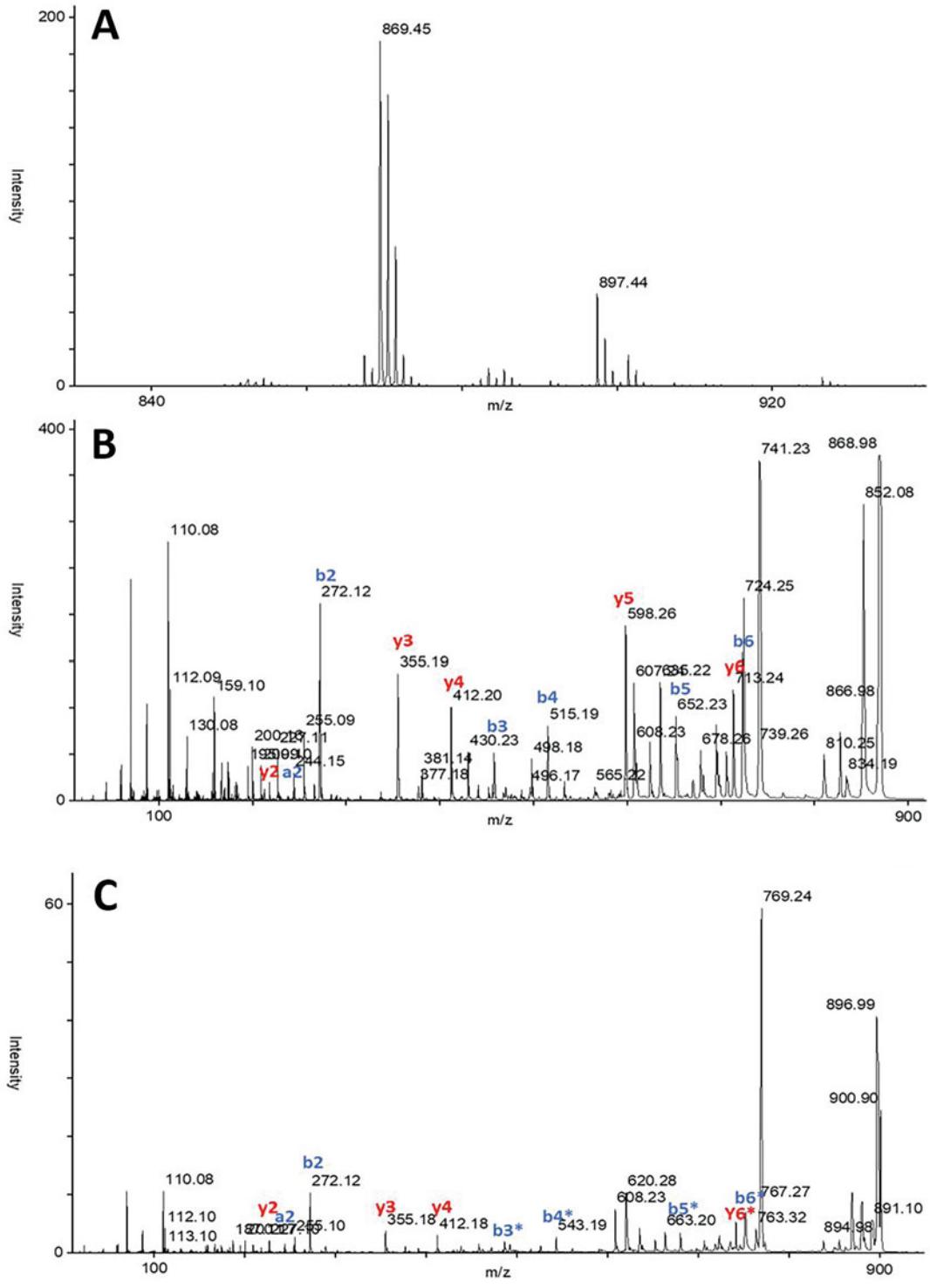

Fig. 1 (**a**) MALDI analysis of a synthetic peptide (Arg-Asp-Trp-Gly-His-Ala-Lys) revealed a correct mass (m/z 869.45) and a modified peptide at +28 Da (m/z 897.44). (**b**) MS/MS analysis of the correct peptide verifying the sequence. (**c**) MS/MS analysis of the modified peptide, locating the modification to the tryptophan. The N-terminal fragment ions (b-ions) are shown in blue and the C-terminal fragment ions (y-ions) in red. The

2. Make a matrix solution depending on the peptide you need to analyze (*see* **Note 1**).
3. For the most sensitive result, prepare a homemade microcolumn (*see* **Note 2**). Alternatively use a C18 Stage Tip ([13], *see* **Note 3**) or a homemade microcolumn (*see* **Note 4**) for desalting and concentration.
4. Micro-purify 0.1–1 μg of sample.
5. Load the sample together with an appropriate matrix on a MALDI-plate (*see* **Notes 2–4**).
6. Run the sample on a MALDI mass-spectrometer (*see* **Note 5**). In addition to the MS1 spectrum, obtain MS/MS (MS2) spectra of appropriate peaks observed in the MS1 spectrum.
7. The spectra can either be analyzed individually using the manufacturers software, exported to external programs in text format (e.g., mMass or GPMAW), or be combined into mgf-files to be analyzed by commercial or open-source search engines (s*ee* **Note 6**).

2.2 LC-MS

1. Dissolve the peptide in milli-Q water or 0.1% TFA.
2. Prepare a homemade microcolumn with a C18 extraction disk (*see* **Note 3**) or use a premade C18 Stage-tip (*see* **Note 4**).
3. Micro-purify 0.1–1 μg of sample.
4. Load the sample on an LC system coupled to an electrospray mass spectrometer (*see* **Note 7**).
5. The resulting raw file should be converted to mgf file format using the manufacturers software prior to analysis.

3 Notes

1. For standard peptides, use α-Cyano-4-hydroxycinnamic acid (8 mg/mL) in 70% acetonitrile, 0.1% TFA as a matrix.
2. Create a slurry of POROS 50 R2 resin (C18) in 100% acetonitrile. Squeeze the end of a gel loader tip by using a tube to make a restriction. Load a small amount of resin in 10 μL of acetonitrile. Use a syringe to push the resin trough the tip by applying air pressure. The length of the resulting column should not exceed 1 cm. Equilibrate your column with 10 μL 0.1% TFA.

Fig. 1 (continued) asterisk indicates a fragment ion modified by +28 Da. Due to the many basic residues, this peptide results in a very complex fragmentation by MALDI but would be very difficult to analyze by LC-MS. The peptide would obtain three to four charges, thus locating it in a difficult to analyze m/z range and still result in a very complex fragmentation. (Data were obtained on an ABI 4700 MALDI MS/MS instrument in positive ion mode using α-Cyano-4-hydroxycinnamic acid as matrix)

Make sure the sample is acidified before it is loaded on the column (pH ≤4). Add the sample in 10 μL 0.1% TFA, and push it slowly through the column. Wash it with 10 μL 0.1% TFA. Elute your sample with 5-μL matrix and make small droplets on your stainless-steel plate/MALDI target. Try to avoid air bubbles.

3. Alternatively, you may use stage tips. Activate the tip with 50 μL 100% acetonitrile and equilibrate with 20 μL 0.1% TFA. Load 0.1–1 μg of sample and wash and equilibrate with 20 μl 0.1% TFA. Elute the sample with 20 μL 50% acetonitrile, 0.1% TFA into a siliconized tube and dry it down. For MALDI, resuspend the sample in 5 μL 0.1% TFA, apply 0,5 μL to a spot on the target, and add 0,5 μL matrix on top. Let the sample dry under a halogen lamp to get a fast and homogeneous crystallization.
4. As an alternative to stage tips, very efficient custom-made tips can be made. Suspend some POROS 50 R2 resin (C18) in 100% acetonitrile. Use a P10 pipette tip and place a C18 plug (C18 Empore 3 M disk) at the bottom of the tip. Place 50 μL 100% acetonitrile on top of the disk and add a small amount of Poros resin slurry. Use a syringe to apply air pressure the drive the solvent through the tip. Do not allow the column to dry out. The length of the column should not exceed 0.5 cm. Equilibrate with 50 μL 0.1% TFA. Make sure the sample is acetified before you load 1 μg on the column (pH ≤4). Push the sample trough and wash the column with 50 μL 0,1% TFA. Elute the sample into a siliconized tube by adding 50 μL 50% acetonitrile, 0.1% TFA, followed by 20 μL 70% acetonitrile, 0.1% TFA. Dry down and resuspend as described in **Note 3**.
5. Load the target into the MALDI mass spectrometer. Depending on the sample, but for standard peptides, run in positive reflector mode. Mass range set to 600–4000 Da. Accumulate spectra until the spectrum quality is good. Use an external calibrant for getting a decent mass accuracy (<50 ppm).
6. Almost all peptide search programs can search for synthetic peptides, but it may not be straightforward to specify a specific peptide instead of a database for searching. In addition, you need to specify a large number of modifications, as you will not find any not specified. You may even need to manually validate spectra to identify unusual modifications (e.g., missing residues). Table 1 lists some of the most common modifications observed in peptide synthesis. For the manual validation of spectra, a program to predict fragment ions from a given sequence is valuable (e.g., ProteinProspector, GPMAW among others).

Table 1
Typical adducts and modifications observed when analyzing synthetic peptides by mass spectrometry

Mass change	Composition	Description
−18	H_2O	Loss of water
−17	NH_3	Loss of ammonia, typically from N-terminal Gln
16	O	Oxidation, typically of Met or Trp
22	Na	Sodium adduct
28	CHO	Formylation
32	O_2	Double oxidation, typically of Trp
38	K	Potassium adduct
42	C_2H_4O	Acetylation
56	C_4H_8	t-Butylation
71	C_3H_6NO	Acetamidomethyl from Cys
96	C_2F_3O	Trifluoroacetylation
242	$C_{19}H_{15}$	Trityl protecting group from Cys, Asn, Gln, or His
252	$C_{13}H_{16}O_3S$	Pbf-protecting group from Arg

In addition to these, errors in synthesis may lead to loss or doubling of an amino acid residue in the sequence

7. Load the sample onto a two-column setup (pre- and analytical column) coupled to an electrospray mass spectrometer. Use a short gradient (22–30 min). Run in data dependent acquisition mode and obtain and acquire the five most abundant peptides with a charge state from 2+ to 4+ for fragmentation.

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Chapter 8

Interpretation of Tandem Mass Spectrometry (MS-MS) Spectra for Peptide Analysis

Karin Hjernø and Peter Højrup

Abstract

The aim of this chapter is to give a short introduction to peptide analysis by mass spectrometry (MS) and interpretation of fragment mass spectra. Through examples and guidelines, we will demonstrate how to understand and validate search results and how to perform de novo sequencing based on the often very complex fragmentation pattern obtained by tandem mass spectrometry (also referred to as MSMS). The focus will be on simple rules for interpretation of MSMS spectra of tryptic as well as non-tryptic peptides.

Key words Mass spectrometry, MS, De novo sequencing, Tandem mass spectra, Validation, Fragmentation

1 Introduction

Mass spectrometry (MS) is a technique that offers many diverse possibilities when the goal is to study and characterize polypeptides of any size, all the way from small peptides loaded onto MHC I to large proteins like immunoglobulins. Traditionally, the determination of the primary structure has been carried out by a bottom-up approach where the protein in question has been cleaved into smaller peptides by a specific protease, whereupon these have been separated and individually analyzed by mass spectrometry (*see* **Note 1**). The purpose of the chapter is not only to demonstrate how sequences can be obtained and validated manually from tandem mass spectrometry data but also to introduce the reader to the complexity of MSMS spectra and demonstrate how simple guidelines can help to judge results determined from automated data analysis by studying specific features of the spectra. We will not give guidelines to automated data analysis but recommend a book chapter [1]. For a description of how to use mass spectrometry-based methods for other kinds of analysis of proteins like immunoglobulins, we can recommend the following articles [2, 3].

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https://doi.org/10.1007/978-1-0716-3914-6_8,

It is often possible to interpret an MSMS spectrum by hand, and we can start by asking the question: should you interpret all of your MSMS spectra manually? The answer would be: No, absolutely not. Today, a long list of software tools for interpreting tandem mass spectra can be generated from a simple Google search. The principles behind most of these tools are based on matching of the experimentally generated data against either theoretically generated data from peptide candidates from a database (e.g., [4, 5]) or against already interpreted spectra (e.g., [6]). Other tools try to automatically deduce the sequence directly from the spectra using, for example, the distance between the ion peaks. This strategy is called de novo sequencing. Then why should you spend time learning how to interpret an MSMS spectrum by hand? For several reasons. First of all, dealing with peptides from more exotic species or from alternative splice variants may not be identified by traditional database-dependent searches if the sequence/variant is not present in the database. You may also find yourself in a situation where you work with nonspecifically cleaved peptides or modified peptides, where a simple search in a search engine may not be fruitful. Some of these situations may be solved by performing error-tolerant searches, but not always (*see* Subheading 3.1.3). Another—and maybe even more important—reason for learning the fragmentation behavior of peptides is to be able to evaluate the results given by the search engines or presented by other scientist (*see* Subheading 3.3). Unless you do a very stringent search in order to avoid any potential false positive hits (and thereby also loosing weak but true peptide hits), you will never be able to avoid false-positive hits. Knowing how you should expect the peptide to fragment will help you evaluate and be critical toward such hits before spending months working on a hypothesis built on these false-positive hits. When performing automated de novo sequencing, you will often be left with several possible partial peptide solutions that you have to judge and choose among and maybe even extend by hand. Here, again, it is essential to have knowledge on manual spectrum interpretation and validation.

In order to obtain detailed information on the primary sequence of a given protein, we often have to start by cutting the protein into smaller pieces of less than 30 residues, which is the size handled most efficiently by mass spectrometers. Trypsin is the most common choice due to a combination of high specificity, high activity, high stability, and low price. Trypsin cleaves C-terminal to lysine and arginine with high specificity, and therefore, we expect the peptides to have a C-terminal lysine or arginine. These are both basic residues having an enhanced proton affinity and therefore a positive effect on the ionization event happening in the mass spectrometer. Often, some of the peptides produced will be too long, too small, or simply too difficult to analyze by conventional MS methods. As a result, strategies involving only one enzyme will

leave regions of the protein not analyzed. Full characterization of a protein therefore requires alternative strategies involving splitting the sample and using different enzymes for digestion. This will provide overlapping peptides covering the remaining of the sequence, hopefully giving us full sequence coverage; trypsin can be combined with more or less specific enzymes like chymotrypsin, Asp-N, Glu-C, and pepsin [6, 7] resulting in peptides that do not have the basic C-terminal characteristic of tryptic peptides. This is also the case if the goal is to analyze small naturally occurring peptides, which have arisen from unknown enzyme specificity, and therefore, we have no prior knowledge of the terminal residues. This provides an extra challenge with respect to de novo sequencing, which we will deal with in Subheading 3.2.

In order to analyze peptides by mass spectrometry, they need to be ionized. This takes place either by electrospray ionization (ESI) [8] or matrix-assisted laser desorption/ionization (MALDI) [9]. Most peptides can be ionized using either of the two methods (*see* **Note 2**). The resulting fragmentation spectra may turn out quite different due to reasons that will be discussed later in this chapter (*see* Subheading 3.3.3).

Inside the MS instrument, the ionized peptides are directed through a mass analyzer and sorted by their mass-to-charge ratio. When run in MSMS mode, peptide ions from a very narrow window of pre-chosen mass-to-charge ratio are isolated and subjected to fragmentation, for example, by colliding the peptide ions with gas molecules, like in collision-induced dissociation (CID). The fragmentation pattern observed for a given peptide is dependent on the amino acid sequence of the peptide (not just the composition) as well as the number of charges carried by the peptide ion, that is, the charge state of the peptide. Unfortunately, the exact structure of the resulting MSMS spectrum can only be partly predicted based on the sequence, as explained later.

The charge state of positive ions produced by ESI is dependent on the numbers of ionizable groups available; that is, the number of basic amino acid residues (His, Lys and Arg) and the N-terminal amino group. The ions will be observed in the spectrum as multiply charged molecular ions $(M + zH)^{z+}$ and often in more than one charge state (e.g., both as doubly and triply charged ions). In contrast, the ions produced in a MALDI-ion source in positive mode are almost always singly charged ions, that is, only one proton is transferred pr. analyte molecule $(M + H)^{+}$. As will be explained, the difference in the number of charges is essential for the fragmentation behavior of the peptides in the two instrument types.

In the collision chamber of the MS instrument, the ions will fragment through cleavage of one or more of the chemical bonds in the peptide, primarily in the peptide backbone. This gives rise to three distinct dissociation pathways as illustrated in Fig. 1 [10], resulting in several series of ions. The ion-series containing the

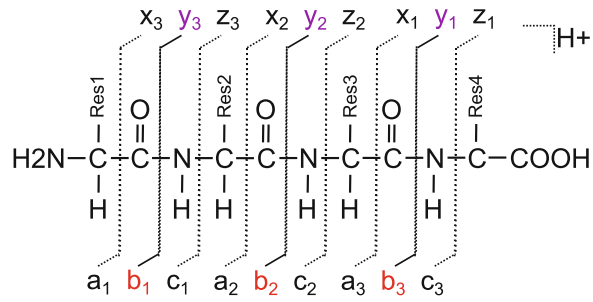

Fig. 1 Fragmentation pattern according to the Roepstorff notation. For CID, fragmentation primarily takes place in the peptide bond giving rise to b- and y-ions. Other fragmentation techniques can yield a, c, x, and z ions

N-terminal part of the peptide are denoted a_n, b_n, and c_n, dependent on which backbone bond is involved in the cleavage. The subscript "n" denotes the number of residues (i.e. alpha carbons) in the resulting fragment ion (*see* Fig. 1). The corresponding ion-series containing the C-terminal part are denoted x_n, y_n, and z_n. For CID fragmentation, the peptide ion will preferably undergo fragmentation in the amide bond, resulting in b- and y-ions depending on which terminus retains the positive charge. Each peptide ion will fragment only once or a few times. However, as we have isolated many copies of the same peptide ion, the resulting spectra will contain fragments representing a large part of the potential fragments (*see* Fig. 2). The exact distribution of various ions (i.e., the peak height) is difficult to predict and will depend both on the exact peptide sequence and on the distribution of positively charged residues.

As the mass difference between the fragment ions in an ion-series in the MSMS spectrum represents the mass of the individual amino acid residues (*see* Fig. 2), the complete peptide sequence can in principle be interpreted directly by calculating the distance/difference between the fragment ions. This is the fundamental principle behind de novo sequencing (*see* Subheading 3.2). However, the process is complicated considerably by several ion series that may coexist in the same spectrum (e.g., y-, b-, and a-ions); some of the ions may be absent; some fragments are unstable and lose small neutral molecules like water or ammonia (18 and 17 Da respectively); some fragments result from two fragmentation events leaving us with fragments having neither the N- nor the C-terminal (internal fragments), and some peaks in the spectra may even be from co-isolated peptides. Some of these fragment types are illustrated in Fig. 2 and introduced in more detail in next section.

The reason why some of the ions are missing or of very low intensity is that cleavage is not equally likely to occur at each individual bond in the peptide; the bonds cleaved in a given

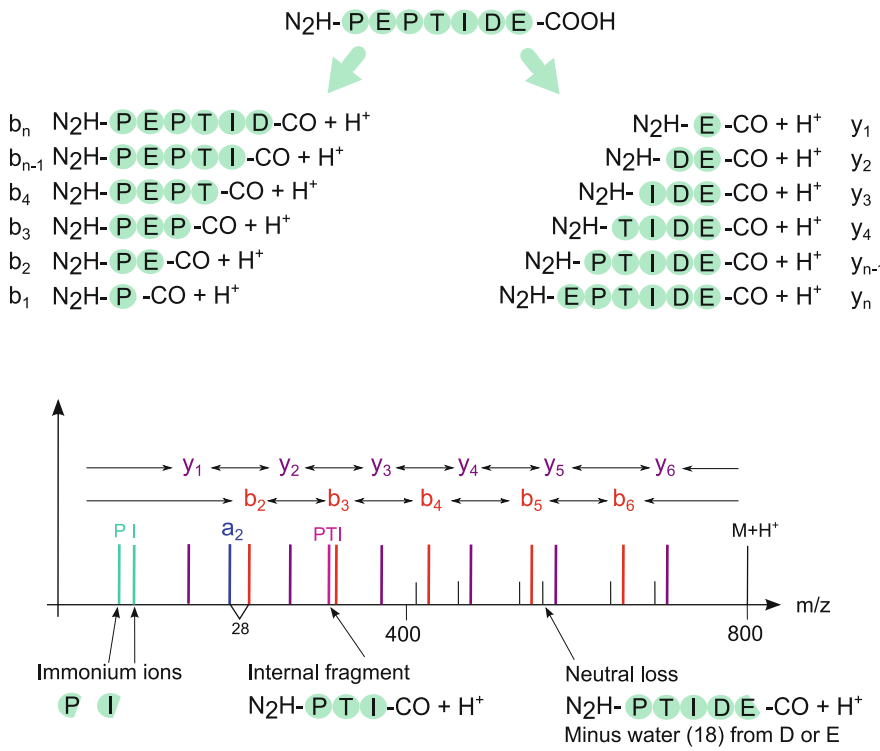

Fig. 2 Typical ions observed in a CID fragmentation mass spectrum. The peptide PEPTIDE gives rise to a number of primary fragmentation ions (the b- and y-ions) as well as satellite peaks arising from loss of water or ammonia. In addition, a few a ions like a2/b2 ion pairs, immonium ions (amino acid side chains) and internal fragments are usually observed

situation can be referred to as the "possible dissociation pathways/channels." Which pathways/channels are followed depends on various factors like, the activation method used, the amino acid side chain adjacent to the dissociation site, the number of protons available, and the rate at which the fragmentation takes place. The stability of the resulting ions will also have an influence on which fragments are observed, as unstable ions can dissociate further into smaller ions. As a result, we can think of some of the dissociation pathways being more populated than others, and some will not be used at all, leading to differences in fragment intensities.

But can it then be concluded which of the pathways will be the preferred pathways and which will not be used at all? Yes and no. There are a few general rules for selectively enhanced fragmentation pathways. As will be described, some of these rules depend on whether or not you have more protons on your peptide ion than you have arginine residues and are especially important for MALDI data. For ESI, the only clear rule involves the presence of the residue proline. These rules will be described in the method section.

Most of the peptide ions will only be subjected to a single backbone cleavage. However, two successive cleavages may take place, resulting in either low-mass ions (e.g., a b_n-ion fragments into a b_{n-1}–ion or an a_n-ion, especially the b_2 and a_2-ions are stable) or in internal fragment ions (containing neither the original C- nor N-terminus). For tryptic peptides, the enhanced proton affinity of the C-terminal residue, offer an explanation as to why the b-ions in the high mass range, are typically of lower abundance (or missing) in a tryptic spectrum. Exceptions from this general finding are seen for peptides containing additional basic sites; b_n-ions containing these residues will be more stable and thereby also more abundant (see examples later, Fig. 3c).

Fragment ions containing only a single residue are called immonium ions. These are of the structure HN=CH-R and range from *m/z* 30 to 159. They can be used as indicators of the presence of specific amino acid residues, *see* Fig. 2 and GPMAW tables.

Satellite peaks connected to the individual fragment ions can be observed in an MS/MS spectrum (*see* Fig. 2) due to loss of small neutral molecules of ammonia (−17 Da, commonly from the side chains of Asn, Gln, Lys and Arg), or water (−18 Da, either from the C-terminal COOH group or the side chains of Asp, Glu, Ser or Thr) (for proposed mechanisms, *see* [11]). *See* **Note 3** for additional neutral losses.

2 Materials

A large amount of programs, tools, and information is available through the Internet. For the manual interpretation of ms/ms spectra, we will mention a few useful tools: Fragment Ion Calculator can be used directly at http://db.systemsbiology.net:8080/proteomicsToolkit/FragIonServlet.html, and the software GPMAW (http://gpmaw.com) has a number of useful features, including the "Fragment Analyzer" tool, which is available as a free download. A number of tables can be downloaded from the GPMAW web page (http://gpmaw.com/html/ms-tables.html). In the following, these tables are referred to as GPMAW tables and comprise elemental mass values, residue mass values, b1- and y1-ions, immonium ions, all a2-b2 ions, and fragment ions of single, double, and triple residues up to 300 Da.

3 Methods

3.1 Data Filtering, Database Searches, and Automated De Novo Sequencing

Before investing a huge effort on identifying the analyzed peptides manually, it is a good idea to filter away low-quality spectra and use relevant software to identify whatever can be easily identified by a database search. The following questions are often relevant for this process.

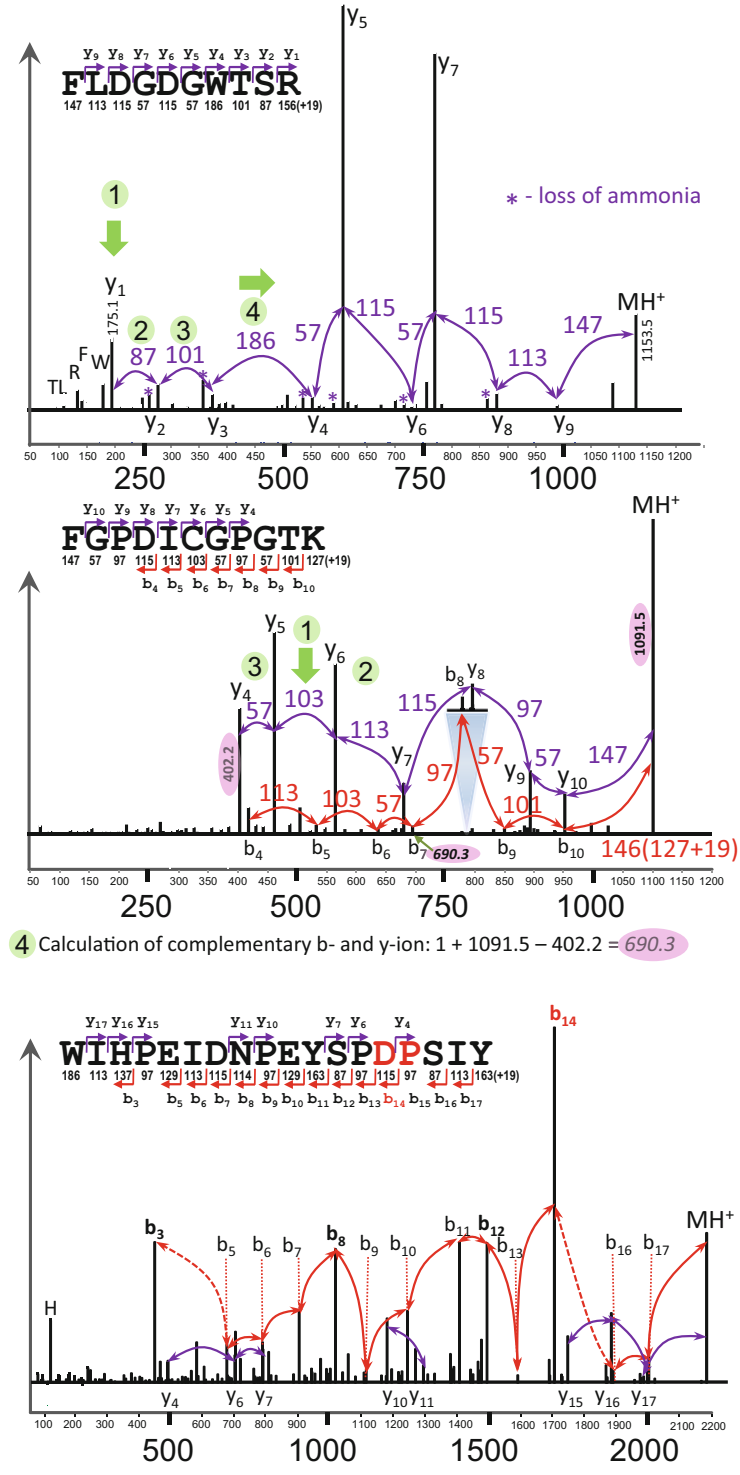

Fig. 3 Typical spectra of singly charged peptides obtained by CID ms/ms fragmentation. (**a**) Arginine-terminated peptide with typical Asp-induced base peaks. (**b**) Lysine-terminated peptide. (**c**) Non-tryptic peptide

3.1.1 Is the Data of High Enough Quality?

It is always worthwhile to take a look at the quality of the data before starting the interpretation. Especially when dealing with large datasets, it is advantageous to use software programs like MassAI to evaluate data quality (http://www.massai.dk). If the goal is to perform de novo sequencing (*see* Subheading 3.2), you can, for example, use the software to confidently exclude all MS/MS spectra with only ten or less fragment peaks as these are less likely to contain enough information for de novo sequencing.

3.1.2 Can the Spectra Be Identified by a Simple Database Search?

Even if one expects that the peptides analyzed are not present in a public or personal database, it is always a good idea to make a database-dependent search using one of the many available search engines. In this step, you also have a fair chance to discover if your sample is contaminated with unexpected proteins, or if very close homologs are present in the database. See reference for a detailed description of how to perform such searches [1].

3.1.3 Does a Second Path Search Reveal Peptides with, for Example, Simple Unexpected Modifications?

For most search engines, it is also possible to make a second path search (sometimes referred to as error tolerant searches). The concept is that when you have identified the proteins present, you can repeat the search one more time, but this time, the search will only be performed against the protein sequences identified in the first run. Your search is now performed in a much smaller search space, and as a consequence, you can allow many more modifications, missed cleavage sites, and even common substitutions. However, be aware that this method often results in many false positives, and manual validation (*see* Subheading 3.3) is often needed.

3.1.4 Are the Spectra Already Identified by Others?

An alternative to traditional database-dependent searches is the spectral library matching strategy [5]. Here, an experimental spectrum is matched against a database of already obtained and assigned experimental spectra. It is then assumed that when a spectrum is nearly identical to an already identified spectrum in the database, then the peptides giving rise to these spectra are identical (*see* **Note 4**).

3.1.5 Can an Automated De Novo Sequencing Program Help?

Yes, but it should be stated that automated de novo sequencing is still in its infancy and manual evaluation of the obtained results are often needed (*see* **Note 5**). It is generally found that automated de novo software is having the hardest time predicting the terminals of the spectra, whereas most of them do an honorable job in the middle part of the peptide, where it often relies on complementary b- and y-ions (*see* later).

3.1.6 Can You Combine De Novo Sequencing and Database Searches?

Yes again. In programs like PEAKS the de novo sequencing, strategy is combined with a database-dependent search strategy to increase the overall output and to cross-validate the results found by both methods [12]. As for database-dependent search strategies,

certain parameters need to be set for automatic de novo sequencing. These are typically enzyme specificity, number of missed cleavages, mass tolerance, modifications, instrument/fragmentation type, and charge state.

3.2 Manual De Novo Sequencing

Equipped with spectra of sufficient quality, a calculator and relevant tables or with a software-assisting tool like the fragment analyzer (*see* **Note 6**) from Lighthouse data, you can train your ability to interpret spectra and sometimes identify spectra not identified by software search tools.

There are several ways to start the manual de novo sequencing. Three of these will be described under Subheadings 3.2.1, 3.2.2 and 3.2.3 and illustrated by the examples in Fig. 3a–c.

3.2.1 Look for the y1 Ion and Extend the y-Ion Series

This is particularly relevant for tryptic peptides where the C-terminal residue is expected to be either arginine (R), or lysine (K). Generally, the y1 ion can be found as the residue mass plus 19 (*see* GPMAW tables).

1. Locate the potential y1 ion/ions. Each of these can be a starting point for the rest of the process. For tryptic peptides, you should start from either m/z 175 (y1 ion for arginine-terminated peptides) or m/z 147 (y1 ion for lysine-terminated peptides). For the spectrum in Fig. 3a, the y1 ion is easily located at m/z 175.
2. The y2 ion can now be found by calculating the mass distance between the y1 ion and the higher mass fragment ions (within a range of 57 and 186, if modified residues are ignored). For the spectrum in Fig. 3a, we find a fragment ion at m/z 262 (y1 + 87). This indicates that the second last residue in the peptide is a serine residue,and we annotate this ion as the y2 ion.
3. When a potential y2 ion is found, the process can be repeated by calculating the distance between this ion and the sequence ions with higher mass in order to find the y3 ion. In the example given this could be the ion which is 101 Da heavier than the y2 ion, indicating a threonine as the next residue. As the y-ion series starts from the C-terminal of the peptide (*see* Fig. 1), the sequence we interpret from the y-ion series is the reverse peptide sequence. So, for now, we have an arginine as the most C-terminal residue, a serine as the second-last residue and a threonine as the third-last residue. The most C-terminal part of the peptide is therefore found to be –TSR.
4. The process is repeated until the end or until no more y-ions can be found. In the latter case, you can try to look for larger distances that may represent a combination of two or three residues. See GPMAW tables for such mass combinations.

Experience tells us that what is often found confusing is the fact that we are looking at both fragment masses and fragment-mass differences. In the example above, we looked for a fragment mass in **step 1**, wanting to find the y1 ion, which we knew should be either 147 or 175 for tryptic peptides. For the rest of the steps, we used mass differences between fragment ions to tell us the nature of the residues in the sequence.

3.2.2 Look for the a2-b2 Ion Pair and Extend the b-Ion Series

An alternative starting point could be to look for the a2-b2 ion pair (illustrated in Fig. 2). The difference between the a2 ion and the b2 ion is CO, that is, a mass difference of 28 (*see* Fig. 1). As the a2 and b2 ions are quite stable, they are often of high intensity, whereas the b1 ion is often missing. The a2-b2 ion pair can therefore be used to kick-start the b-ion series in the same way as we used the y1 ion to kick-starting the y-ion series in the section above. As the b2-ion is composed of two residues, the mass region in which we can find the a2-b2 ion pair is limited by m/z 115 to m/z 373 (i.e., from Gly-Gly + H^+ to Trp-Trp + H^+).

1. Locate any potential a2-b2 ion pairs, that is, two ions in the m/z 115–373 region spaced by 28. In case of several potential a2-b2 pairs, you are now advised to start with the most intense pair (see GPMAW tables for relevant mass values of b2-ions). In the a2-b2 ion pair, the ion with the highest mass will be the b2 ion.
2. The b3 ion can be found by calculating the mass difference between the b2 ion and the higher-mass ions—this is parallel to the process described for y-ions above (*see* Subheading 3.2.1).
3. When a potential b3 ion is found, the process can be repeated by calculating the mass distance to the ions with a higher mass in order to find the b4 ion etc.

Please notice that even though the y- and b-ions are read from each end of the peptide, the smaller (low number) ions (e.g., b2 and y2, each containing two residues) will be in the low-mass region for both ion types, whereas larger ions, like b10 and y10, will be in the higher-mass region, each containing ten residues.

3.2.3 Start in the Middle/High Mass Area of the Spectrum and Extend in Both Directions

Especially if the low mass region is densely filled or contain only very few peaks (as in Fig. 3b), it may not be possible to locate the y1 ion or the a2/b2 ion pair. Instead, interpretation can be started in the middle- or high-mass region of the spectrum. Here, two relatively intense ions separated by the mass of a single residue can serve as a starting point. For tryptic peptides, it is likely that both peaks represent y-ions, as these often dominate in the high-mass area due to the basic residue in the C-terminal (*see* also Subheading 3.3.3). However, there is no guarantee that the peaks are from the same ion series (*see* **Note 7**).

1. Locate two peaks spaced by a distance that corresponds to a single amino acid residue (see GPMAW tables). In the spectrum in Fig. 3b, two peaks (later determined to be y5 and y6) acted as such a starting point. The difference was found to be 103 corresponding to the mass of a cysteine residue (be aware that cysteine is often modified or part of a disulfide bridge).
2. From the fragment ion with the highest mass (y6 in Fig. 3b), you can measure the distance to higher mass peaks and find new members of the ion series. In the example in Fig. 3b, a 113 Da heavier ion was found corresponding to the addition of leucine or isoleucine. From this ion, there was a gap of 212 Da with only very low-intensity ions. According to the GPMAW tables, this difference can correspond to either Asp-Pro or Pro-Asp. A low-intensity ion was found indicating that the correct sequence was Asp-Pro. The ion series could then be extended all the way to the precursor ion (MH^+). The last difference is 147 indicating that the sequence has a terminal phenylalanine (and thus showed that we were analyzing the b-ion series).
3. From the initial lower-mass peak, you can measure the distance to lower-mass peaks to locate members of the ion series containing fewer residues. In the present example, this was only repeated once as the low-mass ions of this spectrum were of very low intensity.

3.2.4 Finding the Corresponding b/y Ion Series

If you have succeeded in finding a partial or complete ion series, you can calculate the mass values of the ions in the complementary ion series. For example, having located the first three y-ions of a peptide of length n and mass MH^+, you can calculate the masses of the corresponding b-ions (i.e., bn-1, bn-2 and bn-3) using the following formula

$$y_m + b_{n-m} = H^+ + MH^+$$

where H^+ is the mass of a proton in units, i.e. 1.007 u.

In the example given in Fig. 3b**,** we can use this to calculate the mass of an ion from the complementary ion series. For this, we use the mass of the peptide ion (MH^+, 1091.5) and the mass of the last ion used in the section above (402.2).

$$M(\text{ion}) = 1 + 1091.5 - 402.2 = 690.3$$

Using this value as a new starting point, we extend the new ion series (the y-ions) in both directions. In the example in Fig. 3b, this was not possible all the way due to lack of fragment ions in the low mass region. However, as the two ion series are overlapping, the combined information provides the entire peptide sequence.

Notice that the difference between the last b-ion and the precursor ion corresponds to the mass of the relevant residue plus 18. This is due to the C-terminal carboxyl acid group.

The sequence for the spectrum in Fig. 3c is also found using the strategy described under Subheadings 3.2.3 and 3.2.4. This sequence is a non-tryptic sequence, and in a few positions, we are missing both the relevant b- and y-ion (e.g., the complementary b4 and y14). We will return to this example at the end of Subheading 3.3.3 and demonstrate how we use this knowledge on peptide fragmentation behavior to deduce the rest of the sequence.

3.3 Manual Validation

Most database-dependent search engines and automated de novo sequencing tools provide you with a confidence score based on how well the peptide matches the experimental data. As discussed in the introduction, it can be beneficial to be able to validate/study the results manually. The same is the case after manual de novo sequencing. The questions below can guide you through the process of manual evaluation, supported by the examples in the figures. The relevance of some of the questions depends on the ionization methods applied and the sequence of the peptide, that is, whether it is a tryptic peptide having a basic residue in the C-terminal or a non-tryptic peptide, which may not even have any basic residues. These differences will be explained as we go along.

3.3.1 Can the Majority of the Intense Peaks Be Explained from a Theoretical Fragmentation of the Suggested Peptide?

Assuming that the spectrum only represents a single peptide, we expect the majority of peaks to be explainable, even though the fragmentation behavior of peptides is not fully understood. Having an intense unexplained peak should make you suspicious. However, such peaks could be other sequence-relevant fragments, for example, internal fragments, a lower charge state of the parent ion, or an ion resulting from a neutral loss of, for example, water or ammonia (*see* Fig. 2). In Fig. 3a, loss of ammonia (indicated by *) explains some of the otherwise non-assigned fragments.

Calculation of the most common ions (a, b, c, x, y, and z) can be done using, for example, Fragment Ion Calculator. Here, you type in the sequence, and the mass values of the selected ion series will be calculated. For a more sophisticated calculation including ions like internal ions, modified residues, and satellite ions resulting from loss of water or ammonia, a software like GPMAW can be recommended (*see* Fig. 4). Be aware that we do not expect to see all potential calculated fragment peaks in the experimental spectrum.

Ions not assigned in the spectrum could also inspire you to check whether rearranging a few residues in the sequence could lead to a better result explaining these peaks. Alternatively, a large residue may mask two smaller residues like whether a tryptophan (W, residue mass 186) should instead be replaced by Ser + Val, Glu + Gly or Asp + Ala and thus explain an otherwise unassigned peak. Looking for the corresponding immonium ions may guide you in the right direction in these relatively rare cases (*see* Subheading 3.3.4).

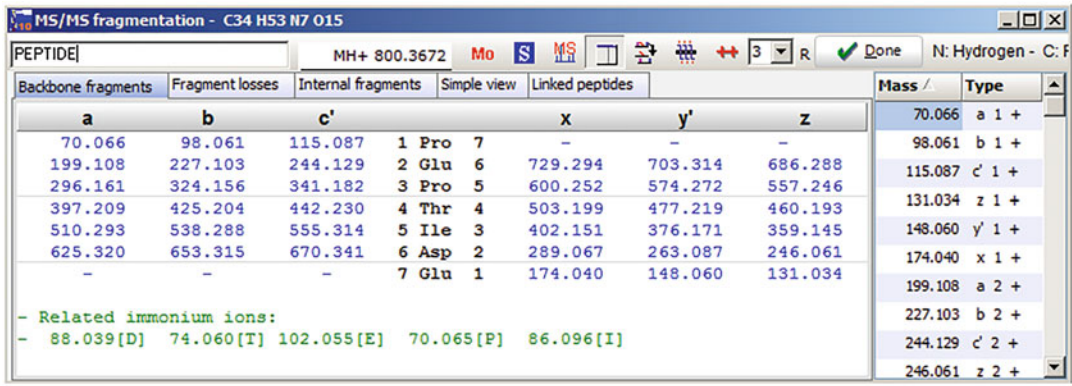

Fig. 4 Calculation of the most common fragments of the peptide "PEPTIDE" as presented by GPMAW. The values presented can be easily modified by the user (e.g., which fragments to show, number of decimals, multiple charges, average/monoisotopic mass etc.). The right-hand panels lists all presented fragments sorted by mass, and other panels show typical fragment losses, internal fragments etc.

3.3.2 Is the Charge State of the Peptide as Expected from the Number of Basic Groups in the Peptide?

For MALDI data, we only expect a single charge on each peptide, independent of the number of basic groups. For ESI, we expect, as a rule of thumb, to observe one proton per basic residue (R, H and K) plus one for the N-terminal amino group. However, each peptide may be detected in more than one charge state in the same ESI experiment, and longer peptides have a tendency to carry a higher charge state, even if they do not contain more basic residues than shorter peptides.

In Fig. 5, we see a peptide identified with a significant score in a database search of ESI CID MSMS spectra from a horse bone marrow sample. However, the spectrum showed to be incorrectly assigned and is instead representing a highly modified collagen peptide with a substitution. The simple search strategy used was not able to identify the correct peptide due to all of these modifications. The peptide suggested by the search engine is a doubly charged peptide with the sequence SDPAGPP$_{ox}$GPPRRSR. The first reason to be suspicious toward the identification is that we would expect this peptide to have more than the two charges due to the three arginines. We will go into more details about this example in Subheading 3.3.3.

3.3.3 Is the Intensity of the Fragment Ions as Expected?

For most of the fragment ions, it is not possible to predict whether they will be of high, medium, or low intensity, or whether they will be detected at all. Sophisticated algorithms can give a hint to the relative intensities [13], but there are no simple rules, except from a few rules of thumb connected to specific residues. However, these few selectively enhanced fragmentation pathways can turn out very useful for manual evaluation as explained below. We will start by describing the proline-induced fragmentation behavior of ESI spectra and then describe the enhanced fragmentation C-terminal to acidic residues seen primarily in MALDI spectra.

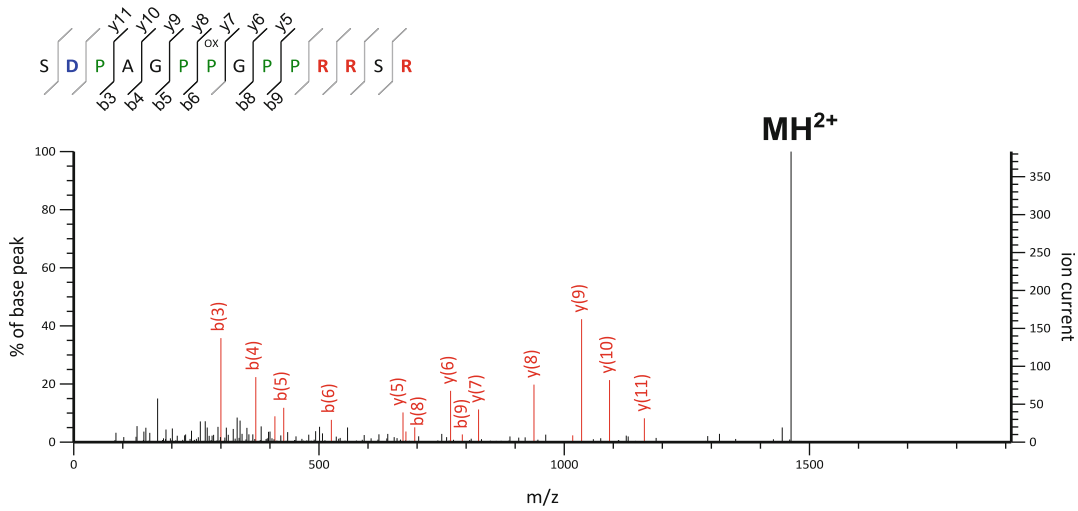

Fig. 5 MSMS spectrum of a peptide initially identified as SDPAGPP$_{ox}$GPPRRSR by the automatic search engine. Careful examination of the spectrum using the rules suggested revealed the peptide to be a modified collagen peptide, for details please see the text

For ESI spectra:

A commonly known feature for ESI data is the enhanced intensity of the fragment resulting from cleavage N-terminal to proline, that is, in the peptide bond Xaa-Pro, where Xaa can be any residue [14]. The effect of proline on the fragmentation pattern seems to be most pronounced when the residue N-terminal to proline (Xaa) is Asp, His, Val, Leu, or Ile. The fragment resulting from cleavage on the other side of the proline (C-terminal to proline) is often found to be of low intensity or even missing.

For MALDI spectra:

The proline-directed fragmentation pattern described for ESI above also applies for MALDI data but only when the peptide does not contain an arginine. In order to understand this, we will have to introduce the mobile proton theory.

When a proton is "free" to move along the backbone to energetically less favored protonation sites, we call it a mobile proton. *See* Fig. 6a, b. Using CID fragmentation, the proton will weaken the amide bonds along the backbone, resulting in "low cost dissociation pathways" giving rise to the dominating b- and y-ion series. For a detailed description of the fragmentation mechanisms, please read [11]. As the proton is involved in the fragmentation, this process is called "charge-directed" or "charge-induced" fragmentation. The energy required to induce the "charge-directed" fragmentation event depends on the composition of the peptide and especially on the basicity of the residues as explained below.

Arginine is the most basic residue in gas-phase reactions having the highest proton affinity. Arginine therefore effectively "immobilizes" or "sequesters" the otherwise available proton. This is the

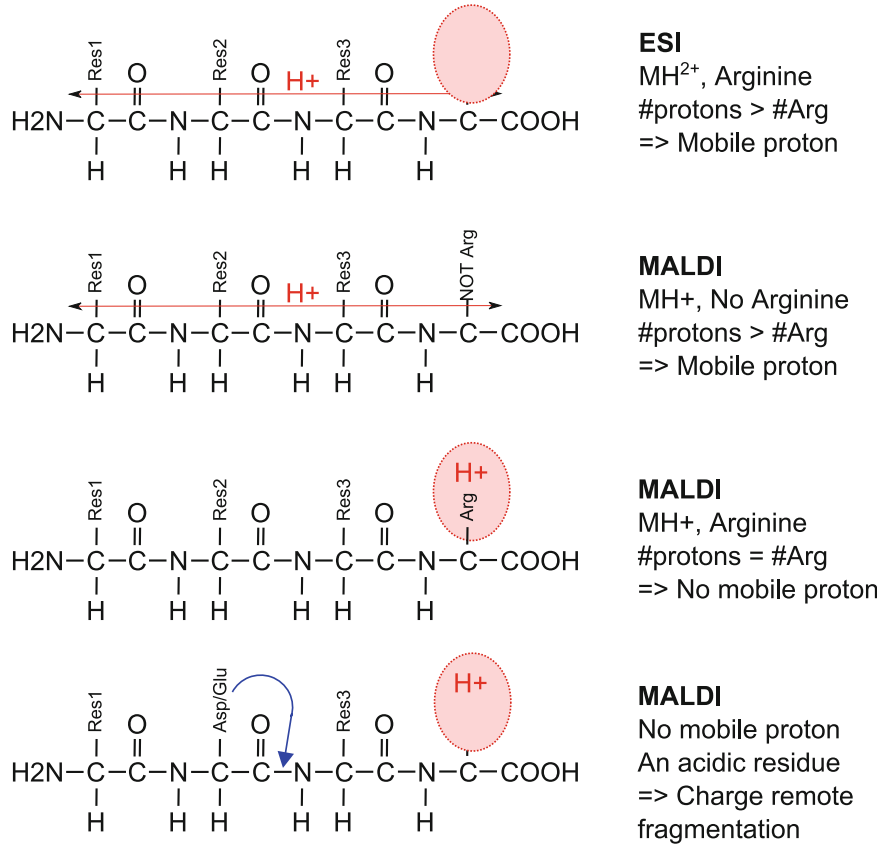

Fig. 6 Mobile and non-mobile protons. When the number of protons is higher than the number of arginine residues, the proton is mobile (**a** and **b**); otherwise, it is non-mobile (**c** and **d**), and charge-remote fragmentation may take place (**d**)

situation in Fig. 6a, c, d. In MALDI, we have only a single proton, so no proton is available for migration at low energies, when an arginine is present, Fig. 6c, d. This opens up for alternative fragmentation pathways.

Such alternative fragmentation channels are called charge-remote fragmentation channels, as they do not involve the proton. The dominating charge-remote channel is selective cleavage C-terminal to acidic residues (Asp and Glu) (*see* Fig. 6d). This phenomenon is described in details in the review on fragmentation pathways of protonated peptides by Paizs and Suhai [11]. The consequence is that for arginine-containing peptides, intense ions can be expected from fragmentation C-terminal to aspartic acid (D) and glutamic acid (E), with D having a higher impact than E (Asp-Xaa > Glu-Xaa).

In the spectrum of the peptide FLDGDGWTSR (*see* Fig. 3a), this phenomenon can be observed, as the two very dominating ions, y5 and y7, are the result of charge-remote fragmentation C-terminal to Asp (D). As seen in this example, the fragment

containing the arginine will be the dominating ion, and for tryptic peptides, this will typically be the y-ion, as the arginine will be the C-terminal residue and has high proton affinity. If, instead, the arginine is located in the N-terminal part of the peptide, b-ions will be dominating. In addition to these acidic-induced intense fragments, arginine-containing peptides will often result in alternative fragments involving fragmentation in the side chain of residues as well as in the backbone (*see* **Note 3**). The effects of these charge-remote fragmentation pathways have a huge influence on the resulting MALDI MSMS spectra.

But what about the prolines then? For arginine-containing peptides, the fragmentation N-terminal to proline is still enhanced but mainly if the previous residue is D (e.g., [15]). Confused? That is understandable. But let us add one more rule of thumb before summing up.

The proton affinity of histidine is not as high as for arginine but still higher than for lysine. Histidine therefore plays a role in the relative abundance of the resulting b- and y-ions as the histidine attracts the proton, but does not sequester it to the same degree as arginine does, and the fragmentation pattern is often unpredictable. This is illustrated in the example in Fig. 6c, where b-ions are dominating the spectrum, due to the histidine being located as the third residue. Especially the b-ion resulting from cleavage C-terminal to D and N-terminal to P is found to be dominating the spectrum.

So, to sum up:

- For MALDI, you should distinguish between situations where you have an arginine-containing peptide and an arginine-deficient peptide.
- For arginine-containing peptides, you should expect acidic residues to result in fragmentation C-terminal to these residues with D giving fragments of higher intensity than E.
- If the peptide contains the dipeptide PD, you should expect the fragmentation between these two residues to be of the highest intensity.
- For arginine-deficient peptides, you should not expect fragmentation C-terminal to acidic residues to dominate.
- Expect that the basic residues have a high influence on the intensity of the relevant ion series—basic residues in the C-terminal part will support the formation of y-ions, whereas basic residues in the N-terminal part support formation of b-ions.

Let us go back to the example in Fig. 5, the one with the non-correctly identified peptide. We see that we have three arginines and only two protons. We know that this is not something we

would expect, but for now, let us follow the idea. As the arginines are holding on to the two protons, we are left with absolutely no mobile protons. Therefore, we should check to see whether we should expect a charge-remote fragmentation event to dominate our spectra. And indeed yes. As the second residue, we have an aspartic acid, and it is even followed by a proline. According to the rules described above, we should expect an enhanced fragmentation event here. But should we expect the enhanced fragmentation to result in a b2 ion or a y12 ion? As the arginines are located in the C-terminal part and they have sequestered the protons, we would expect a dominating y12 ion—mainly as a doubly charged ion but probably also as a singly charged ion. But we see neither! This is an example of how we can use the rules above to verify or, as in this case, reject a search result.

We can also use the guidelines to support the de novo sequencing process. Look at the spectrum in Fig. 3c. Here, we miss some fragment ions, for example, the corresponding b4/y14 ion pair. However, we see a very intense b3 ion indicating a potential proline. The difference between the b3 and the b5 corresponds to a PE dipeptide. The same is the case C-terminal to the very intense PD-directed b14 ion. Here, the difference between b14 and b16 supports the finding of a PS dipeptide. Matching our obtained sequence against a database of relevant proteins supports the resulting peptide sequence (*see* **Note 8**).

3.3.4 Is the Sequence Confirmed by the Immonium Ions/Diagnostic Ions?

Immonium ions (*see* Fig. 2) are found in the very low-mass region (below m/z 172), and some residues can give rise to more than one immonium ion (*see* GPMAW tables). The immonium ions can provide a hint as to whether the correct residues are identified, but be aware that immonium ions are not always present, and they are not always proof of presence, as other ions can co-exist in the same region. See GPMAW tables for the mass values of relevant residues and their immonium ions. For the peptide FLDGDGWTSR (*see* Fig. 3a), the immonium ions for Thr, Leu/Ile, Arg, Phe, and Trp are present in the low-mass region. Immonium ions of modified residues are often used as diagnostic ions to confirm the presence of the given modification. Examples can be found in [16].

3.3.5 Are There Any Modification-Specific Ion Peaks Present?

If the identified peptide holds some modified residues, some of these may give rise to additional peaks confirming the presence of such modification.

For peptides containing an oxidized methionine, enhanced side-chain cleavage due to a neutral loss of CH_3SOH (64 Da) can be observed [17]. Such an intense ion is valuable as a diagnostic ion in order to distinguish between phenylalanine and oxidized methionine, as they have the same nominal mass. All signals,

corresponding to fragments containing this oxidized methionine, will have a corresponding satellite ion with a 64 Da lower mass (the signal-to-noise ratio influence the chance of observing these ions).

Other examples of neutral losses are those of 80 or 98 Da from ions containing a phosphorylated residue, and several different neutral losses can be observed for peptides with N-linked glycans [16, 18].

4 Notes

1. Recent advances in top- and middle-down MS analysis show great promise as to quickly analyze even large proteins like immunoglobulins [19, 20]. However, these techniques still only work efficiently when the primary structure is known, and even then, only part of the primary structure is verified. These problems are exaggerated when the protein is modified, and for sequences like the variable region of IgG, as such modifications and variable regions cannot always be deduced from the corresponding DNA. In these cases, you have to use the bottom-up approach, and even then automated sequence determination will fail to identify the sequence in many cases.
2. Traditionally, ESI MSMS instruments are coupled to LC separation systems and can be used for analysis of very complex samples resulting in thousands of MSMS spectra from a single MS experiment. In contrast, MALDI MSMS is often used for the analysis of less complex samples, for example, characterization of peptides from a single protein.
3. Other neutral losses are due to intramolecular rearrangements involving both a backbone cleavage and the loss of a side-chain of the adjacent amino acid residues. The resulting fragments are denoted v_n-, d_n-, and w_n-ions [21]. These side-chain reactions require high-energy activation and an immobilized proton to occur and are therefore primarily observed in high-energy CID experiments on arginine containing peptides.
4. A limitation in this strategy is that the appearance of the spectra in terms of fragments observed and the intensities of the fragments vary with the ionization method used, the type of analyzer, and the fragmentation method applied. As a consequence, only spectra obtained under similar conditions should be compared, and the database should contain verified spectra of sufficient quality.
5. In the review by Allmer [22], the different types of de novo algorithms are presented and discussed. Also, the benefit of combining several spectra of either the same type (e.g., grouping similar CID spectra) or different types (e.g., CID and ETD spectra of the same peptide) are described.

6. Fragment Analyzer is a small free-ware utility supplied by Lighthouse data (http://www.gpmaw.com look in the download section). When manually annotating a spectrum during de novo sequencing, you select a peak and enter the mass into the program. This will then suggest matching peaks for the next amino acid residue in the ion series moving up or down in mass. If you locate a residue, just double-click on the residue, and the program will suggest the next residue in the chosen direction. The program is designed to be small and unobtrusive and sit on top of the mass spectrum.
7. By applying different experimental strategies involving labeling of one of the peptide terminals it is possible to get a hint as to whether two ions belong to the same or different ion series. Examples on such strategies are tryptic digestion in O18 water selectively labeling y-ions [23] and combining Lys-N digestion with differential isotopic dimethyl resulting in labeling of the N-terminal containing fragments, the b-ions [24].
8. Having obtained one or more peptide sequences by de novo sequencing, it is often of interest to match these against sequences from protein databases in order to get an idea of the function of the protein based on homologous proteins. A traditional BLAST search engine is not optimized for such short sequences, and it is advisable to use a specialized program like MSBlast for this purpose [25]. This program allows combined searches, where several peptide sequences are searched together in order to find proteins matching to some or all of the query sequences. The program can even deal with gaps and ambiguities in the peptide sequences.

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Chapter 9

Cyclic Peptide Conjugate Vaccines and Physically Mixed Cyclic Peptide Vaccines for Subcutaneous Immunization

Wenbin Huang, Harrison Y. R. Madge, Istvan Toth, and Rachel J. Stephenson

Abstract

Immune stimulants (adjuvants) enhance immune system recognition to provide an effective and individualized immune response when delivered with an antigen. Synthetic cyclic deca-peptides, co-administered with a toll-like receptor targeting lipopeptide, have shown self-adjuvant properties, dramatically boosting the immune response in a murine model as a subunit peptide-based vaccine containing group A *Streptococcus* peptide antigens.

Here, we designed a novel peptide and lipid adjuvant system for the delivery of group A *Streptococcus* peptide antigen and a T helper peptide epitope. Following linear peptide synthesis on 2-chlorotrityl chloride resin, the linear peptide was cleaved and head-to-tail cyclized in solution. The selective arrangement of amino acids in the deca-peptide allowed for selective conjugation of lipids and/or peptide antigens following cyclisation. Using both solution-phase peptide chemistry and copper-catalyzed azide-alkyne cycloaddition reaction were covalently (and selectively) ligated lipid and/or peptide antigens onto the cyclic deca-peptide core. Subcutaneous administration of the vaccine design to mice resulted in the generation of a large number of serum immunoglobulin (Ig) G antibodies.

Key words Cyclic peptide, Lipopeptide, Cyclic physical mixture, Copper-catalyzed alkyne-azide cycloaddition click reaction, Self-assembly, Structure design and synthesis

1 Introduction

The gram-positive bacterium, group A *Streptococcus* (GAS), is responsible for a wide range of diseases, from minor irritations, such as pharyngitis and impetigo, to fatal invasive infections, such as toxic shock syndrome and necrotizing fasciitis. In addition, post-infectious sequelae may occur due to repeated infection, such as glomerulonephritis and rheumatic fever [1]. Rheumatic heart diseases primarily caused by GAS are a significant burden on world health, with around 34 million young adults and children suffering from severe illness and 320,000 fatalities annually [2]. Vaccine

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development against GAS is a viable therapeutic strategy for the treatment and/or prevention of this economically burdensome GAS infection [3, 4].

Vaccines are typically designed to induce an adaptive immune response, such as cellular or humoral responses, and are regarded as one of the most effective medical interventions for preventing bacterial and viral infections, inhibiting the development of tumors, and lowering disease prevalence [5]. Subunit vaccines, in contrast to traditional vaccines based on live-attenuated or inactivated pathogens, contain only the pathogen's minimal antigenic components (e.g., protein, peptide, etc.). Consequently, subunit vaccines have a number of benefits, including the simplicity of vaccine production and storage, the reduction of toxicity, and, most crucially, decrease of autoimmune responses and allergic reactions [6]. Nevertheless, subunit vaccinations often require co-administration with powerful adjuvants to improve immune system recognition and to elicit a more effective immune response [7].

Lipidic compounds have been developed as popular adjuvants due to their abilities to activate toll-like receptors that facilitate the detection and uptake of a vaccine. These lipids are usually nontoxic and stabile and have the ability to induce a powerful immune response [8]. For example, palmitic acid was demonstrated to activate toll-like receptor (TLR) 2 and TLR 4, enhancing both humoral and cellular immune responses [9, 10]. In addition, 2-aminohexadecanoic acid (C16), a 16-carbon alpha-amino fatty acid, dramatically improved humoral immune responses in mice by acting as a TLR 2 agonist [11, 12]. 2,3-Dipalmitoyl-S-glycerylcysteine (Pam2Cys) led to a more robust immune response than 2-aminohexadecanoic acid when evaluated as an adjuvant in mice, due to a greater affinity for TLR 2 [13, 14]. Dipalmitoyl serine (DPS), an analogue of Pam2Cys, exhibited a similar selectivity for TLR 2 binding and elicited a significant humoral immune response [13].

As an adjuvant delivery system, cyclic peptides provide a promising platform for peptide subunit vaccines. In comparison to a linear analogue, cyclic cell-penetrating peptides (CPPs) conjugated with antigen have several advantages. For example, cyclic CPPs facilitated antigen transport into cells due to higher binding affinities to the plasma membrane [15]. In addition to enhancement of stable conformations and biological activity, cyclic CPPs prolonged vaccine persistence in serum by increasing resistance to endogenous protease degradation [16]. A physical mixture of three components, a cyclic decapeptide, a TLR 2 targeting lipopeptide, and a GAS B cell epitope (J8) chemically conjugated to a universal T helper cell epitope (PADRE), generated significant IgG antibody titers in the sera of mice with high opsonization potential against clinical GAS isolates when administered subcutaneously [17–19].

In this protocol, components of cyclic mixed peptide were synthesized using solid-phase peptide synthesis. Cyclic peptide contained a lysine amino acid pre-functionalized with 4-pentynoic acid on its side chain to facilitate the copper-catalyzed click reaction between an azido-functionalized antigen. Additional lysine amino acids containing an amine group were also included in the sequence to allow for the selective conjugation of lipids following Boc-deprotection using strong acid (TFA).

2 Materials

All chemicals utilized in this study were of an analytical grade.

2.1 Synthesis of Peptide BB1 to BB10

1. Dichloromethane (DCM).
2. 3 Å molecular sieves.
3. 2-Chlorotrityl chloride resin (2-CTC) 1% DVB (100–200 mesh; resin substitution: 1.14 mmol/g).
4. N-α-Fluorenylmethoxycarbonyl (Fmoc)- L-amino acids.
   Fmoc-Gly (G)-OH; Fmoc-Pro (P)-OH; Fmoc-Ala (A)-OH; Fmoc-Lys (K)-[Boc]-OH, Fmoc-Lys (K_1)-[N-4-pentynoic acid]-OH synthesis refers to the protocol [20]; Fmoc-Ser (S)-[tBu]-OH; Fmoc-Ser (S)-OH; Fmoc-Leu (L)-OH; Fmoc-Thr (T) –[tBu]-OH; Fmoc-Trp (W)-[Boc]-OH; Fmoc-Val (V)-OH; Fmoc-Phe (F)-OH; Fmoc-Gln (Q)-[Trt]-OH; Fmoc-Asp (D)-[Otbu]-OH; Fmoc-Glu (E)-[Otbu]-OH; Fmoc-Arg (R) –[Pbf]-OH.
   The chemical group of the branch chain is located between square brackets. Boc: tert-butyloxycarbonyl protecting group; tBu: tert-butyl protecting group; Trt: trityl protecting group; Otbu: tert-butyl ester protecting group; Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl protecting group.
5. Diisopropylethylamine (DIPEA).
6. N,N-Dimethylformamide (DMF).
7. Piperidine.
8. Methanol (MeOH).
9. 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b] pyridinium 3-oxid hexafluoro phosphate (HATU), 9.5 g dissolve in 50-mL DMF to prepare 0.5-M solution.
10. Acetic acid.
11. 2,2,2-Trifluoroethanol (TFE).
12. N-Hexane.
13. Diethyl ether.

14. Acetonitrile.
15. Trifluoroacetic acid (TFA).
16. N,N′-Diisopropylcarbodiimide (DIC).
17. 1-Hydroxybenzotriazole (HOBT).
18. Palmitic acid.
19. 4-(Dimethylamino)pyridine (DMAP).
20. Triisopropylsilane (TIPS).
21. 4-(2′,4′-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl (Rink Amide) MBHA resin (100–200 mesh; resin substitution: 0.65 mmol/g).
22. 2-[[1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)ethyl]amino]-hexadecanoic acid (Dde-C16). Synthesis of product is referred to the protocol [21].
23. Acetic anhydride.
24. Hydrazine hydrate.
25. Azido-acetic acid synthesis is referred to the protocol [22].
26. 1-Pentanol.
27. Copper sulfate.
28. Sodium ascorbate.
29. Balance.
30. Scintillation vial.
31. Shaker.
32. 15-mL Falcon tubes.
33. Solid-phase peptide synthesis vessel.
34. Ultraviolet-visible (UV-vis) spectrophotometers.
35. Cuvette.
36. Magnetic stirrer mixer with hot plate.
37. Round-bottom flask (250 mL and 100 mL).
38. Spk40 polyethylene frits.
39. 20-mL syringe.
40. Rotary evaporator.
41. High vacuum evaporator.
42. Desiccator.
43. Freeze dryer.
44. Centrifuge.
45. Aluminum foil.
46. Oil bath.

2.2 Immunization

1. Black mice (C57BL/6, 4–6 weeks old, female).
2. Phosphate-buffered saline (PBS) 1×.
3. Freund's Complete Adjuvant (CFA).
4. 1-mL syringe
5. 18 G × 38 mm blunt needle
6. Ear puncher/notcher.
7. 1.5 mL Eppendorf tubes
8. Ultrasonic bath.
9. −80 °C freezer
10. Insulin syringe and needle.
11. Shaker.

3 Methods

3.1 Synthesis of Cyclic Peptides BB1 and BB2 (Figs. 1, 2 and 3)

1. Prepare dry DCM by drying with 3 Å molecular sieves overnight (*see* **Note 1**).
2. Weight 175 mg 2-CTC resin (0.2 mmol, 1.14 mmol/g)) into a scintillation vial and swell resin in dry DCM for 2 h at R.T.
3. Pre-activate the first amino acid (Fmoc-Glycine, 71.4 mg, 1.2 eq) with DIPEA (139.4 μL, 4.0 eq) in dry DCM for 5 min and add to the scintillation vial containing the 2-CTC resin for 3 h moving continuously on a rotary display at R.T. (*see* **Note 2**).
4. Wash the resin-peptide with DMF and DCM and place it in a desiccator for 20 min.
5. Loading efficiency calculation (derived from the protocol [23]):

   Sample: Add 1 mL of 20% piperidine in DMF solution to 7 mg of resin-peptide from **step 4** (Subheading 3.1) into a 1.5-mL Eppendorf tube and gently rotate at room temperature for 5 min. Shake the tube and wait 30 s for the resin to precipitate. Extract the reaction supernatant (50 μL) and dilute this supernatant to 5 mL with DMF in 15 mL Falcon tubes.

   Blank: Dilute 20% piperidine (50 μL) with DMF to a volume of 5 mL.

   At 301 nm, measure the UV absorption of both the blank and sample, and use Eq. 1 to calculate the resin loading efficiency (*see* **Note 3**).

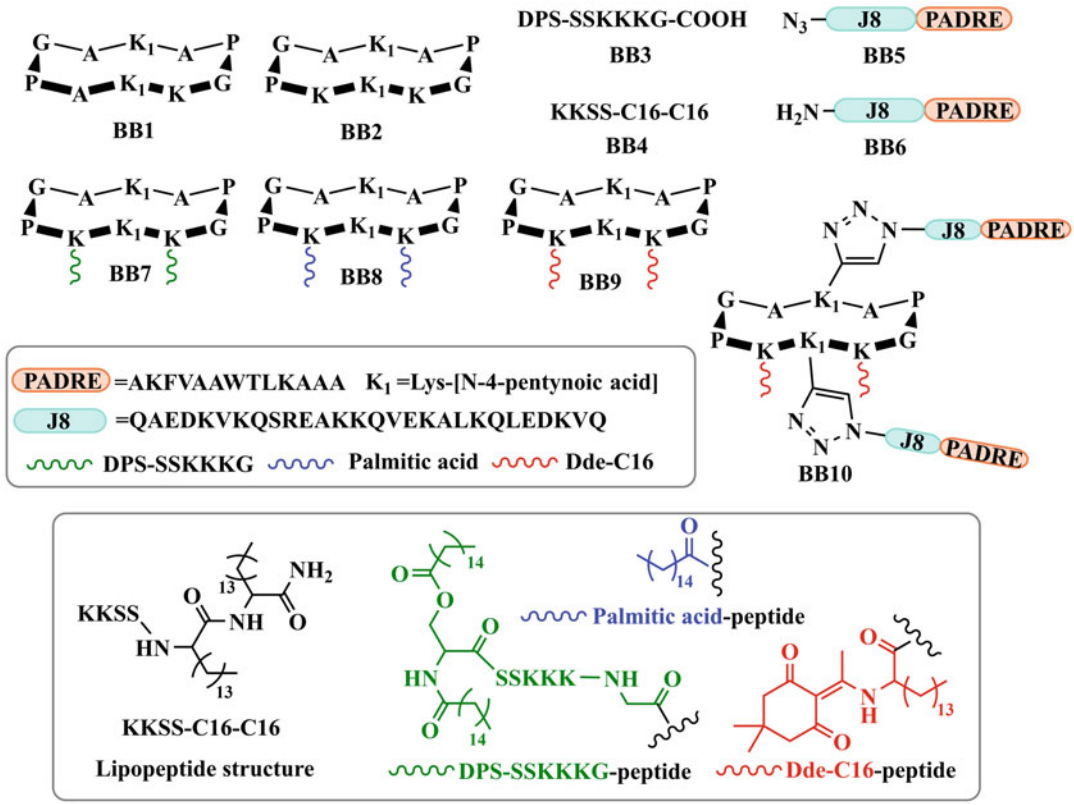

Fig. 1 Chemical structures of building blocks from **BB1** to **BB10** and **lipopeptide**

$$\text{Loading capacity}\left(\frac{\text{mmol}}{\text{g}}\right) = \left(\frac{A_{301}}{M \times 7800\ \text{mol.L}^{-1}.\text{cm}^{-1} \times 1\ \text{cm} \times 0.01}\right) \times 100\% \quad (1)$$

Equation 1. 2-CTC resin loading capacity calculation.

A_{301}: UV absorbance of the reaction minus the blank; M: mass of resin (g); 7800 mol. L^{-1}. cm^{-1}: extinction coefficient for Fmoc fragment; 1 cm: length of the cuvette; 0.01: reaction dilution factor mentioned (dilute 50 μL to 5 mL) before.

6. Endcap the unreacted chloride groups of 2-CTC resin for 2 h in methanol.
7. Swell resin in DMF for 30 min.
8. Wash the resin with DMF three times.
9. Remove the Fmoc protecting groups by using a solution of 20% piperidine in DMF (5 mL, 2 × 10 min). Wash resin two times with DMF between each deprotection.
10. Wash the resin with DMF three times to remove any remaining piperidine solution.

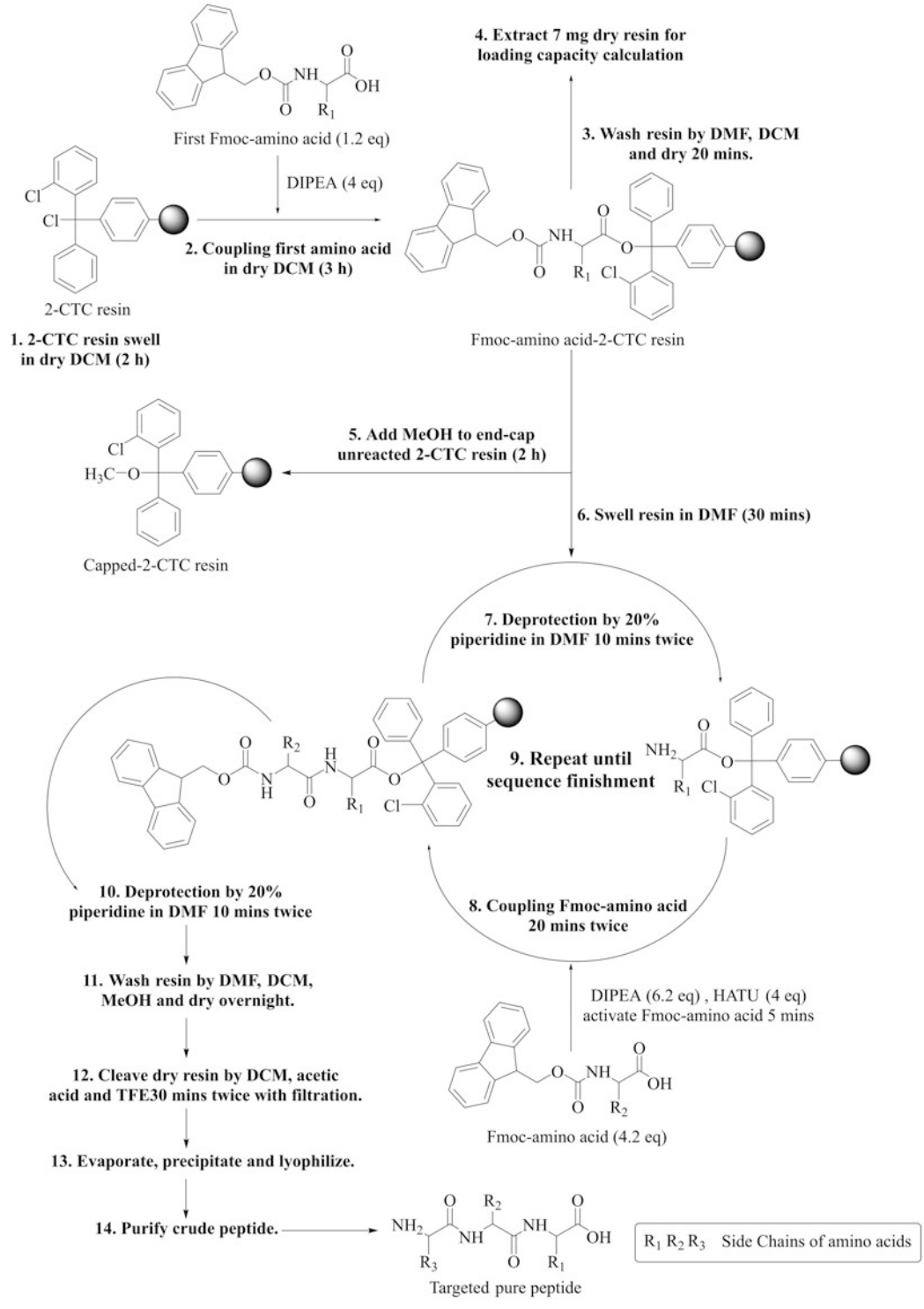

Fig. 2 Linear confirmation of cyclic peptide synthesis on 2-CTC resin (**steps 1**–**23**, Subheading 1)

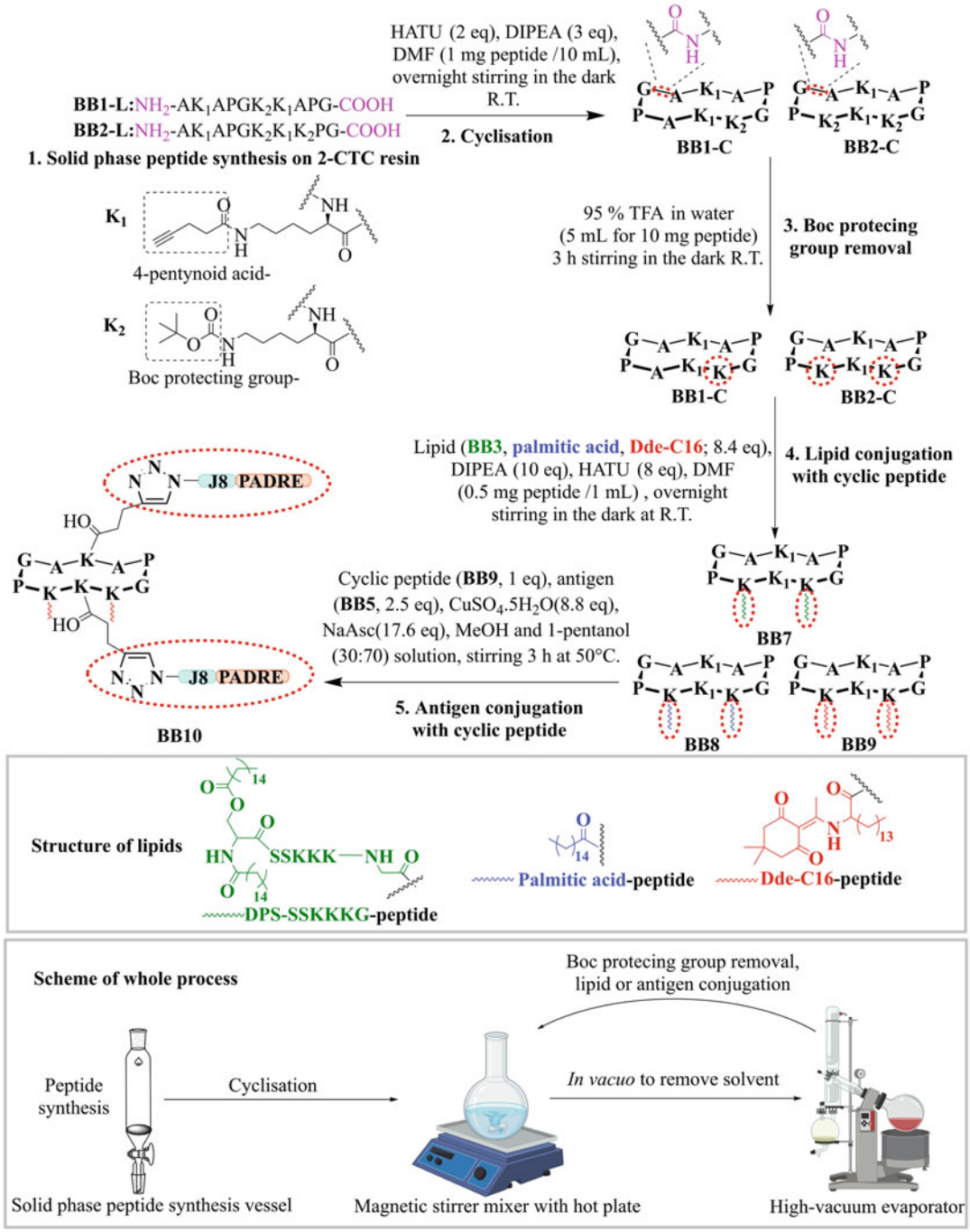

Fig. 3 The process of cyclisation; Boc protecting group removal; lipid conjugation with cyclic peptide and antigen conjugation with cyclic peptide

11. Activate *N*-a-Fmoc protected amino acids (4.2 eq, refers to the loading capacity calculation; **step 5**, Subheading 3.1) for a minimum of 5 min with HATU in DMF solution (4 eq) and DIPEA solution (6.2 eq).
12. Add amino acid twice for a minimum of 20 min/coupling. However, Fmoc-Lys-[N-4-pentynoic acid]-OH must be coupled twice for a minimum of 3 h (synthesis of Fmoc-Lys-[N-4-pentynoic acid]-OH is referred to the protocol [20]). The resin is washed with DMF between each coupling to eliminate any remaining amino acid.
13. Repeat **steps 8–12** (Subheading 3.1) until the peptide sequence is complete.

    BB1-L (**NH_2-AK_1APGK_2K_1APG-COOH**; K_1: Lys-[4-pentynoic-acid], K_2: Lys-[Boc]). **BB2-L** (**NH_2-AK_1APG$K_2K_1K_2$PG-COOH**, K_1: Lys-[4-pentynoic-acid]; K_2: Lys-[Boc]).

    The chemical group of the branch chain is located between square brackets.
14. Once the peptide sequence is finished, deprotect with 20% piperidine in DMF (5 mL, 2 × 10 min).
15. Wash the resin with DMF (three times), DCM (two times), and MeOH (three times) and dry under vacuum overnight.
16. Cleave the resin by using a cocktail solution (DCM: acetic acid: TFE, 8:1:1, 5 mL, for 0.5 g resin) with stirring (30 min, R.T.) (*see* **Note 4**).
17. Filter the resin.
18. Repeat **steps 16** and **17** (Subheading 3.1).
19. Combine the filtered solvent and concentrate the liquid in vacuo.
20. Add n-hexane into the cleaved liquid, and then concentrate the liquid in vacuo until no liquid (*see* **Note 5**).
21. Add cold diethyl ether (4 °C) to precipitate the crude peptide and discard the supernatant (*see* **Note 6**).
22. Dissolve the peptide in water solution containing 30% acetonitrile. Lyophilize the crude peptide.
23. Purify the linear peptide **BB1-L** or **BB2-L** by RP HPLC and lyophilize (Fig. 2; *see* **Note 7**).
24. Dissolve the pure linear peptide **BB1-L** or **BB2-L** (10 mg) in DMF in a flask covered with foil (100 mL, 1-mg peptide in 10-mL DMF) (*see* **Note 8**).
25. Add HATU (2 eq; refer to the **BB1-L**, 33.8 μL; refer to the **BB2-L**, 29.8 μL) and DIPEA (3 eq; refer to the **BB1-L**, 3.9 μL; refer to the **BB2-L**, 4.4 μL) with overnight stirring in the dark at R.T. (Fig. 3).

26. Concentrate the liquid in vacuo to remove extra DMF and create an orange oil (*see* **Note 9**).
27. Dissolve the orange oil in water solution containing 40% acetonitrile and lyophilize.
28. Purify samples **BB1-C** or **BB2-C** using RP-HPLC (*see* **Note 10**).
29. Add 95% TFA in water (5 mL for 10-mg peptide) to the remove Boc protecting group in **BB1-C** or **BB2-C** by 3 h stirring in the dark at R.T. (Fig. 3).
30. Concentrate the liquid in vacuo to remove extra TFA and create a translucent oil.
31. Purify samples using RP-HPLC to get the final purified cyclic peptides **BB1** or **BB2** (Fig. 1).

3.2 Synthesis of Lipopeptides and Antigens

3.2.1 DPS-SSKKKG-COOH (BB3) Synthesis (Fig. 1)

1. Use the same synthesis procedure from **steps 1** to **13** (Subheading 3.1) to synthesize the lipid-peptide sequence on 2-CTC resin, Fmoc-SSKKKG-2-CTC resin (*see* **Note 11**).
2. Deprotect the lipid-peptide by using a solution of 20% piperidine in DMF (5 mL, 2 × 10 min). Wash resin two times with DMF between each deprotection.
3. Wash the resin with DMF three times to remove any traces of piperidine.
4. Add the Fmoc-Ser-OH (4.2 eq, refers to the loading capacity calculation; **step 5**, Subheading 3.1) amino acid onto the SSKKKG-2-CTC resin with DIC (4.2 eq) and HOBT (4.2 eq) in DMF for 1 h moving continuously on a rotary display at R.T. (*see* **Note 12**).
5. Repeat **steps 2** and **3** (Subheading 3.2.1).
6. Add palmitic acid (20 eq) with DIC (25 eq) and DMAP (2.0 eq) in dry DCM to the resin and rotate it in the dark overnight.
7. Once the peptide sequence has finished, wash the resin with DMF, DCM, and MeOH and dry under vacuum overnight.
8. Cleave the lipid peptide from the resin using a mixture of 95% TFA, 2.5% water, and 2.5% TIPS (10 mL for 1 g resin) for 3 h with stirring at R.T.
9. Filter the resin.
10. Add 20 mL of cold diethyl ether (hydrophilic peptide) or 50:50 diethyl ether and hexane (hydrophobic peptide) to the filtered cleavage solution (*see* **Note 13**).
11. Centrifuge at 2000 rpm for 10 min at R.T. to isolate the precipitate.
12. Discard the supernatant and dissolve the precipitated pellet in the mixture solution of 80% acetonitrile and 1% TFA in water. Then lyophilize the crude peptide.

13. Purify the crude lipid peptide using RP-HPLC to obtain a pure peptide **BB3** (*see* **Note 14**).

3.2.2 KKSSC16C16 (BB4) Synthesis (Fig. 1)

1. Swell 308 mg Rink amide MBHA resin (0.2 mmol, 0.65 mmol/g) in DMF for 3 h at R.T.
2. Deprotect the resin using a solution of 20% piperidine in DMF (5 mL, 2 × 10 min). Wash resin with DMF between each deprotection.
3. Wash the resin with DMF three times.
4. Activate Dde-C16 (4.2 eq, 366 mg, synthesis of compound is referred to the protocol [21]) for a minimum of 5 min with HATU in DMF solution (4 eq, 1.6 mL) and DIPEA solution (6.2 eq, 0.216 mL).
5. Add Dde-C16 twice for a minimum of 8 h/coupling. Wash resin with DMF between each coupling to eliminate any remaining amino acid.
6. Wash the resin with DMF three times.
7. End-cap the unreacted resin by adding a mixture of 0.25-mL acetic anhydride, 0.25-mL DIPEA, and 4.5-mL DMF (2 × 10 min). Wash resin with DMF between each end capping.
8. Wash the resin with DMF three times.
9. Deprotect Dde protecting group by using a solution of 5% hydrazine hydrate in DMF (10 mL, 6 × 30 min). Wash resin with excess DMF between each deprotection (*see* **Note 15**).
10. Repeat **steps 3–5** and **8–9** (Subheading 3.2.2) until another C16 lipid is successfully coupled.
11. Wash the resin with DMF three times.
12. Follow **steps 11–13** (Subheading 3.1) until the peptide sequence is complete.
13. Follow **steps 7–13** (Subheading 3.2.1) but dissolve the precipitated pellet in the mixture solution of 50% acetonitrile and 1% TFA in water.
14. Purify the crude lipid peptide using RP-HPLC to obtain a pure peptide **BB4**.

3.2.3 Antigen N_3-PADRE-J8 (BB5) and NH_2-PADRE-J8 (BB6) Synthesis (Fig. 1)

The synthesis of the antigen, **BB5** and **BB6**, is similar to the synthesis of KKSSC16C16, with the exception of Fmoc-amino acid used as the initial two amino acids coupled instead of the lipid, Dde-C16. Further, the final amino acid coupling for **BB5** is azido-acetic acid (synthesis of compound is referred to the protocol [22]), which takes 8 h at R.T.

3.3 Conjugation Between Cyclic Peptide, Lipopeptide, and Antigen (Fig. 3)

1. Add cyclic peptide (1 eq, **BB2**) and lipopeptide (8.4 eq, **BB3**, palmitic acid or Dde-C16) to a solution of DIPEA (10 eq) and HATU (8 eq) in DMF (0.5-mg cyclic peptide/mL) with stirring at R.T. overnight protected with foil.
2. Concentrate the liquid in vacuo to remove DMF.
3. Dissolve crude reaction in the mixture solution of 70% acetonitrile and 1% TFA in water and lyophilize.
4. Purify samples using RP-HPLC to get pure **BB7**, **BB8**, and **BB9,** respectively.
5. Dissolve pure **BB9** (1 eq, 1 μmol, 2 mg) and **BB5** (2.5 eq, 2.6 μmol, 12 mg) in a MeOH and 1-pentanol (30:70) solution, respectively (*see* **Note 16**).
6. Dissolve copper sulphate pentahydrate (8.8 eq, 9 μmol, 2.2 mg) and sodium ascorbate (17.6 eq, 18 μmol, 3.6 mg) in water, respectively.
7. Mix **BB9** and **BB5** solution in small flask.
8. Add copper sulfate and sodium ascorbate water solution into the mixed solution in **step 7** (Subheading 3.3).
9. Stir reaction at 50 °C for 3 h (*see* **Note 17**).
10. Quench reaction by addition to five times water and lyophilize.
11. Purify the crude reaction using RP-HPLC to get pure **BB10**.

3.4 Immunization (Subcutaneous Administration)

1. Acclimate mice in their environment for 7 days prior to the start of the experiment.
2. Divide the mice into different groups and perform ear notching to label each mouse.
3. Collect a blood serum sample (20 μL) from each mouse by a tail bleed (day −1, 20, 27, 34) and dilute it to 200 μL in PBS (1×).
4. Collect the supernatant after centrifuging the blood for 20 min at 3600 rpm, R.T.
5. Store the supernatant at −80 °C for further analysis.
6. Administer 30-μg J8-PADRE emulsified in CFA and PBS (1:1, total 50 μL) to each mouse in the positive control group subcutaneously (day 0) (*see* **Note 18**). Administer three injections on days 21, 28, and 35 of J8-PADRE (30 μg) in PBS (50 μL).
7. Inject 50 μL of PBS to each mouse in the negative control group on days 0, 21, 28, and 35.
8. Inject a single vaccine dose in 50-μL PBS to each mouse in different experimental group on days 0, 21, 28, and 35 (Tables 1 and 2, Fig. 1, *see* **Note 19**).

Table 1
Vaccine dosage in a single mouse for Study 1 (50 μL PBS 1×)

Study 1					
Vaccine groups	**Physical mixture A**	**VC-1**	**VC-2**	**VC-3**	**VC-4**
BB1(1 eq) MW: 1066.3	2.8 μg	2.8 μg	2.8 μg	–	–
BB3(2 eq) MW:1197.7	–	6.3 μg	–	–	–
BB4(1 eq) MW: 954.4	2.5 μg	–	–	–	–
Palmitic acid (2 eq) MW: 256.4	–	–	1.4 μg	–	–
BB6(2 eq) MW: 4611.4	24.4 μg	24.4 μg	24.4 μg	24.4 μg	24.4 μg
BB7(1 eq) MW: 3482.7	–	–	–	9.2 μg	–
BB8(1 eq) MW: 1600.2	–	–	–	–	4.2 μg

Table 2
Vaccine dosage in a single mouse for Study 2 (50 μL PBS 1×)

Study 2			
Vaccine groups	**Physical mixture A**	**VC-5**	**VC-6**
BB1(1 eq), MW: 1066.3	2.8 μg	–	–
BB4(1 eq), MW: 954.4	2.5 μg	–	–
BB5(2 eq), MW: 4694.4	24.8 μg	24.8 μg	–
BB9(1 eq), MW: 1958.6	–	5.2 μg	–
BB10(1 eq), MW: 11347.5	–	–	30 μg

9. Euthanize all mice on day 41 and collect cardiac blood.
10. Collect serum following centrifugation (4000 rpm, 20 min, R. T.) and keep the supernatant at −80 °C freezer for further analysis (*see* **Note 20**).
11. Perform an enzyme-linked immunosorbent assay (ELISA) to measure antigen-specific IgG in serum, according to the previously reported procedure [24] (Fig. 4).

4 Notes

1. Put 3 Å molecular sieves into the 70 °C oven overnight before usage.
2. The coupling procedure is required to be degassed by nitrogen. Moist air will affect the resin loading capacity.

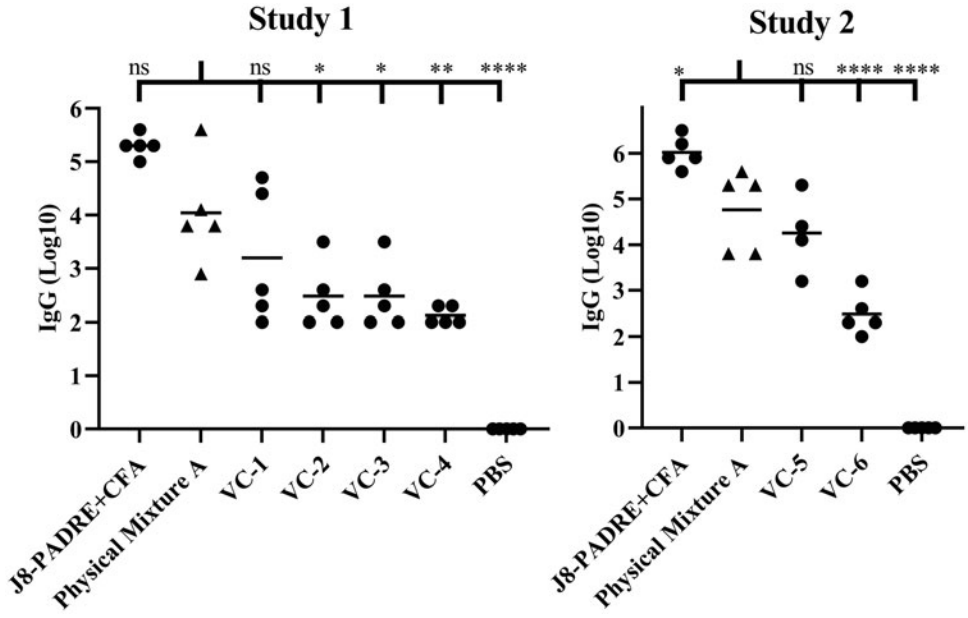

Fig. 4 J8-specific serum IgG antibody titers following four subcutaneous immunizations

3. Due to the poor loading capacity and potential hydrolysis of 2-CTC resin, it is essential to examine the loading capacity to reduce the waste of amino acids during synthesis.
4. TFE can promote peptide cleavage from 2-CTC resin and stabilize the structure of the peptide [25].
5. It is difficult to evaporate acetic acid in vacuo. Due to the crude peptide's high solubility in acetic acid, its precipitation in cold diethyl ether will be hindered, resulting in a lower product yield. Nevertheless, hexane can help evaporate acetic acid.
6. Cold diethyl ether can dissolve the residual TFE and acetic acid while precipitating the peptide, which can improve the purity of crude peptide.
7. It is necessary to purify because impurities will adhere to the targeted peptide after cyclisation. As a consequence of the McLafferty rearrangement of the Boc protecting group, the Boc protecting group (MW: 100.5 g/mol) is readily ionized in ESI-MS, resulting in the appearance of additional peaks on the ESI-MS [26]. So, an extra signal without 100.5 g/mol will appear. Reducing declustering potential during MS measurements will improve this issue.
8. Low concentrations of the linear confirmation of cyclic peptide in DMF prevent the production of dimers or polymers during cyclization.

9. DMF is difficult to completely evaporate and exhibits a large solvent peak on the analytical RP-HPLC, which reduces the purification efficiency. Without a high vacuum evaporator, it will require more than week's lyophilization on a freeze dryer.
10. Only RP-HPLC purification can completely remove DMF, where any remaining DMF reduces the effectiveness of TFA in cleaving the Boc protecting groups.
11. Fmoc-SSKKKG-2-CTC resin contains the tBu side chain protecting group on the serine and the Boc side protecting group on lysine.
12. The carboxylic group in the main chain of Fmoc-Ser-OH that will react with the hydroxyl group in the branch chain of Ser-OH when HATU and DIPEA are used.
13. Hydrophilic peptide will precipitate in cold diethyl ether, but the hydrophobic cleaved protecting group and residual TFA (avoid TFA adduct formation) will dissolve in cold diethyl ether. So, this step will improve the purity of crude peptide.
14. Lipopeptide is a hydrophobic peptide that requires the C4 column for purification, whereas hydrophilic cyclic peptides use the C18 column.
15. Determines the absorbance of the filter solution at 460 nm (blank solution with 1 mL of hydrazine hydrate). If the absorbance value is more than zero. Dde-hydrazine adducts are present, and the Dde deprotection process must be repeated [21].
16. Check the solubility of peptide first. If the solubility is poor, a small amount of dimethyl sulfoxide can be added to ensure that all of the reacting lipids and peptides dissolve properly.
17. The initial color of the solvent is brown-yellow. After the reaction, the solution will become light green or light blue with cloudy white gels. The final product will precipitate indicating reaction completion.
18. Dissolve 0.6 g antigen (J8-PADRE, 20 mice dosage) into 0.5-mL PBS (1×). Resuspend the CFA using a vortex. Add 0.5-mL CFA into the antigen solution. Use 1-mL syringe with 18 G × 38 mm blunt needle to repeat pumping the solution up and down for at least 30 min to form an emulsion.
19. For physical mixture A, VC-1 or VC-2, dissolve antigen into mL PBS (one time) first. Then add cyclic peptide and lipopeptide successively. Finally, sonicate the solution at least 10 min for self-assemble.
20. By centrifugation at more than 3000 rpm, the cardiac blood will be separated into two components: serum as supernatant and precipitation as plasma. In addition, it is essential to avoid

freeze-thaw cycles since they are harmful to numerous serum components such as the IgG antibodies. As a result, it is required to prepare the separate aliquots (20 μL and dilute to 200 μL by PBS 1×) for ELISA measurement.

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Chapter 10

Polyclonal Peptide Antisera

Tina H. Pihl, Kristin E. Engelhart, and Gunnar Houen

Abstract

Polyclonal antibodies are relatively easy to produce and may supplement monoclonal antibodies for some applications or even have some advantages.

The choice of species for production of (peptide) antisera is based on practical considerations, including availability of immunogen (vaccine) and animals. Two major factors govern the production of antisera: the nature of adaptive immune responses, which take place over days/weeks and ethical guidelines for animal welfare.

Here, simple procedures for immunization of mice, rabbits, sheep, goats, pigs, horses, and chickens are presented.

Key words Peptide antisera, Polyclonal antibodies, Mice, Rabbits, Sheep, Goats, Pigs, Horses and chickens, Immunoassays, Immunization, Bleeding, Coagulation, Serum

1 Introduction

Polyclonal antibodies (pAbs) are relatively easy to produce and may supplement monoclonal antibodies (mAbs) for some applications or even have some advantages [1–3].

Traditionally, rabbits have been the species of choice. Goats and sheep have also been much used, and horses have been used for production of antitoxin sera for therapeutic applications, where large amounts of serum are needed. Chickens may be selected for the following reasons: (1) greater evolutionary distance to mammals, (2) ease of pAb production from egg yolk, or (3) low reactivity with human rheumatoid factors, which are present in many human sera and may compromise immunoassays (e.g., sandwich immunoassays) [3, 4]. On the other hand, pigs may be chosen due to high similarity with humans [5].

Here, simple procedures for immunization of mice, rabbits, sheep, goats, pigs, horses, and chickens are presented. The choice of species for production of (peptide) antisera is based on practical considerations, including availability of immunogen (vaccine) and

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animals. Two major factors govern the production of antisera: the nature of adaptive immune responses, which take place over days/weeks and ethical guidelines for animal welfare.

2 Materials

1. Ammonium sulfate.
2. Polyethylene glycol (6000).
3. Adjuvant: aluminum hydroxide ($Al(OH)_3$) 2%, autoclaved (*see* **Note 1**).
4. Saline (0.15 M NaCl), sterile.
5. Sterile needles and syringes or vacutainer system and blood collection tubes with no anticoagulant.
6. For large animals: sterile needles (14G), infusion tube, 1-L sterile glass bottles if a large volume of blood needs to be collected.
7. Autoclave.
8. Centrifuge.
9. Animals.
10. Mice (any strain, e.g., Balb/c).
11. Rabbits (any strain, e.g., New Zealand White).
12. Pigs (any breed, e.g., "mini pigs").
13. Chickens (any breed, e.g., New Hampshire).
14. Sheep (any breed).
15. Goats (any breed).
16. Horses (any breed).
17. Animal facilities suitable for the species in question (*see* **Note 2**).

3 Methods

3.1 Vaccine Preparation

Mix peptide carrier conjugate (*see* Chaps. 6 and 7) 1 mg/mL 1:1 (v:v) with autoclaved aluminum hydroxide adjuvant (2%) (optionally incubate with end-over-end rotation overnight at 5 °C or at room temperature).

See Table 1 for recommended amounts depending on the species in question.

3.2 Immunization

All procedures involving animals must be carried out by trained and authorized personnel in approved facilities and following current ethical guidelines for animal welfare.

Table 1
Immunization and bleed volumes (mL) for various species

Species	Injection route	Injection volume[a]	Bleed volume[b]
Mouse	sc	0.1	0.2–0.5
Rabbit	sc	0.5	10–50
Sheep	sc	1	50–500
Goat	sc	1	50–500
Pig	sc	1	50–500
Horse	sc	1	50–500
Chicken	sc	0.5	10–20[c]

[a]Per animal/per site (usually two sites per animal)
[b]Per animal
[c]Eggs also useful (*see* **Notes 3** and **9**)

Table 2
Immunization and bleeding schedule

Week/day	Action
0/0	Pre-bleed (optional but recommended) (*see* **Note 8**)
1/1	1′ immunization
2/8	1′ bleed
3/15	2′ immunization
4/22	2′ bleed
5/29	3′ immunization
6/36	3′ bleed
7/43	4′ immunization
8/50	4′ bleed
$n/7(n-1)+1$	n′ immunization (*see* **Note 9**)
$n+1/7n+1$	n′ bleed
−/−	Terminal bleed (*see* **Note 10**)

See Table 1 for volumes

See Table 2 for immunization schedule. We recommend the same basic schedule for all species, basically consisting of subcutaneous (sc) immunization every second week and bleeding every second week in between.

The immunization course may be followed by testing for carrier and peptide antibodies by, for example, ELISA as often as required during the process (*see* Subheading 3.4).

3.3 Bleeding

See Table 2 for approximate bleed volumes per animal for each type of animal. Maximal volumes are dependent on the weight of the individual animal [6], which can vary significantly in larger animals such as horses.

Mice are bled from the tail vein.

Rabbits are bled from the ear marginal vein.

Sheep are bled from the jugular vein.

Goats are bled from the jugular vein.

Pigs are bled from the cranial vena cava.

Horses are bled from the jugular vein.

Chickens are bled from a wing vein (*see* **Note 3**).

3.4 Testing

Preliminary testing is done by ELISA (*see* **Note 4**). The production of antibodies may be followed by the increase in absorbance as a function of immunization times or by determination of serum titers (*see* **Note 5**) defined as the dilution of the serum which yields half maximal response in the test system (*see* **Note 6**).

Sera should be analyzed as soon as possible after collection in a test system suitable for the intended use (e.g., immunoblotting, inhibition immunoassay, immunodiffusion, immunoprecipitation, sandwich immunoassay, etc.).

3.5 Processing

Large volumes of serum can be separated from the blood cells by letting the blood sample stand to precipitate by itself, followed by collection of the serum by aspiration and optionally filtering through a cheesecloth and/or centrifugation. Preparation of smaller volumes of serum can be made by centrifugation of the blood samples at 1000 ×*g* for 10–15 min. Blood samples should be left at room temperature for at least 30 min before centrifugation [7]. Optional: Immunoglobulins may be purified from the serum by ammonium sulfate precipitation, ion exchange chromatography, or protein A/G/L affinity chromatography (*see* **Note 7**).

3.6 Storage

Store at −20 °C, −50 °C, or −80 °C (years).

For short term storage at 5 °C: (add sodium azide to 0.02%) (weeks to months).

4 Notes

1. We recommend the use of aluminum hydroxide ($Al(OH)_3$) as the adjuvant of choice. It is a safe adjuvant, also approved for human use, and has few side effects. $Al(OH)_3$ is known to activate the alternative complement pathway as a major mechanism of action [8]. Other adjuvants, for example, Freund's complete/incomplete adjuvant may be used for special purposes but are not recommended.

2. If you do not have access to an animal facility, many companies offer this as a service.
3. For egg-laying hens, antibodies may also be collected and purified from egg yolk (IgY).
4. See [1, 2] or Chaps. 12 and 21.
5. Make a dilution series starting at 1:10 and dilute further ten times from this, etc. Further testing may be done from one of these dilutions by making two-fold titrations.
6. Sometimes, the endpoint titer is used, which is the dilution required to reach background signal.
7. See Chap. 15 and [2].
8. If a pre-immunization bleed is not available, normal serum from nonimmunized animals or a serum from an animal immunized with another (irrelevant) antigen may be used as a control.
9. Polyclonal antibodies (IgY) are easily obtained from egg yolk by ammonium sulfate/PEG fractionation and/or ion exchange chromatography [9, 10] (*see* Chap. 15).
10. When the titer is sufficiently high, immunization may be discontinued but bleeding continued, if larger volumes of serum are required. The volumes and methods depend on the species and must follow local procedures and ethical guidelines.

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Chapter 11

Production of Antibodies to Peptide Targets Using Hybridoma Technology

Nicole Hartwig Trier and Tina Friis

Abstract

Hybridoma technology is a well-established and indispensable tool for generating high-quality monoclonal antibodies and has become one of the most common methods for monoclonal antibody production. In this process, antibody-producing B cells are isolated from mice following immunization of mice with a specific immunogen and fused with an immortal myeloma cell line to form antibody-producing hybridoma cell lines. Hybridoma-derived monoclonal antibodies not only serve as powerful research and diagnostic reagents but have also emerged as the most rapidly expanding class of therapeutic biologicals. In spite of the development of new high-throughput monoclonal antibody generation technologies, hybridoma technology still is applied for antibody production due to its ability to preserve innate functions of immune cells and to preserve natural cognate antibody paring information. In this chapter, an overview of hybridoma technology and the laboratory procedures used for hybridoma production and antibody screening of peptide-specific antibodies are presented.

Key words Enzyme-linked immunosorbent assay, Fusion, Hybridoma enhancing reagent, HybER, Immunization, Isotype determination, Monoclonal antibody, Peptide, Screening, Selection

1 Introduction

Monoclonal antibodies have become a key component in biomedical research and are important for the identification of various targets [1]. Antibodies are among the most used research tools used in multiple setups such as western blotting, enzyme-linked immunosorbent assay (ELISA), immunoprecipitation, immunohistochemistry, and quantitative immunofluorescence, which has advanced the knowledge of protein functions, structures, and interactions, and contributed to improve the understanding of immune responses and infectious diseases [1, 2]. In addition to this, the specific nature of monoclonal antibodies allows them to be used in diagnostic settings (ELISA and flow cytometry), in anatomic pathology (immunohistochemistry), and in treatment of various diseases [3–9].

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Originally, proteins or larger protein domains were used for antibody production; however, for practical reasons, peptides can be used for immunization as well, for example, in cases where the protein is unavailable or when requiring proteins that are difficult to prepare in large amounts [10–15]. Peptide antibodies have the advantage that the antigenic target already is well-defined and may among others recognize posttranslational modified targets, N- or C-terminal regions, conserved regions, intracellular or extracellular domains, cleavage sites, tags, or secondary structures, which can be challenging to generate when using proteins for immunization [3–5, 10, 11, 15–19]. In addition, peptide antibodies may recognize denatured and native proteins and may even be directed to toxic proteins, which can be difficult to use in traditional antibody production [16, 18, 20]. Collectively, peptide antibodies have become powerful tools, not only in immunological research but also for clinical diagnostic purposes as well [3–5, 15, 20, 21].

Antibodies targeting proteins as well as peptides are commonly produced by traditional immunization as originally presented by Köhler and Milstein, who described the combination of the ability of a plasma cell to produce antibodies with the ability of B cell tumors to divide limitless, generating an immortal cell line producing monospecific antibodies [22, 23]. Moreover, they developed an efficient method to select for newly fused hybridomas in a mixture of hybridomas, B cells, and non-fused tumor cells [22]. The development of this technique has become a well-established and proven method, which is used for production of highly specific antibodies, in theory offering limitless production of antibodies specific to the immunized target [22, 24, 25]. Moreover, the technique has gained revolutionary influence, not only in relation to immunologic research but also in biological and medical research as well [23, 24].

Generation of hybridoma-derived peptide antibodies can be divided into several main steps: peptide selection, immunization, fusion, cloning and screening, and characterization (Fig. 1) [26].

One of the first critical steps for generation of peptide antibodies is to select an appropriate peptide and a potential carrier, as immunization with short peptides generally does not induce an immune response with antibodies in high titers [11, 12, 15, 18, 27–30]. Therefore, a peptide of sufficient size, alternatively coupled to a carrier protein, for example, keyhole limpet hemocyanin (KLH), ovalbumin (OVA), or bovine serum albumin (BSA), is often used to induce a sufficient immune response in the selected animal [12, 15, 27–30].

Several variations of the immunization process originally described by Kohler and Milstein exist, which can be used for production of antibodies [11, 12, 15, 18, 31–33]. In general, most protocols yield satisfactory antibody levels, which mainly varies with respect to the immunogen, carrier complex, and

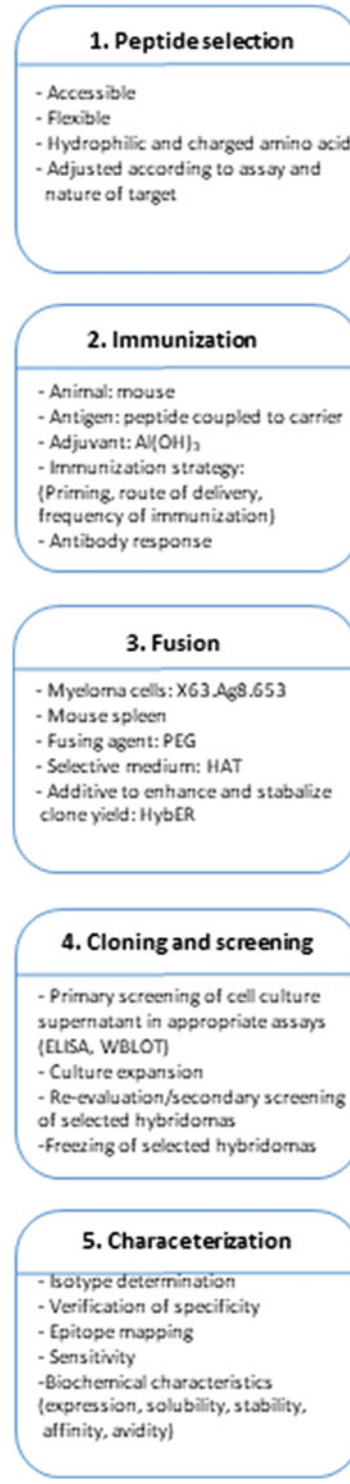

Fig. 1 The generation of hybridoma-derived peptide antibodies can be divided into five steps: (1) Peptide selection, (2) immunization, (3) fusion, (4) cloning and screening, and (5) characterization

immunization strategy [15, 18, 19, 34]. The choice of method used may depend on the nature of the antigen and the type of antibody requested [10, 12, 20, 26, 33].

Produced peptide antibodies are commonly validated by determining antibody specificity, referring to the ability of antibodies to recognize specific targets [16, 18, 26, 35]. Several techniques for screening of antibody specificity exist. The most basic approach employs the antigenic peptide coated to a microtiter plate or a carrier surface, where antibody reactivity is determined using a colorimetric substrate, for example, by ELISA [15, 16, 32]. It is unpredictable whether a peptide antibody will recognize the native protein due to conformational/structural differences between synthetic peptides and peptide epitopes in the native protein and whether the antibody will recognize its target in different assay systems [32]. During the screening and selection phase, it is critical that the peptide antibodies are screened in the assay setup they are intended to be used in and that they are screened toward the target they are supposed to recognize [18, 20, 32].

This chapter describes a straightforward approach for production of monoclonal antibodies using mice, which is designed to give optimal results with minimal injury to the host animal, which has been used extensively and successfully for several years [15, 32, 35]. In addition, this chapter describes the final screening process to ensure generation of assay-specific peptide antibodies.

1.1 Selection of Peptide for Immunization

Several factors such as amino acid composition, peptide structure, peptide length, and final application should be considered before selecting a peptide for production of peptide antibodies [10–12, 16, 18, 36].

Peptides used for immunization are usually 10–25 amino acids long [15, 16, 18, 19, 34, 36], although shorter peptides occasionally are used as well [37]. Peptides shorter than ten amino acids and longer than 25 amino acids are commonly not preferred, as they may elicit antibody responses of low titers or even low affinity or specificity, especially when targeting native protein structures, as short peptides fail to fold, whereas longer peptides may misfold [15, 21, 38, 39]. In addition, the application of shorter peptides may in theory increase the probability of generating a cross-reactive antibody, which may cross-react with other proteins or alternatively intramolecular sequences [40].

Peptides originating from protruding regions, exposed termini and flexible regions such as turns, loops, and connecting regions are often favored [10, 11, 19, 20, 36, 41–43]. Moreover, terminal structures and posttranslational modified sequences are often selected for generation of peptide antibodies as well, whereas hidden structures, transmembrane domains, and conserved regions should be avoided, as these have reduced accessibility or may result in cross-reactivity to other proteins of similar composition [16, 18, 36, 40].

Accessibility is often directly associated with the application of the peptide antibody and ultimately a key term in the identification of an optimal peptide target for peptide antibody production. If the peptide antibodies are designed to recognize a native target to be used in, for example, immunoprecipitation or immunoassays, protruding, surface oriented, and alternatively secondary structures are often selected for immunization, whereas linear peptides or flexible nature often are preferred for generation of peptide antibodies, recognizing denatured targets, for example, in western blotting and ELISA [10, 11, 16, 20, 32, 36, 41–43].

For practical reasons, peptides selected for immunization may often be identical to sequences identified by antibodies, especially if the peptide antibody is to be used for detection of native structures. Based on this, computational analyses and algorithms may be applied for identification of peptide candidates for antibody production, although many of these commonly are based on peptide antigenicity rather than immunogenicity [41, 42, 44–47]. As a consequence, selected peptides used for production of peptide antibodies are often overrepresented in hydrophilic and charged amino acids [15, 18, 19, 34].

Finally, some technical considerations should be taken into account when selecting peptide targets. An additional Cys residue may be located in the N- or C-terminus of the peptide, which is essential for conjugation of the peptide to a carrier [15, 18, 19]. To circumvent cyclization through disulfide bond formation, a peptide without additional Cys residues may be selected, unless deliberately used for specific conformational reasons [19]. Similarly, some amino acids are prone to cyclize in their side chains or to form aggregates, for example, peptides that contain high number of amino acids processing hydrophobic side chains [48, 49]. Similarly, to avoid that the generated peptide antibodies recognize the terminal ends of the peptides, the terminals should be masked or tagged.

1.2 Immunization and Hybridoma Technology

Generation of useful monoclonal antibodies with the desired antigen specificity is dependent on a number of factors such as type of animal used for immunization, choice of immunogen, choice of carrier, choice of adjuvant, immunization schemes and route, source of fusion partner, B cell immortalization procedure, and selection of appropriate methods to test for antibody reactivity.

1.2.1 Choice of Animal

Different animals can be used for production of monoclonal antibodies, but the mouse has been the preferred and most commonly used animal specie for monoclonal antibody production. BALB/c has often been the strain of choice, since the majority of the murine myelomas used for cell fusion was derived from this strain, including X63.Ag8.653, Sp2/0-Ag14, FOX-NY, and NSO/1 [50]. The age and the genetic background of the mouse strain used for immunization have been demonstrated to have an impact on the

antibody response [51–53]. A number of studies have found that the inbred mice strains BALB/c and C57BL/6 with haplotype H-2^b and H-2^d, respectively, elicit weaker antibody responses than other inbred mice strains such as A/J haplotype H-2^a and C3H/He with haplotype H-2^k NMRI. Moreover, outbred mice strains have been found to elicit stronger antibody responses compared to inbred strains [51–53]. The antibody response elicited by immunization with the same antigen in young, adult, and/or old mice of various inbred and outbred mice species has been observed to be highly species dependent. In addition, an antigen that elicits a high antibody response in young mice might only give rise to a weak antibody response in adult or old mice, whereas immunization with the same antigen in another mice strain might show the opposite result [54]. In general, outbred mice seem to be more prone to induce an antibody response with high magnitude than inbred mice. This variation in antibody response has to be taken into consideration, when choosing the source of mice for monoclonal antibody production. Using a combination of different mouse strains for immunization might increase the repertoire of specific antigen bindings sites of the developed antibodies.

1.2.2 Vaccine Formulation

Proteins and peptides that are longer than 15 amino acids can be excellent inducers of an immune response, whereas small peptides less than 15 amino acids cannot induce an immune response. However, to circumvent this and produce effective peptide vaccines that elicits an immune response, small peptides less than 15 amino acids can be conjugated to a carrier protein with superior immunogenicity such as KLH, OVA, or BSA [55, 56]. Alternatively, an immune response to peptides less than 15 amino acids can be induced by conjugating the peptide to the highly immunogenic and effective carrier S3 (secreted proteins from cultures of Bacillus Calmette-Guérin (BCG)) [57]. The immune response to S3 and the conjugated small peptide can be enhanced by priming the mice with an intraperitoneal (IP) injection of BCG vaccine 3–4 weeks prior to administration of the peptide-S3 conjugate.

In addition to conjugation of the small peptide to a carrier with superior immunogenicity, an adjuvant is added to the vaccine in order produce a vaccine with the capacity of inducing sufficient activation of the innate immune system and antibody production [58]. A vaccine adjuvant is a compound that enhances and improves the immunogenicity without having any antigenic effect itself [59, 60], and the choice of adjuvant may impact the affinity, specificity, and functional profile of the induced antibody response [61–63].

Use of squalene-based oil-in-water emulsion (SE), AddaVax™, has been demonstrated to facilitate rapid antibody response upon a single subcutaneous (SC) immunization due to rapid translocation

of the antigen to follicular B cells in the draining lymph node. In contrast, when either of the depot-forming adjuvants aluminum hydroxide or the cationic liposome-based adjuvant (CAF01) was used as adjuvant, the antigen was sequestered at the site of injection, and the presentation of antigen to follicular dendritic cells and B cells was delayed, thus causing a reduction or delay of B cell activation, germinal center formation, and antibody response. However, the depot-forming adjuvants were found to promote higher antibody responses than non-depot forming adjuvants, probably due to a promotion of affinity maturation caused by the constant delivery of antigen to germinal centers. Moreover, the use of aluminum hydroxide as adjuvant has been demonstrated to enhance activation of all three complement pathways [60], and it has been suggested that it can induce and enhance activation of the adaptive immune system by acting on dendritic cells [61].

When choosing an adjuvant, one has to consider, whether the antigen is hydrophilic or hydrophobic. Depot-forming oil-in-water emulsions are recommended for hydrophobic antigens, whereas water-in-oil emulsions are suggested for hydrophilic antigens and antigens containing both hydrophobic and hydrophilic properties [64].

1.2.3 Immunization Route and Time Intervals

The route of vaccine administration and delivery as well as the antigen composition of vaccines have great impact on the immune response, the subclass swift of developed antibodies, and vaccine-induced protection [65–67]. Vaccines based on protein antigens not only tend to swift the subclass of the antibody toward IgG1 or IgG3 but also can induce swift to IgG4 or IgE, whereas vaccines with carbohydrate antigens, for example, bacterial capsular polysaccharide antigens, induce a swift toward subclass IgG2 [68]. The route of administration has been described by Mohanan D. et al. (2010) [67] to be highly important, since they found a strong association between the magnitude of the IgG2a antibody response associated with the Th-1 immune response, whereas the route of administration only had minimal influence on the Th-2 type immune response and the magnitude of the IgG1 antibody response. Despite from the antigen composition and the immunization route, the number of vaccine injection sites, the antigen dose per vaccine, and the number of booster injections also have an impact on the strength of the immune response [64, 69].

If the only purpose of the immunization is generation of antibody production, the route of immunogen administration seems less important, since activation of the humoral immune response with production of IgG1 antibodies with comparable titers have been obtained after intramuscular (IM), IP, intravenous (IV), and SC injections of different vaccines composed of different immunogens and adjuvants [65–67].

For immunization with peptide conjugated to a carrier protein, Harlow and Lane suggested to choose the SC route for the primary immunization and all subsequent boosters except the last booster [1]. Harlow and Lane described that SC injection of the last booster vaccine composed of antigen in the absence of adjuvant only resulted in a poor immune response, whereas IP injection resulted in a fair response and IV injection in the best response. We recommend to use the IP administration route for priming, repeated SC injections for immunization with antigen and adjuvant every 2–3 week until an antibody titer of at least 1600 is obtained and IP injection for the last boosting with antigen in the absence of adjuvant 4 days prior to fusion.

1.2.4 Fusion Partner and Fusion Method

Different methods are suitable to stimulate fusion of B cells and immortalized myeloma cells. In 1975, Köhler and Milstein used Sendai virus for the first hybridoma fusion. Afterward, the use of Sendai virus has been replaced by polyethylene glycol (PEG) and other more efficient fusogens or by electrical pulses [70].

The murine myeloma cell lines X63-Ag 8.653 and SP2/0-Ag 14 origin from the BALB/c mouse strain and do not secrete immunoglobulins, and both are sensitive to hypoxanthine, aminopterin, and thymidine (HAT) medium due to a deficiency mutation in the salvage pathway of purine nucleotide biosynthesis. If either of these two myeloma cell lines are used for fusion, fused B cell-myeloma hybridomas can be selected by cultivation in HAT medium. Only successfully fused B cell-myeloma hybridomas will growth unlimited in HAT medium. Unfused B cells will die due to a limited lifespan, and unfused myeloma cells will die due to the HAT sensitivity [1, 22, 50, 70]. Use of a fusion partner from the same species as the B cell donor promotes formation of hybridomas that are more stable than interspecies hybridomas [55].

1.2.5 Feeder Cells and Growth Media Additives

Immediately after fusion, the hybridoma cells are very instable and fragile. A number of different solutions have been developed to stabilize and rescue the hybridoma cells. Addition of murine peritoneal macrophages has been used as feeder cells in the hybridoma cultures. Use of feeder cells is associated with a number of disadvantages such as use of animals and batch-to-batch variations. Moreover, the feeder cells represent a risk of contamination, and they may interfere with or even overgrow and kill the hybridoma cells growth as they metabolize nutrients in the culture medium [71]. Even though addition of conditioned medium from murine macrophages or fibroblasts can replace feeder cells, the conditioned medium is associated with potential large and unpredictable batch-to-batch variations [71, 72]. Moreover, addition of conditioned medium will dilute the culture medium. These disadvantages can be circumvented by use of the lyophilized hybridoma enhancing reagent, HybER, that can be solubilized in culture medium.

1.3 Antibody Screening

Following fusion, B cell-myeloma hybridomas are cultured in 96-well plates, where only one hybridoma cell initially is found in each well, also referred to as the limiting dilution method [73]. Upon culturing, screening of culture supernatants is performed, where hybridomas that produce the desired peptide antibodies are selected for further culturing [70]. If more than one cell originally was found in each well, it may be necessary to split the cells and conduct another round of screening. Cells that produce specific peptide antibodies are cloned to produce identical daughter clones, thereby expanding the number of positive hybridomas and neutralizing nonspecific hybridomas [26, 50, 70, 73]. This may be followed by a second round of confirmatory tests to verify the specificity of the antibodies produced and to ensure a stable antibody production by the initially selected positive hybridomas [70, 73].

The most applied screening methods include antibody capture assays, antigen capture assays, and functional assays [1, 32, 50, 74, 75]. However, as the peptide antigen used for generation of peptide antibodies often is available in relatively large amounts, it is seldom necessary to use highly sensitive immunoassays or complicated screening approaches initially to check for peptide-specific antibodies. In fact, for many purposes, a colorimetric antibody capture immunoassay is adequate [15, 19, 34], where the peptide originally used for immunization is passively adsorbed to the bottom of microtiter wells, either as a free peptide or as a peptide conjugated to an irrelevant carrier. Next antibodies in the hybridoma culture supernatant are allowed to bind to the peptides, which are detected with an appropriate second-layer reagent, typically an enzyme-linked antibody, and the assay is developed with a colorimetric substrate. Carriers such as streptavidin, resins, or alternatively NH-linked microtiter plates are especially needed, in cases where amino acid residues essential for antibody binding are not accessible either due to direct contact with the microtiter plate upon adsorption or due to inappropriate folding or the peptide [32, 34, 37, 76]. However, when selecting for peptide antibodies solely based on peptide screenings, it is possible that the chosen peptide antibodies only recognize the denatured form, when testing for reactivity to the complete protein. To circumvent this, it is recommended that peptide antibody screenings should be confirmed by screening for reactivity to native or denatured proteins as well, especially if the peptide antibodies are intended to react to the denatured or native forms of the complete protein [32]. In fact, one of the critical points when generating peptide antibodies is that they may not recognize the native protein due to structural differences between the synthetic peptide and the peptide epitope in the native protein structure. Hence, if the intended use of the peptide antibody is to react to the native protein structure, antibodies should be screened for reactivity toward the native protein structure as well.

A crucial aspect of peptide antibody screening relates to profiling in different assay systems as well. This especially relates to antibodies, which are to be used in different systems. This phenomenon, termed assay restriction [31, 77, 78], relates to how an antibody recognizes its target epitope in the context of the assay system used. In this case, the epitope could be masked, denatured, or rendered inaccessible by the immobilization procedure adopted within a given technique. Thus, because the peptide-antibody recognizes its target in ELISA does not necessarily mean that is will recognizes its target in other immunoassays. Since peptides usually do not have the same conformation when they are free in solution, coupled to a protein carrier or adsorbed to microtiter plates, the antigenic activity of synthetic peptides/proteins may vary between immunoassay formats [79, 80]. Thus, it is essential to test the peptide antibody in a variety of formats and most appropriate in the format of intended use before any conclusions are drawn. In addition to selection of the appropriate assay for antibody selection, it may be essential to test for reactivity to various protein structures in the specific assay as well, especially if the structure of the given protein is prone to structural changes [32]. For example, when coating proteins to the surface of microtiter plates through passive adsorption, a molten globule intermediate may be generated, which is structurally different from the native and a completely denatured state [32]. As a result, the antibody may not recognize the protein or peptide in question.

1.4 Antibody Characterization

Following selection of stable antibody-expressing clones expressing antibodies to the selected target, peptide antibodies are characterized in terms of reactivity, specificity, and cross-reactivity, which can be achieved using cell culture supernatants or purified antibodies [1, 19, 34, 37, 50]. The simplest approach for determination of these factors is titration assays, where antibody reactivity to the peptide antigen and a panel of related peptides and various forms of the original protein is determined [2, 19, 32, 37]. Alternatively, western blotting may be conducted to determine antibody specificity, where specific antibodies commonly are determined by the visualization of a single band at the known molecular size of the target protein, whereas the presence of multiple bands may indicate nonspecific reactivity [2].

Moreover, biochemical characteristics such as solubility, stability, and binding characteristics (e.g., performance in antibody affinity chromatography) should be determined [26, 81–83]. In addition to this, the antibody's isotype should be determined. Isotype determination not only serves to define the immunoglobulin class or subclass but also confirms the presence of a single isotype and is required for selection of appropriate isotype-matched control antibodies in different assays [84]. An easy and fast way to determine the isotype is by using commercially available subtyping strips.

Alternatively, ELISA or bead-based immunoassays may be conducted, where capture antibodies that specifically recognize the heavy chain of each isotype and kappa and lambda light chains are conjugated to beads or coated into the wells of microtiter plates, whereafter the reactivity of the peptide-specific antibodies to each isotype is determined [84].

Although antibodies usually are specific for a single target, some antibodies may occasionally cross-react with other targets or exhibit dual specificity [85]. This may occur when the antibody recognizes more than one antigenic determinant, either because of sequence similarity or structural similarity [34, 40]. In addition to this, peptide antibodies to protein isoforms should be characterized carefully, as these antibodies may be cross-reactive as well. To circumvent this, antibodies like these should be characterized by analyzing for reactivity to all isoforms [34].

Peptide antibodies can be characterized through epitope mapping using synthetic peptides, where key amino acid residues essential for antibody recognition are identified, by replacing all amino acids in the peptide immunogen with either alanine or amino acids with similar side chain functionality followed by analysis of antibody reactivity to the substituted peptides [19, 34, 37, 76]. Other approaches such as X-ray crystallography provide high-resolution information of peptides antibody epitopes as well, although this approach is rather time-consuming [85].

Further characterization of peptide antibodies may include affinity measurements of peptide-antibody interactions using bio-layer interferometry; surface plasmon resonance technology, for example, BIACore or other techniques [86–88]. Once characterized, peptide-specific monoclonal antibodies can serve as investigative research tools, or may be applied in diagnostic assays or as therapeutic agents.

2 Materials

2.1 Immunizations, Fusion, Cell Cultivation, and Cloning

1. Syringes.
2. Needles.
3. Scissors.
4. Forceps.
5. Eppendorf tubes.
6. EDTA coated tubes.
7. Gaze.
8. Counting tubes.
9. Pasteur pipets.

10. Serological pipets.
11. 96-well culture plates with lids.
12. CO_2 incubator.
13. Laminar flow cabinet.
14. Homogenizer (mortar and pestle).
15. Microscope.
16. Centrifuge.
17. Counting chamber.
18. Stop watch.
19. −80 °C freezer.
20. −135 °C freezer or N_2 tank.
21. Female mice, for example, NMRI strain or Balb/c strain.
22. BCG vaccine for priming, if S3 is used as carrier.
23. 1 mg/mL carrier (e.g., S3, KLH, OVA or BSA) conjugated with peptide. Store at −20 °C.
24. Adjuvant, for example, 10 mg/mL aluminum hydroxide. Store at Store at room temperature (RT).
25. Merthiolate for conservation of the prepared ready to use vaccine. Store at 4 °C.
26. Saline, 4 °C.
27. 70% Ethanol. Store at RT.
28. Crystal violet in acetic acid for leucocyte staining. Store at RT.
29. 0.2% Nigrosine in saline or 0.4% Trypan blue. Store at RT.
30. Myeloma cells, for example, X63.Ag8.653.
31. Serum-free medium:

    Dulbecco's Modified Eagle Medium (DMEM) with high glucose and GlutaMax and 1% penicillin/streptomycin (Pen/-Strep, 10.000 µ/mL Pen/10.000 µg/mL Strep). Store at 4 °C and use within a week.
32. PEG: (PEG, 7.5% v/v DMSO):

    14.1-mL melted polyethylenglycol (PEG) (47% v/v), 2.25-mL dimethylsulfoxide (DMSO) (7.5% v/v), and 13.65 mL DMEM with high glucose and GlutaMax. Store at 4 °C and use within a week.

    HAT+HybER medium: DMEM with high glucose and GlutaMax +10% fetal calf serum (FCS), HAT (0.038 mM hypoxanthine, 0.4-µM aminopterin, 0.1-mM thymidine), 0.5% HybER, and 1% Pen/Strep. Store at 4 °C and use within a week.

This medium is used for cultivation of fused cells.

Cloning medium: DMEM with high glucose and GlutaMax +10% FCS, 0.038-mM hypoxanthine, 0.1-mM thymidine, 1% HybER, and 1% Pen/Strep. Store at 4 °C and use within a week.

This medium is used for cultivation of the cells during the cloning process.

HT medium: DMEM with high glucose and GlutaMax +10% FCS, 0.038-mM hypoxanthine, 0.1-mM thymidine, and 1% Pen/Strep. Store at 4 °C and use within a week.

This medium is used for cultivation of the cells, when the cloning process is completed; cells have to be expanded in order to be able to establish a cell bank composted of, for example, eight vials each containing approximately 5×10^5 *hybridoma cells.*

Culture medium: DMEM with high glucose and GlutaMax +10% FCS, and 1% Pen/Strep. Store at 4 °C and use within a week.

This medium is used for cultivation of hybridomas.

Freezing medium: Either DMEM with high glucose and GlutaMax +10% FCS, 1% Pen/Strep, and 5% DMSO or FCS + 5% DMSO. Store at 4 °C and use within a week.

2.2 Antibody Screening

1. Coating buffer, for example, carbonate buffer: 15-mM Na_2CO_3, 35-mM $NaHCO_3$, 0.001% phenol red, pH 9.6. Store at 4 °C. Discard if changes in pH occur (*see* **Note 1**).
2. Peptide solution/suspension at 1 mg/mL in phosphate-buffered saline (PBS). The free peptides are supplied as a lyophilized product. Dissolve the free peptides according to the manufacturer's instructions to a concentration of 1 mg/mL (*see* **Notes 2** and **3**).
3. Cell culture supernatants/ peptide antibody. Store at 4 °C.
4. 96-well microtiter plates.
5. Alkaline phosphatase (AP)-substrate buffer: 1-M diethanolamine, 0.5-mM $MgCl_2$, pH 9.8. Store at 4 °C.
6. Tris-Tween-NaCl buffer: 0.05-M Tris, 0.3-M NaCl, 1% Tween 20, pH 7.5 (*see* **Note 4**). Store at 4 °C.
7. Secondary antibody: AP-conjugated anti-mouse IgG_{1-4}/IgA/IgM antibody.
8. AP buffer: Dissolve phosphatase substrate tablets (4-nitrophenyl phosphate) in AP-substrate buffer to a final concentration of 1 mg/mL. The substrate buffer is light-sensitive and should be prepared immediately before use and kept in the dark. Remains should be discarded (*see* **Note 5**).

3 Methods

3.1 Immunization

1. In three to five mice, inject intraperitoneally 0.2-mL Bacillus Calmette-Gúerin (BCG) vaccine per mouse (~ 2 human doses), 3–4 weeks before the first immunization with peptide coupled to the S3 carrier.
2. Bleed the mice immediately before the first immunization with the peptide vaccine (use EDTA-containing tubes). Centrifuge the tube at 600 $\times g$ for 5 min and harvest plasma. This plasma sample, called Bleed 0, will serve as a baseline control to use for assay setup and during the immunization course for test of antibody reactivity.
3. Prepare a vaccine containing 20–50 μg/mL S3 conjugated with peptide and 2 mg/mL $Al(OH)_3$ by diluting S3 conjugated with peptide in saline and add it drop wise to the Al $(OH)_3$ while stirring the mixture slowly. Vaccines for all immunizations except the booster vaccine can be prepared simultaneously if 0.05% Merthiolate is added for preservation. Store at 4 °C.
4. Inject subcutaneously 0.5 mL of the peptide vaccine.
5. Repeat immunizations every 2–3 week.
6. Bled the mice 10 days after the third immunization, harvest plasma as described above and test for antigen reactivity in ELISA or the assay system for intended antibody use.
7. Repeat testing for antigen reactivity 10 days after all subsequent immunizations until the antibody titer is at least 1600. The antibody titer is defined as the reciprocal value of the dilution that gives an OD value that is half the maximal measured OD value.
8. After approximately four to six immunization, when the antibody titer is above 1600, inject IP 0.5-mL peptide-S3 conjugate in the absence of adjuvant in order to boost the number of antigen-specific B cells in the spleen.
9. Sacrifice the mouse 3 days after the booster injection.

3.2 Preparation of Myeloma Cells for Cell Fusion

Harvest of the spleen cells from an immunized and boosted mouse results in approximately 2×10^8 antigen-specific B cells. Myeloma cells and B cells should be fused in a ratio of 1:5, thus requiring a minimum of 4×10^7 myeloma cells.

1. Thaw the murine myeloma fusion partner, X63.Ag8.653, 1–2 weeks prior to cell fusion.
2. Calculate the cell population doubling time by daily cell counting.

3. Three to four days before cell fusion dilute the X63.Ag8.653 cells according to the calculated population doubling time to obtain 4×10^7 exponentially growing myeloma cells with high viability on the day of fusion.

3.3 Fusion

1. Prior to cell fusion, preheat 1-mL PEG, 5-ml serum-free medium, and 225 mL HAT+HybER medium to 37 °C and cool 100-mL serum-free medium to 4 °C.
2. Preparation of the X63.Ag8.653 myeloma cells:
   (a) Count the number of cells and determine the viability.
   (b) Transfer 4×10^7 cells to a 50-mL tube and centrifuge for 10 min at 400 $\times g$ at RT.
   (c) Resuspend the cells in serum-free medium at a density of 1×10^6 cells/mL, and store at 37 °C, 6.5% CO_2 until use.
3. Preparation of spleen cells:
   (a) Transfer 2–3 mL cold serum-free medium to a homogenizer.
   (b) Dip the mouse in 70% ethanol and transfer it to a laminar flow cabinet.
   (c) Open the mouse by using sterile scissors and forceps.
   (d) Immediately, transfer the spleen to the cold serum-free medium in the homogenizer.
   (e) Open the heart and transfer heart blood to an EDTA-containing tube. Centrifuge the tube at 600 $\times g$ for 5 min, harvest plasma, and store it at—20 °C for later use.
   (f) Grind spleen tissue with a mortar and pestle to obtain spleen cells in suspension.
   (g) Filtrate the spleen cell suspension into a 50-mL tube through sterile gaze.
   (h) Rinse homogenizer and gaze with 10-mL cold serum-free medium and transfer to the spleen cell suspension.
   (i) Add cold serum-free medium to a total volume of 50 mL.
   (j) Centrifuge the cells for 10 min at 400 $\times g$.
   (k) Resuspend cell pellet in 10-mL cold serum-free medium.
   (l) Use methyl violet acetic acid for leucocyte counting to count the number of viable B cells.
4. Mixture of spleen cells and myeloma cells.
   (a) Add myeloma cells to the tube with spleen cells in a ratio of one myeloma cell to five spleen cells, and add cold serum-free medium to 50 mL.
   (b) Centrifuge for 10 min at 400 $\times g$.
   (c) Remove all supernatant from the pellet.

5. Fusion.
   (a) Add slowly 1 mL of 37 °C warm PEG to the cell pellet, while carefully stirring with a 1-mL serological pipette.
   (b) Continue the slow stirring for 2 min.
   (c) During a 3 min period, add slowly 2 mL of 37 °C warm serum-free medium while stirring.
   (d) During a period of 0.5–1 min, add 7 mL of 37 °C warm HAT+HybER medium.
   (e) Dilute to a density of approximately 1×10^6 cells/mL in HAT+HybER medium.
   (f) Transfer 225-μL cell suspension per well to 96-well cell culture plates.
   (g) Culture the cells at 37 °C, 6.5% CO_2 and 90% humidity for 7 days.
   (h) Replace the medium with freshly prepared HAT+HybER medium and continue cultivation for another 4 days.
   (i) Harvest 150-μL culture supernatant from cell-containing wells and replace with 150-μL HT medium.
   (j) Test hybridoma supernatants for antibody production in preferred assays.
   (k) Select a number of wells with cells producing antigen-specific antibodies and transfer cells to T25 flasks.
   (l) Follow the cell density and add fresh HT medium when the medium turns yellow or the cell density reaches $4–5 \times 10^5$ cells/mL (the maximal volume in a T25 flask is 15 mL).
   (m) Test the hybridoma supernatants for antibody production again after 2 weeks of cell propagation in culture flasks.

3.4 Use of the Limiting Dilution Technique to Obtain Hybridoma Monoclonality

(a) Prepare the cloning medium.
(b) Count the number of living and dead cells (use, e.g., 0.2% nigrosine staining).
(c) Dilute the cells in 5–10 mL cloning medium to a density of five cells/mL, 2.5 cells/mL, and 1.25 cell/mL.
(d) Seed 200 μL of cell suspension into 20 wells per cell density (use the 60 center wells) in 96-well-culture plates.
(e) Incubate the cells at 37 °C, 6.5% CO_2, and 90% humidity for up to 14 days or until clones appear in the wells.
(f) Test culture supernatants from, for example, 25 cell-containing wells in selected assays.

(g) Expand antibody-producing cells from selected wells, and repeat the cloning step of antibody-producing cells until positive antibody-specific reaction is obtained in culture supernatant from all tested wells. Then the first cloning step is considered finished.

(h) Subclone the cells as described above except that the cells in the lowest density should be seeded in 60 wells and cells in two other densities should each be seeded in 30 wells.

(i) Test culture supernatant from, for example, 60 cell-containing wells in selected test assays.

(j) Expand the monoclonal hybridoma cells in HT medium in culture flasks in order to create a cell bank composed of, for example, eight vials each containing 5×10^6 cells with a viability of at least 80%.

(k) Count the number of viable cells by using 0.2% nigrosine.

(l) Centrifuge the cells for 10 min at 400 $\times g$.

(m) Resuspend the cells at a density of 5×10^6 cells/ml in freezing medium and immediately transfer the cells to a − 80 °C freezer in a specialized freezing container to secure a controlled freezing process of −1 °C per min.

(n) Transfer the cells to a −135 °C freezer or liquid N_2 for storage.

3.5 Enzyme-Linked Immunosorbent Assay for Determination of Peptide-Specific Antibodies

1. Dilute free peptides or peptides coupled to another carrier than the one used for immunization to a final concentration of 1 μg/mL in coating buffer (*see* **Notes 1–3**).
2. Coat microtiter plates with 100 μL of the peptide solution in each well (*see* **Note 6**). Incubate overnight at 4 °C (*see* **Note 7**).
3. Remove any non-adsorbed peptides and wash the plates three times with TTN buffer (250 μL/well) (*see* **Note 7**).
4. Add 250-μL TTN as blocking buffer to each well to block free binding sites and incubate at RT for 20 min (*see* **Note 4**).
5. Empty the wells and add 100 μL of plasma or cell culture supernatants (*see* **Note 8**).
6. Incubate for 1 h at RT on a platform shaker.
7. Wash the wells three times as described in **step 3**.
8. Add 100 μL of AP-conjugated secondary antibody reagent diluted in TTN to a final concentration of 1 μg/mL (*see* **Note 5**).
9. Incubate the microtiter plate on a platform shaker for 1 h at RT.
10. Following incubation with secondary antibody repeat washing steps described in **step 3**.

11. Detect the presence of bound antibodies by adding 100 μL of freshly prepared *p*-NPP substrate in AP buffer solution to each well. Place the plates on a platform shaker and read the plate when the solution turns yellow (*see* **Note 5**).
12. The absorbance is measured at 405 nm, with background subtraction at 650 nm on a microtiter plate reader or on an equivalent instrument measuring the wavelength of 405 nm and a reference wavelength of 650 nm (*see* **Note 9**).

4 Notes

1. Various coating buffers may be applied, such as carbonate buffer, PBS, tris buffer, etc.
2. A common issue with synthetic peptides, especially those containing hydrophobic amino acid residues, is insolubility in aqueous solutions. Other solvents recommended for peptide solvation include dimethylformamide (DMF), DMSO, or different mixtures of DMF and water or DMSO and water. However, note that DMSO may oxide SH groups to disulfides. Some peptides may also be soluble in acetonitrile/water mixtures.
3. After lyophilization, peptides retain significant amounts of water. Peptides are oxidized over time at −20 °C and slowly degrade. Thus, the peptide stock solution should be stored in small aliquots upon arrival to prevent degradation caused by repeated freezing and thawing.
4. Alternatively, TTN buffer can be replaced with PBS with Tween 20.
5. Alternatively, other color reagents may be applied for detection of antibody reaction, for example, appropriate peroxidase-conjugated secondary antibody in combination with o-phenylenediamine and stop solution.
6. Coat with the peptide originally used for immunization (but without conjugation to the carrier used for immunization) or alternatively the peptide conjugated to an irrelevant carrier protein or the whole protein, possibly in a denatured version depending on the original location of the immunogen in the protein structure.
7. Alternatively, coat the plates with antigens for 2 h at RT.
8. Suitable starting dilutions are 1:100 for plasma and 1:10 for hybridoma culture supernatants.
9. Measure the absorbance at an appropriate wavelength according to the selected color reaction detection system.

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Chapter 12

Affinity Chromatography for Anti-Glucosylated Adhesin Antibody Purification: Depletion of Nonspecific Anti-Protein Antibodies and Antibody Recovery with Unconventional Elution Solutions

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Abstract

Antibodies from sera of a multiple sclerosis (MS) patient subpopulation preferentially recognize the hyperglucosylated adhesin protein HMW1ct(Glc) of the pathogen *Haemophilus influenzae*. This protein is the first example of an N-glucosylated native antigen candidate, potentially triggering pathogenic antibodies in MS. Specific antibodies in patients' sera can be isolated exploiting their biospecific interaction with antigens by affinity chromatography. Herein, the proteins HMW1ct and HMW1ct(Glc) were first immobilized on appropriately functionalized supports and further used to purify antibodies directly from MS patients sera. We describe a protocol to obtain an antibody fraction specifically recognizing the glusosylated residues on the HMW1ct(Glc) adhesin protein depleting antibodies to the unglucosylated HMW1ct sequence. Different elution solutions have been tested to recover the purified antibody fraction, strongly bound to the immobilized HMW1ct(Glc) adhesin protein.

Key words Antibody isolation, Adhesin protein HMW1ct, Elution solutions, Affinity chromatography

1 Introduction

We have recently shown that the hyperglucosylated adhesin protein HMW1ct(Glc) of non-typeable *Haemophilus influenzae* (NTHi) specifically detects IgM and IgG in multiple sclerosis (MS) patient sera, discriminating between MS patients and controls [1]. At variance, antibodies against the non-glucosylated protein HMW1ct were identified in both patients and control sera, and therefore, they were not able to discriminate between MS patients and controls. The relatively high titers observed for both antigens

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HMW1ct and HMW1ct(Glc) in NBD sera is not surprising as NTHi is a rather ubiquitous human pathogen to which a high percentage of the population has been exposed. In any case, these results suggest that the hyperglucosylation of this bacterial protein favorably affects antibody recognition in MS.

Next, with the hypothesis in mind that N-Glc modification is a key determinant only for specific MS serum antibodies, we focused our research on the isolation of specific antibodies against glucosylated residues on HMW1ct(Glc), depleting those directed to the amino acid sequence epitopes shared with the non-glucosylated HMW1ct. To this purpose, we set up an affinity chromatography protocol to purify antibodies exploiting the biospecific interaction between antigen and antibody. Affinity chromatography is a widely used method for antibody purification, due to its high selectivity and rapidity [2]. Its efficacy is based on the binding characteristics of the antibody and the ligand used for antibody capture. In the case of HMW1ct(Glc), antibodies directed to the glucosylated residues showed a particularly high affinity with a $K_D = 2.8 \times 10^{-8}$ M [1]. In fact, the most widely used standard affinity chromatography protocols usually applied in antibody purification need modifications in order to break the high affinity binding of antibodies to adhesin protein HMW1ct(Glc). For this purpose, the evaluation of different elution solutions can sensibly improve the antibody purification protocol, increasing the yield of specific anti-HMW1ct (Glc) antibodies. As far as the elution process is concerned, there are different conditions to perform it in the best way, also based on the type of ligand present in the resin.

Herein, we report a protocol to deplete anti-HMW1ct antibodies using sequential immunoaffinity columns with unglucosylated HMW1ct and glucosylated HMW1ct(Glc) adhesin proteins, testing three different elution solutions in order to improve the yield of specific anti-HMW1ct(Glc) antibodies. These optimization tests were then carried out using minimal amounts of sepharose and protein, selecting the most efficient protocol for detaching the antibodies of the resin.

2 Materials

CNBr-sepharose resin and reagents ware commercially available. Proteins HMW1ct and HMW1ct(Glc) are obtained in-house following the previously reported protocol [1]. All solutions and buffers are prepared with milliQ water and filtered daily with a 0.22-μm filtering system. All affinity chromatography steps are performed at ambient temperature.

2.1 Immunoaffinity Chromatography

1. CNBr-sepharose resin.
2. Pipettes (10–100 and 100–1000 μL volumes) and their corresponding tips.
3. HCl, 1 mM solution.
4. Coupling buffer: $NaHCO_3$ 0.1 M, NaCl 0.5 M at pH 8.3. Weigh out 4.2 g $NaHCO_3$ and 14.6 g NaCl; add 400 mL of milli-Q water, adjust pH at 8.3 and then add milli-Q water till 500 mL final volume (*see* **Note 1**). Store at +4 °C.
5. Glycine 0.2 M pH 8.0 solution. Weigh out 1.5 g Glycine add 80 mL of milli-Q water, adjust pH at 8.3 and then add milli-Q water till 100 mL final volume. Store at +4 °C.
6. Acetate buffer: Sodium acetate (AcNa) 0.1 M, NaCl 0.5 M pH 4.3. Weigh out 0.82 g AcNa and 2.92 g NaCl; add 0.8 L of milli-Q water, adjust pH at 9.6, and then add milli-Q water till 100 mL final volume. Store at +4 °C.
7. Dulbecco-PBS pH 7.2.
8. Elution buffer 1: Glycine 0.2 M pH 2.6. Weigh out 1.5 g glycine add 80 mL of milli-Q water, adjust pH at 2.6, and then add milli-Q water till 100 mL final volume. Store at +4 °C.
9. Elution buffer 2: NaOH 40 mM, NaCl 1 M. Weigh out 0.16 g NaOH and 5.84 g NaCl, add milli-Q water till 100 mL final volume.
10. Elution buffer 3: NaOH 100 mM, NaCl 2 M. Weigh out 0.4 g NaOH and 11.68 g NaCl, add milli-Q water till 100 mL final volume.
11. Neutralization solution $NaHCO_3$ 0.5 M: Weigh out 4.2 g $NaHCO_3$ and add milli-Q water till 100 mL final volume.
12. Centrifuge able to work at 4000 rpm.

2.2 ELISA

1. Clear flat-bottom immuno 96-well polystyrene plates.
2. Pipettes (1–10 and 10–100 μL volumes) and multichannel pipette (100–200 μL volumes).
3. Microplate washer, optional.
4. Coating buffer: Dulbecco's phosphate buffer saline (D-PBS) (*see* **Note 1**). Store at +4 °C.
5. Use saline solution 0.9% NaCl containing 0.05% of Tween-20 as washing buffer (*see* **Note 2**).
6. Blocking solution: 10% FBS in washing buffer (*see* **Notes 3** and **4**).
7. Sera of previously diagnosed multiple sclerosis patients and controls (*see* **Note 5**).

8. Secondary antibody solution: alkaline phosphatase-conjugated anti-human IgG antibody diluted 1:3000 in dilution buffer (*see* **Note 6**).
9. Substrate: 1 mg/mL of 4-nitrophenyl phosphate disodium salt hexahydrate (*p*NPP) in coating buffer (*see* **Note 7**).
10. Stop solution: 1 M NaOH.
11. ELISA reader: A microplate reader equipped with a 405 nm filter.

3 Methods

3.1 Sequential Antibody Purification Design

With the idea in mind that a N-Glc modification is a key determinant for specific MS serum antibodies, the isolation of specific antibodies against glucosylated residues on HMW1ct(Glc) depleting those directed to the amino acid sequence epitopes shared with the non-glucosylated HMW1ct is mandatory. Then, using the purified antigens HMW1ct(Glc) and the non-glucosylated HMW1ct, an immunoaffinity-based fractionation is performed for representative MS (MS 1) and control (control 1) sera, as outlined schematically in Fig. 1. For this purpose, proteins are immobilized on agarose resins, specifically CNBr-Sepharose, which consists of 4% cross-linked agarose beads.

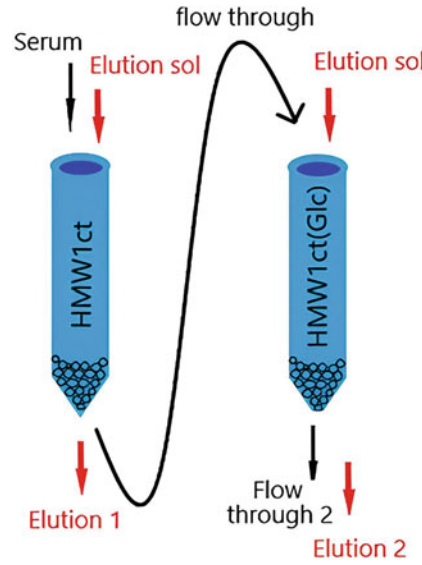

Fig. 1 Schematic process of antibody fractionation during the immunoaffinity purification of antibodies from sera samples

3.2 Antigens Immobilization on the Sepharose Resin

1. Wash the resins twice with HCl 1 mM (1 mL/100 mg resin) and centrifuge at 200 $\times g$ for 3 min. Remove the upper solution and add fresh HCl 1 mM, mix with beads an centrifuge repeating this washing step with Milli-Q water and then with coupling buffer.
2. Dissolve proteins separately in coupling buffer [1 mg/mL] and apply it to the resin overnight at room temperature under agitation.
3. Check the absorbance at 280 nm of each protein dissolved in coupling buffer before and after the reaction with the resin to quantify the antigen bound to the resin.
4. Wash the resin with coupling buffer twice and block free sites on resin by adding glycine 0.2 M pH 8.0 (1 mL/100 mg of resin) for 2 h at room temperature.
5. Wash the resin with coupling buffer and then with acetate buffer (1 mL/100 mg of resin).

3.3 Antibody Purification Protocol by Affinity Chromatography

1. Dilute sera samples 1:10 in D-PBS pH 7.2 independently, pass it through a 0.22 μm filter, and apply it first to the Sepharose column in which HMW1ct is immobilized. Recover the flow through (not retained fraction) and apply it again for other two times to the column. Then repeat the step in the second column containing HMW1ct(Glc) protein.
2. Wash the columns with D-PBS pH 7.2 and coupling buffer (1 mL/1 mL of diluted serum).
3. Elute specific antibodies of both columns with Elution buffer 1, 2, and/or 3 (1 mL/1 mL of diluted serum) and collect fractions of around 1 mL volume separately. Collected fractions should be immediately neutralized by adding 10% of $NaHCO_3$ 0.5 M.
4. If required, concentrate the fractions by centrifuging with an amicon centrifugal filter 50 KDa and recover antibodies with D-PBS pH 7.2, or dialyzed.
5. Check sample absorbance of each fraction at 280 nm to calculate IgG concentrations (A280 nm/1,4 = Ab concentration [mg/mL]).

3.4 ELISA

1. Test each antibody fraction against each protein separately.
2. Add 100 μL/well of a solution 10 μg/mL of protein in coating buffer of a 96-well immunoplate and coat overnight at +4 °C.
3. Wash with 250 μL of washing solution (3×) (*see* **Note 8**). Empty completely the plate, add 100 μL/well of blocking solution, and incubate for 1 h at room temperature.

4. Dilute the antibody fractions 1:10 (*see* **Note 9**). Add the diluents to the wells and incubate for 3 h at ambient temperature (*see* **Notes 10** and **11**). Triplicates of each sera sample and controls should be tested.
5. Wash the plates with washing buffer three times. Add 100 μL/well of secondary antibody solution and incubate plate for 2 h at ambient temperature.
6. Wash the plates with washing buffer (3×). Apply 100 μL/well of substrate solution and stop the reaction after 30 min approximately with 50 μL/well of stop solution.
7. Read absorbance at 450 nm using a micro-plate ELISA reader.
8. Evaluate data for each protein and fraction separately. Subtract blanks for each sera absorbance to remove nonspecific signals. Compare evaluated results and plot all data in the graph highlighting differences among fractions (Fig. 2).

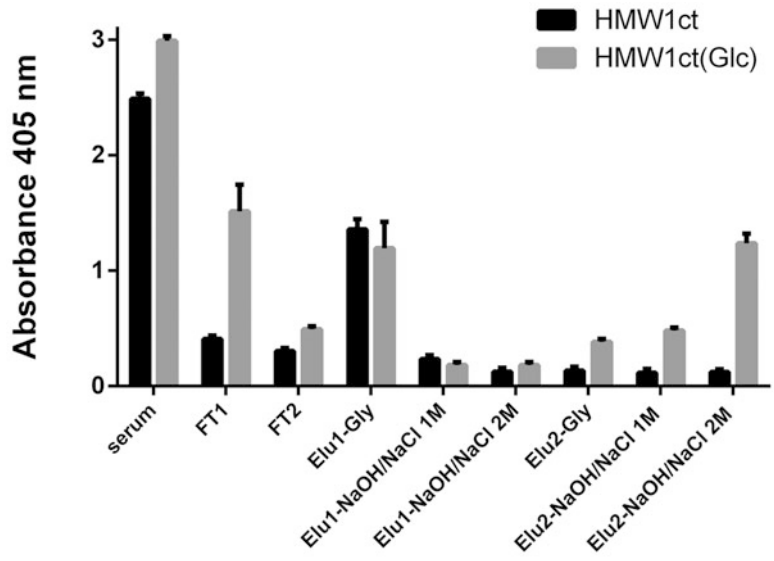

Fig. 2 SP-ELISA of antibody fractions obtained from two sequential sepharose columns bearing the immobilized non-glucosylated HMW1ct and hyperglucosylated HMW1ct(Glc). Serum of multiple sclerosis patient was flowed and fractions flow through 1 (FT1), flow through 2 (FT2), and the eluted fractions from column with HMW1ct (Elu1) and column with HMW1ct(Glc) immobilized (Elu2) using the following elution solutions: Gly 0.2 M pH 2.6 (Gly), 40 mM NaOH/1 M NaCl (NaOH/NaCl 1 M), and 100 mM NaOH/2 M NaCl (NaOH/NaCl 2 M) were collected. Each point is calculated as mean ± standard deviation for $n = 3$ independent experiments

4 Notes

1. Filter all buffer solutions in 500 mL flask with PES membranes (0.22 μm).
2. Alternatively, saline solution 0.9% NaCl can be replaced with PBS buffer.
3. Prepare this solution fresh each time.
4. Alternative blocking solutions exist such as BSA, casein or porcine gelatine. Also, commercially available blocking agents are available. Select and optimize the blocking solution for each ELISA system.
5. Recruit patients according to a shared inclusion protocol. The most extended protocol employed for multiple sclerosis is established international diagnostic criteria [3, 4].
6. It is strongly recommended to optimize reagents and concentrations such as antigens and antibodies for each ELISA system.
7. Prepare fresh prior to use and employ a darkness bottle (or kept in the dark or wrapped) because substrate is light sensitive.
8. The use of a microplate washer helps reducing washing times. Be careful that the washer completely empty the wells; otherwise, it can be a cross-contamination faking the experiment results.
9. Note that dilution works for antibody concentration around 0.05 mg/mL. The ELISA absorbance in serum should be higher considering that IgG concentration in the whole sera (10 mg/mL) is 300 times higher than in eluted fractions (0.03 mg/mL).
10. It's highly recommended to test also blanks and sera controls for a correct background evaluation.
11. Sera samples can be incubated overnight at 4 °C alternatively.

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Chapter 13

Identification and Validation of Peptides Specifying SARS-CoV-2 B-Cell Epitopes Eliciting Neutralizing Antibodies

Chit Laa Poh, Abdul Aziz Al-Fattah Bin Yahaya, Huey Tyng Cheong, and Hui Xuan Lim

Abstract

Vaccination is an effective means of inducing immune protection to prevent transmissible diseases. During the Covid-19 pandemic, immunizations using traditional and novel vaccine platforms such as the inactivated SARSCo-V-2 vaccine, adenoviral-vectored, and nucleic acid-based mRNA vaccines have been relatively successful in controlling the rates of infection and hospitalizations. Nevertheless, the danger posed by the emergence of SARS-CoV-2 variants would set the stage for the design of next generation vaccines. To overcome the lack of efficacy of current vaccines against emerging SARS-CoV-2 variants, new vaccines must be able to overcome the reduced effectiveness of the current vaccines. Since the current Covid-19 vaccines are dependent on the whole S-protein of Wuhan strain as the antigen, mutations have rendered the current Covid-19 vaccines less effective against variants of concern (VoCs). Instead of using the whole S-protein, peptide-based epitopes could be predicted using immunoinformatic approaches, simulation of the 3D structures, overlapping peptides covering the whole length of the S-protein or peptide arrays based on synthetic peptide combinatorial libraries comprising peptides recognizable by monoclonal antibodies. B-cell epitopes were predicted, and immunogenicity of peptides was validated in mice by immunizing mice with peptides conjugated to keyhole limpet hemocyanin (KLH) mixed with Montanide 51 as an adjuvant. The immunogenicity of epitopes that could elicit peptide specific IgGs was determined by peptide-based ELISA. Neutralizing activities were determined by cPass and pseudovirus-based neutralization assays.

Key words B-cell epitopes, SARS-CoV-2, Neutralizing antibodies

1 Introduction

1.1 The Role of Bioinformatics in Development of Next-Generation SARS-CoV-2 Vaccines

Whole virus particles are used in the production of inactivated vaccines. For manufacturing of inactivated vaccines, large amounts of live viruses are required to be cultured before being inactivated with chemical compounds such as formaldehyde or S-caprolactone. Therefore, there is concern with production of the inactivated

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SARSCo-V-2 vaccines due to the necessity to grow large amounts of live viruses and process them [1].

A new concept in vaccination is incorporating peptides specifying B- and T-cell epitopes into a peptide-based multi-epitope vaccine that could elicit both humoral and cellular immune responses. Designing such epitope-based vaccines would rely on immunoinformatic approaches. Protein sequences are downloaded from the NCBI database (www.ncbi.nlmnih.gov). Linear B-cell epitopes are predicted using multiple software such as BepiPred, ABCPred, BCPREDS, and ElliPro [2, 3]. Linear epitopes predicted by BepiPred 2.0 in IEDB had recommended cutoff values above 0.5 (with a specificity of 57% and sensitivity of 59%), and they were subjected to antigenicity analysis by the VaxiJen v2.0 program, which was an alignment-independent prediction software based on physicochemical properties of proteins [2]. The antigenicity of predicted B-cell epitopes was set at VaxiJen score above the default cutoff of 0.4. Besides antigenicity scores, other properties such as hydrophilicity determined by the Parker method (Parker et al. 1986); surface accessibility by Emini, Kolaskar, and Tongaonkar; surface prediction through the IEDB (http://tools.iedb.org/bcell/); and 3D-structure based on Pymol 2.3.4 [4] could be utilized to predict B-cell epitopes with high accuracy.

The rationale behind a vaccine that incorporates peptides carrying SARS-CoV-2 epitopes is to introduce antigenic regions of the virus instead of the whole protein that could trigger the immune response. When these peptides are processed by antigen-presenting cells such as dendritic cells, the epitope-specifying peptides are presented on the surface, primarily in complex with MHC II and activate T helper cells, which in turn can interact with naïve B cells to activate them and trigger the humoral immune system. The peptides specifying these epitopes are also involved in the activation pathways of cytotoxic T cells (CTLs) and helper T cells (TH cells). The existence of these epitopes provides vaccine researchers with a new tool for the design of novel vaccine constructs that comprise peptide sequences specifying these immunogenic epitopes, which would confer strong immune response and protection while eliminating the inclusion of potentially toxic or allergenic components. The technology required to select and identify viral antigenic sequences already exists in the form of online computational resources used in bioinformatics studies [5].

Databases containing the genomic sequences of SARS-CoV-2 viruses derived from patients globally allow researchers to gain further insights into the functionality and application of SARS-CoV-2 B-cell epitopes. Due to the variables concerning the epitopes to be incorporated into a working vaccine prototype, all peptides should have potent antigenicity and high levels of genetic conservation to ensure a strong and long-lasting immune response in immunized individuals. In silico analysis of virus genes and

corresponding proteins would be instrumental in determining the loci of these epitopes in the SARS-CoV-2 proteome and cut down the time necessary to identify them through computational algorithms in computers and software, which would allow researchers to rapidly identify regions within a genome that could predict B-cell epitopes, surface accessibility, antigenicity, and hydrophilicity. These properties, when analyzed simultaneously, could determine if a predicted B-cell epitope is able to provide the protective effects desired in a peptide-based vaccine.

The purpose of incorporating B-cell epitopes in a vaccine construct is to facilitate the activation of B cells to elicit antibody production. This is crucial to halt viral spread by binding to virus particles to neutralize them and prevent them from infecting other cells. Immunoglobulin-γ (abbreviated to IgG) is the most common type of serum antibody present in humans. Its function is primarily the opsonization of pathogens for engulfment by phagocytes and activation of the complement system. IgG is most concentrated in the blood and extracellular fluid, and it is ideal for interacting with the pathogen as it passes through body tissues via the circulatory system. The incorporation of epitopes that could also trigger the production of neutralizing antibodies would bolster the B-cell response as well.

1.2 Overlapping Peptide Library

The overlapping peptide library is a common strategy for identifying and mapping of B cell epitopes. Peptides are generated by dividing the entire sequence of a specific protein antigen into many overlapping peptides of equal length. Poh et al. (2020) identified two immunodominant linear B-cell epitopes, S14P5 and S21P2, by using pools of overlapping linear B-cell peptides spanning the entire S glycoprotein of SARS-CoV-2. Two linear epitopes in SARS-CoV-2 S glycoprotein were strongly detected by probing sera from COVID-19 patients. Sera depleted of antibodies targeting either peptides S14P5 or S21P2 led to a >20% reduction in pseudotyped lentivirus neutralization, validating that antibodies targeting these two linear S epitopes were important for neutralizing SARS-CoV-2 [6]. Profiling B-cell epitopes using sera from animals immunized with overlapping peptides spanning the receptor binding domain (RBD) revealed the molecular determinants of antigenicity. Three linear peptides specifying B-cell epitopes (R345, R405, and R465) were shown to elicit strong and specific IgG antibody responses from the SARS-CoV-2 S1 protein [7]. A peptide library was employed to identify antigenic determinants recognized by monoclonal antibodies (mAbs). Three conserved linear B-cell epitopes, S12, S19, and S49, were identified as the immunodominant epitopes in the study of Wang et al. (2022), which reported the binding of six monoclonal antibodies to 49 overlapping peptides that covered the extracellular region of S protein. The actual length of these epitopes could be further

truncated to S12.2286TDAVDCALDPLS297, S19.2464FERDISTEIYQA475, and S49.41202ELGKYEQYIKWP1213 [8].

1.3 Peptide Arrays

Two linear epitopes in the RBD were identified in the study of Makdasi et al. (2021) through the binding of six antibodies to an overlapping peptide array that cover the entire RBD of SARS-CoV-2. One of these epitopes spanning amino acids S376–390 (TFKCYGVSPTKLNDL) was targeted by antibodies 24 and 67, while the second epitope S396–410 (YADSFVIUGDEVRQI) was targeted by antibodies 69 and 90 [9]. Based on peptide arrays, Farrera-Soler et al. (2020) identified three immunodominant linear epitopes (S655–672, S787–822, and S1147–1158), which were recognized in >40% of COVID-19 patients. Two of these epitopes (S655–672 and S787–822) corresponded to key proteolytic sites on the spike proteins S1/S2 and S2, which have been shown to play a critical role in efficient viral entry [10]. A peptide array was used to map the dominant linear B-cell epitopes recognized through screening of convalescent sera. Yi et al. (2020) used a peptide array of 20-mer overlapping peptides covering the entire spike (S), membrane (M), and envelope (E) proteins to identify five dominant epitopes that reacted with the majority of COVID-19 convalescent sera. Only three of the epitopes were shown to compete for the neutralization activity of convalescent sera, implying that antibodies elicited by these epitopes were involved in the neutralization of SARS-CoV-2 [11].

1.4 Monoclonal Antibodies

The majority of monoclonal antibodies isolated to date specifically target the RBD on the spike protein, which allows SARS-CoV-2 to interact with the ACE2 receptor. Three monoclonal antibodies (15G9, 12C10, and 10D2) targeting the peptides R345, R405, and R465, respectively, were shown to inhibit the RBD–ACE2 interaction with an inhibition rate of 20–60% [7]. Among the three mAbs, 12C10, which targeted the peptide R405 (DEVRQIAPGQTGKIADYNYK), could strongly bind to both the SARS-CoV and SARS-CoV-2 S proteins, indicating that 12C10 was a cross-reactive antibody [7]. Makdasi et al. (2021) identified two linear epitopes in the RBD through the binding of six antibodies to an overlapping peptide array that covered the entire RBD of SARS-CoV-2. One of these epitopes spanning amino acids S376–390 (TFKCYGVSPTKLNDL) was targeted by antibodies 24 and 67, while the second epitope S396–410 (YADSFVIUGDEVRQI) was targeted by antibodies 69 and 90 [9]. A broadly neutralizing beta-coronavirus mAb, S2P6, was isolated from a COVID-19 convalescent patient and used in the investigation by Pinto et al. (2021). S2P6 targeted the highly conserved S protein region spanning amino acids S1148-1156 (FKEELDKYF) in the S2 subunit stem helix. S2P6 was shown to completely neutralize the infection of authentic SARS-CoV-2 in Vero-E6 cells as well as other SARS-

CoV-2 related pseudotyped viruses through the inhibition of membrane fusion [12].

1.5 Validation of the Immunogenicity of B-Cell and T-Cell Epitopes in Mice

The vaccine candidate would be prepared as an emulsion of peptides adjuvanted with either Freund's adjuvant or Montanide ISA-51. BALB/c mice were used for validating the immunogenicity of the peptides representing SARS-CoV-2 epitopes. Mice were divided into groups ($n = 5$), each receiving a specified dose (50 μg/mouse) of the vaccine that was injected subcutaneously at set intervals (day 0, day 14, and day 28). Validation of the immunogenicity of B-cell epitopes was conducted using sera collected from blood. Binding antibodies were detected by ELISA, while neutralizing antibodies were determined by either pseudotyped lentivirus or cPass neutralizing antibody detection assays. Immunogenicity of T-cell epitopes is conducted by isolating splenocytes from the mice spleens for use in flow cytometry after intracellular staining (ICS).

2 Materials

2.1 ELISA for Measuring Peptide-Specific IgG Antibody

Immulon 2HB (high binding) 96-well flat bottom microtiter plate (Thermo Fisher Scientific, Pittsburgh, PA, USA).

Doubly distilled water or deionized water.

Carbonate-bicarbonate buffer: 50-mM carbonate-bicarbonate buffer, pH 9.6. Weigh 1.59-g Na_2CO_3 and 2.93-g $NaHCO_3$. Add deionized water to a volume of 1 L. Adjust to pH 9.6. Store at 4 °C.

Phosphate-buffered saline (PBS): Dissolve each 5-g Gibco™ PBS Tablet in 500 mL of deionized water. The pH will be 7.45 and requires no adjustment.

Phosphate-buffered saline with Tween® detergent (PBST): 0.05% Tween 20 in PBS.

Blocking buffer (1% BSA in PBS).

Dilution buffer (0.5% BSA in PBS).

HRP-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA).

3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution (Thermo Fisher Scientific, Waltham, MA, USA).

1 M sulfuric acid H_2SO_4: Add 56 mL of 95% sulfuric acid stock solution into a bottle containing 500 mL of deionized water. Add deionized water to a volume of 1 L.

Microplate reader (Infinite M200 PRO, TECAN, Männedorf, Switzerland).

2.2 Plaque Reduction Assay for Measuring Neutralizing Antibodies in Immune Sera

Multi-well culture plate, 6-well (SPL, #30006).

Vero E6 cells (ATCC CRL-1586).

Dulbecco's Modified Eagle's Medium (DMEM, Nacalai Tesque, Kyoto, Japan).

FBS (Gibco, Boston, MA, USA).

Penicillin and streptomycin (Nacalai Tesque, Japan).

1.5% of carboxymethyl cellulose (CMC) in DMEM with 2% FBS and 1% of PSA: Weight 15 g of CMC powder (Sigma-Aldrich, St Louis, MO, USA) into the glass bottle. Add deionized water to a volume of 450 mL. Allow the CMC powder to dissolve overnight in a shaking incubator at 220 rpm, 37 °C. Add 500 mL of double concentrated DMEM (2×) containing 20 mL of FBS and 10 mL of penicillin and streptomycin. Store at 4 °C.

0.5% crystal violet solution: Weight 2.5 g crystal violet powder (Sigma-Aldrich, St Louis, MO, USA) into a glass bottle. Add 100 mL of methanol. Add PBS to a volume of 500 mL.

CTL Immunospot S6 Versa (Cellular Technology Limited, United States).

2.3 Pseudotyped Lentivirus Neutralization Assay

Multi-well culture plate, 96-well (SPL, #30096).

ACE2-293 T cells (Takara Bio USA, CA, USA).

Passive lysis buffer (Promega, #E1941).

Luciferase Assay System (Promega, #E1510).

Promega GloMax Luminometer.

2.4 Genscript cPass Surrogate Virus Neutralizing-Antibody Test

The cPass sVNT kit includes all the necessary reagents required for the assay, which are listed in Table 1.

3 Methods

3.1 ELISA for Measuring Peptide-Specific IgG Antibody

1. Add each KLH-conjugated peptide dissolved with double distilled water into the carbonate-bicarbonate coating buffer (pH 9.6) at a concentration of 10 μg/mL.
2. Add 100 μL/well of peptide solution in triplicates into wells within an Immulon 2HB (high binding) 96-well flat bottom microtiter plate (Thermo Fisher Scientific, Pittsburgh, PA, USA) and incubate for 12 h at 4 °C.
3. Block the plate with 200 μL/well of blocking buffer (1% BSA in PBS) for 1 h at 37 °C to prevent nonspecific antibody binding.

Table 1
Components included in Genscript cPass sVNT kit

Item #	Component
1	96-Well capture plate pre-coated with hACE2
2	Positive control reagent
3	Negative control reagent
4	SARS-CoV-2 RBD conjugated with HRP
5	HRP dilution buffer
6	Sample dilution buffer
7	20× wash solution
8	TMB substrate
9	Stop solution
10	Plate sealer

4. Wash the plate four times with 200 μL/well of 0.05% Tween 20 in PBS (PBST).
5. Heat inactivate all the immune sera for 30 min at 56 °C.
6. Dilute heat-inactivated immune sera collected from mice immunized with each KLH-conjugated peptide at 1:1000, 1:10,000, and 1:100,000 dilutions in dilution buffer (0.5% BSA in PBS).
7. Add the diluted immune sera at 100 μL/well in triplicates and incubate for 1 h at 37 °C.
8. Wash the plate four times with 200 μL/well of 0.05% Tween 20 in PBS (PBST).
9. Add 100 μL/well of HRP-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) at 1:1500 dilution in dilution buffer and incubate for 1 h at 37 °C.
10. Wash the plate four times with 200 μL/well of 0.05% Tween 20 in PBS (PBST) to remove any unbound IgG.
11. Add 100 μL/well of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution (Thermo Fisher Scientific, Waltham, MA, USA) and incubate for 15 min at room temperature in the dark.
12. Add 50 μL/well of 1 M sulfuric acid (H_2SO_4) to stop the enzymatic reaction.
13. Measure the absorbance at 450 nm by using a microplate reader.

3.2 Plaque Reduction Assay for Measuring Neutralizing Antibodies in Immune Sera

1. Seed 7×10^5 Vero E6 cells per well in 2 mL of complete Dulbecco's Modified Eagle's Medium (DMEM, Nacalai Tesque, Kyoto, Japan) as culture media in each well of a 6-well plate and culture overnight to reach 80% confluency.
2. Heat inactivate all the immune sera collected from mice immunized with each KLH-conjugated peptide for 30 min at 56 °C.
3. Incubate a fixed dose (60PFU/well) of SARS-CoV-2 pseudotyped virus (pseudotyped lentiviral particles expressing SARS-CoV-2 S protein) with serially diluted immune sera 37 °C for 1 h to ensure the potential interactions between the neutralizing antibodies and RBD occur prior to RBD-ACE interaction.
4. Add the virus-antibody complex to infect Vero E6 monolayers at 37 °C for 1 h.
5. Discard the inoculum and overlay the infected cells with 1.5% of carboxymethyl cellulose (CMC) (Sigma Aldrich, MO, USA) in DMEM with 2% FBS and 1% penicillin-streptomycin. Incubate the plate for 72 h at 37 °C.
6. Discard the media. Fix and stain the cells with 3 mL/well of 0.5% crystal violet solution.
7. Determine the number of plaques in each well by counting plaques manually against a white background and calculate the reduction in infection relative to the control wells containing only surrogate viruses without immune sera. Take the image of plaques in each well using the CTL Immunospot S6 Versa (Cellular Technology Limited, United States).

3.3 Pseudotyped Lentivirus Neutralization Assay

1. Seed 2×10^4 ACE2-293 T cells in 100 μL of complete DMEM media in each well of a 96-well flat bottom cell culture plate (SPL, #30096).
2. Heat inactivate all the immune sera collected from mice immunized with each KLH-conjugated peptide for 30 min at 56 °C.
3. Incubate an equal volume of pseudotyped lentivirus (pseudotyped lentiviral particles expressing SARS-CoV-2 S protein) at the final volume of 50 μL with serially diluted immune sera for 1 h at 37 °C.
4. Add the virus-antibody complex to infect ACE2-293 T-cell monolayers for 1 h at 37 °C.
5. Add 150 μL of culture media to each well and incubate the cells for 48 h at 37 °C.
6. Harvest the cells and remove the culture media.
7. Wash the cells with sterile PBS twice and lyse the cell in 20 μL of 1 × passive lysis buffer (Promega, #E1941) with gentle shaking at 400 ×*g* for 30 min at 37 °C.
8. Measure the luciferase activity by using Luciferase Assay System (Promega, #E1510) in a Promega GloMax Luminometer.

3.4 Neutralizing Antibody Detection Using the Genscript cPass Surrogate Virus Neutralizing-Antibody Test

The Spike (S) protein on the SARS-CoV-2 virus contains the receptor binding domain (RBD) that recognizes and binds to the cell surface receptor, human angiotensin-converting enzyme 2 (hACE2), which in turn triggers viral uptake into the host cell. Infection also initiates the production of neutralizing antibodies by B cells.

The receptor binding domain of the SARS-CoV-2 S protein presents a significant target for B-cell antibody production as the antibodies generated have strong immunogenic potency. By mimicking the interaction of the RBD region to a human host cell in vitro, it is possible to determine the presence of neutralizing antibodies in a sample of sera while removing the requirement for cell culture, live viruses, and biosafety level 3 facilities. Thus, this principle has been used to develop the Genscript cPass surrogate Virus Neutralising Test (sVNT) kit, which determines neutralizing antibody levels by incorporating the principle of antigen-antibody binding between the RBD and the antibodies in a sample of sera with an enzyme-linked immunosorbent assay (ELISA) (*see* **Note 1**).

The Genscript cPass SVNT kit is designed around the concept of semiquantitatively detecting antibody levels. The kit comes with two main components: a recombinant SARS-CoV-2 RBD peptide conjugated to horseradish peroxidase (HRP) and a 96-well plate pre-coated with human ACE-2 receptors. During the assay, a sera sample would be diluted in a 1:10 factor and incubated with the recombinant SARS-CoV-2 RBD-HRP conjugate. The mixture of RBD-HRP with diluted sera would then be transferred into the wells of the hACE-2 coated 96-well plate and incubated further. The TMB substrate would be added to each well to develop the sample before the plate is read at an optical density (OD value) of 450 nm.

Interaction between an antigen, the SARS-CoV-2 RBD conjugated to HRP with neutralizing antibodies present in a sera sample, is the working principle behind the cPass assay. As shown in Fig. 1, the neutralizing antibodies in the sera would bind to the HRP-conjugated RBD, which would prevent the binding of the RBD to the hACE2 receptors present in the wells of the pre-coated plate. When the TMB substrate is added, it would react with the HRP resulting in a color change that can be enumerated through the OD value. Neutralizing antibody titers for each sample is determined through their respective OD values; a percentage of inhibition is calculated using the following formula:

$$\%\text{of inhibition} = \left[1 - \frac{\text{OD value of sample}}{\text{OD value of negative control}}\right] \times 100\%.$$

Inhibition (in %) represents the neutralizing antibody titer present in the respective dilution of the sera. A threshold value of

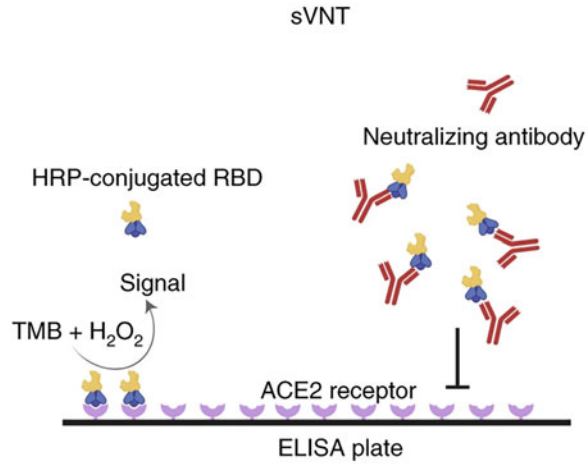

Fig. 1 Illustration of general principle in the Genescript cPass sVNT assay

>30% indicates that there is a sufficient titer present to confer immune protection in the sera (positive result) and anything below the threshold would indicate low antibody titer (negative result).

3.5 Genscript cPass sVNT Assay Experimental Protocol

1. Preparation of samples.
   (i) Blood samples obtained must be stored at 4 °C until required for use.
   (ii) Each sample should be centrifuged at 1000 ×*g* for 15 min at 4 °C to separate the sera from the whole blood.
   (iii) A pipette is used to transfer the sera from each tube into a fresh tube, which will be refrigerated until required for the assay (*see* **Note 2**).
2. Preparation of reagents.
   The reagents should be taken out of refrigeration and allowed to gradually warm to room temperature before the assay is carried out and vortexed. They must be promptly put back into refrigeration after use.
   (i) SARS-CoV-2 RBD and HRP conjugate preparation:
   The RBD-HRP conjugate is diluted in a 1:1000 dilution in accordance to the number of samples to be tested. That is, 10 μL of RBD-HRP conjugate with 10 mL of dilution buffer to test 96 samples.
   (ii) Wash solution preparation:
   The wash solution supplied with the kit has a concentration of 20× and requires dilution to 1× using deionized water. To prepare 1 L of 1× wash solution, add 760 mL of deionized water to 40 mL of 20× wash solution.

(iii) Sample and control preparation.

Each sample and the included positive and negative controls must be diluted in a 1:10 dilution factor. To prepare a diluted sample for 1 well, 10 μL of sera or control must be diluted with 90 μL of the included sample dilution buffer.

3. Conducting the assay.

(i) Mix each individual sample and the positive and negative control with the diluted HRP-RBD conjugate in a 1:1 ratio. To prepare each sample and HRP-RBD conjugate mixture, add 60 μL of sample to 60 μL diluted HRP-RBD conjugate. Multiple the quantity of each part by the number of replicates to prepare enough mixed sample and HRP-RBD conjugate for the desired number of replicates.

(ii) Incubate the samples and the positive and negative controls at 37 °C for 30 min.

(iii) Add 100 μL of the positive and negative control and each sample to their respective wells in the ACE-2 pre-coated 96-well plate.

(iv) Cover the plate with the plate sealer and incubate for 15 min at 37 °C.

(v) Wash the plate four times with 260 μL of the prepared 1× wash solution.

(vi) Invert and pat the plate onto a dry paper towel to remove any residual liquid.

(vii) Add 100 μL of TMB substrate to each well and incubate at 20–25 °C in the dark for 15 min. Timing begins after addition of TMB substrate to the first set of wells,

(viii) Stop solution (50 μL) is added to each well after the elapsed incubation time.

(ix) The absorbance of the plate is read at 450 nm (*see* **Note 3**).

4. Interpretation of results.

(i) In order to ensure the accuracy of the assay, the positive and negative results must fall within an OD range of <0.3 and >1.0, respectively. The assay results are invalid if the control parameters are not met.

(ii) The OD value obtained from each sample and the negative control is used to determine the % of inhibition induced by the sera. The % of inhibition is calculated as follows:

$$\%\text{of inhibition} = \left[1 - \frac{\text{OD value of sample}}{\text{OD value of negative control}}\right] \times 100\%.$$

(iii) The results of the assay are interpreted semiquantitatively as positive or negative based on the % of inhibition obtained from each sample. Percentage of inhibition above 30% indicates detection of neutralizing antibodies, while ≤30% indicates no detectable neutralizing antibodies.

Items	Cutoff	Result	Interpretation
SARS-CoV-2 neutralizing antibody test	≥30%	Positive	SARS-CoV-2 neutralizing antibody detected
	<30%	Negative	No detectable SARS-CoV-2 neutralizing antibody

4 Notes

1. The unopened kit is stable at 2–8 °C until its expiry date and the opened kit is stable for up to 1 month from the date of opening at 2–8 °C.
2. Sera samples should be kept on ice or at 4 °C until the assay is to be conducted. Storage of sera at −80 °C is required for long-term storage. All sera should be handled as if it is capable of transmitting infectious agents.
3. Read the plate immediately after adding the stop solution.

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Chapter 14

Characterization of Peptide Antibodies by Epitope Mapping Using Resin-Bound and Soluble Peptides

Nicole Hartwig Trier

Abstract

Characterization of peptide antibodies through identification of their target epitopes is of utmost importance, as information about epitopes provide important knowledge, among others, for discovery and development of new therapeutics, vaccines, and diagnostics.

This chapter describes a strategy for mapping of continuous peptide antibody epitopes using resin-bound and soluble peptides. The approach combines three different types of peptide sets for full characterization of peptide antibodies; (i) overlapping peptides, used to locate antigenic regions; (ii) truncated peptides, used to identify the minimal peptide length required for antibody binding; and (iii) substituted peptides, used to identify the key residues important for antibody binding and to determine the specific contribution of key residues. For initial screening, resin-bound peptides are used for epitope estimation, while soluble peptides subsequently are used for final epitope characterization and identification of critical hot spot residues. The combination of resin-bound peptides and soluble peptides for epitope mapping provides a time-saving and straightforward approach for characterization of antibodies recognizing continuous epitopes, which applies to peptide antibodies and occasionally antibodies directed to larger proteins as well.

Key words Continuous epitopes, Epitope mapping, Enzyme-linked immunosorbent assay, Peptide antibodies, Resin-bound peptides, Synthetic peptides

1 Introduction

Peptide antibodies are powerful tools and are, in contrast to many other antibodies, usually directed to a specific peptide region limited in size [1–5]. Peptide antibodies were originally described four decades ago, where peptides coupled to carrier proteins were used for generation of peptide-specific antibodies [6].

Characterization of peptide antibodies is essential in relation to their applications, as peptide antibodies can be applied in various situations, for example, as diagnostic tools and for research purposes [1, 4, 5, 7]. Peptide antibodies have been proven to be especially useful for identification of single point mutations in proteins related to detection of various cancer forms [7–9].

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Characterization of peptide antibodies is usually accomplished by epitope mapping, which determines the antibody binding sites on their target antigens [2, 3, 10, 11]. Among several techniques, the use of synthetic peptides in combination with immunoassays has emerged as a powerful tool for identification and characterization of peptide antibody epitopes [2, 3, 11–15], although this relatively simple approach competes with more high-resolution approaches such as X-ray crystallography [16].

Nevertheless, the use of synthetic peptides as targets has become an alternative method in identifying individual epitopes, because the majority of peptides are easily synthesized, and the assays used for testing provide reliable results in little time. Moreover, the synthetic peptide fragments of short size can fold into similar structures of the native antigen structure, thus allowing the antibody to interact with the peptide antigen. Moreover, peptide antibodies are easily characterized using synthetic peptides, because the immunogen used for antibody generation is known, and as the immunogenic peptide used to generate the antibody is limited in size, favoring continuous epitopes [1].

An often applied approach for initial epitope estimation originally described by Geysen is to employ overlapping peptides of varying length and overlap, where antibody reactivity is analyzed to peptides covering a protein or an antigenic region of interest [10, 11, 17–21]. This approach, referred to as peptide scanning or pepscan, is primarily used to identify continuous epitopes, where the key amino acids that mediate contact to the antibody most often are located within the primary structure, usually not exceeding 15 amino acids in length. Occasionally, pepscans can be applied to indicate components of discontinuous epitopes in the event that two distant peptides each contain sufficient structural elements that allow them to bind the antibody separately, indicating that these peptides contribute to the intact epitope [22].

The combination of peptide scanning and screening of truncated and substituted peptides has been employed with success for systematic mapping of continuous epitopes and epitopes recognized by peptide antibodies [18, 20, 21, 23]. This approach may entail combinations of free peptides in solution and resin-bound peptides, where the peptides are anchored to a solid support in the C-terminal end of the peptide. For initial screening steps, overlapping resin-bound peptides are employed for rough epitope estimation, while identification of the final epitope and key amino acids is performed using systematically truncated and substituted peptides in solution.

Design of peptide set and details of the procedures for characterization of continuous epitopes are described in the following paragraphs. Mapping of continuous epitopes using this approach provides a rapid, straightforward, and cost-effective approach for characterization of peptide antibodies.

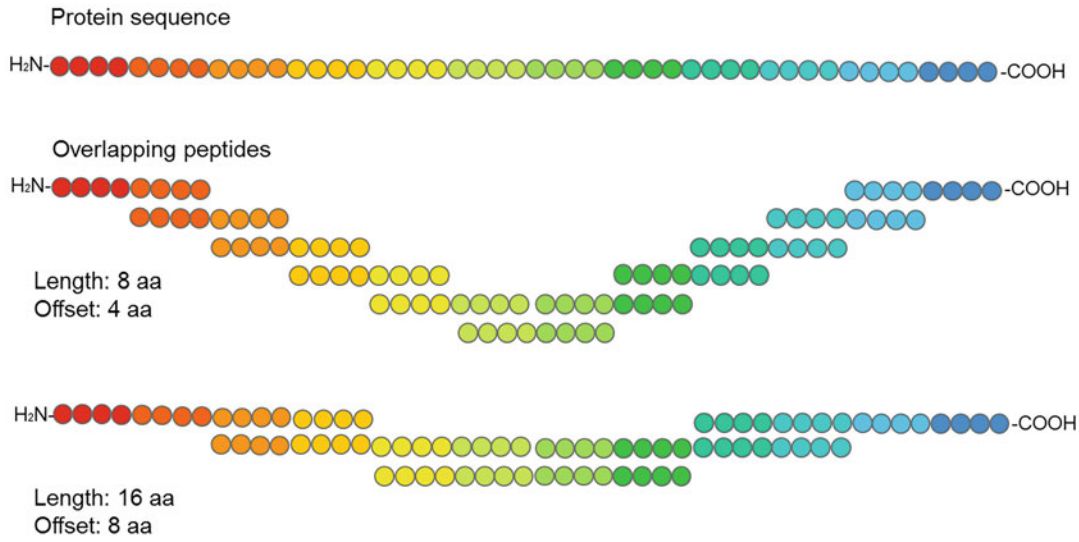

Fig. 1 Schematic illustration of an overlapping peptide ensemble. The generation of a peptide ensemble by reduction of the original protein or peptide domain into overlapping amino acid fragments of equal length. Dependent on the number of amino acids within the peptide and the amino acid offset, varying numbers of peptide fragments are necessary for complete protein coverage. aa: amino acid

1.1 Peptide Sets

1.1.1 Overlapping Peptides

Application of overlapping peptides can be used for identification of antigenic regions. For this, the amino acid sequence of the target used for generation of the antibody must be known, either by using protein sequence databases or by sequencing, before epitopes can be mapped using synthetic peptides [17–21, 23]. Given the amino acid sequence, overlapping peptides are generated (Fig. 1). Critical is the length of the peptide and the number of residues that is shifted along the protein sequence, also referred to as the offset number or overlap. 15mer peptides have been recommended for these types of studies, since most continuous epitopes do not exceed this length [24], although 20mer overlapping peptides often have been used with success for initial identification of antigenic regions [17–21]. The advantages of using longer peptides, for example, peptides of 15–20 amino acids, are that longer peptides often adopt a structure similar to the native structure [15, 25, 26]. The most critical disadvantage in employing longer peptides is that they are more difficult to synthesize, which may result in lower purity. Studies using peptides of four to eight amino acids have also been reported for identification of short epitopes [10], although peptides of this size may experience problems in obtaining stable conformations. As many epitopes are longer than eight amino acids, the application of these peptide sets may be limited [23, 24]. Moreover, a potential disadvantage of applying shorter peptides is that the number of peptides necessary for coverage of the antigenic region may increase significantly, unless small overlaps are employed [10, 11].

Dependent on the location of the epitope within the protein, it may be an advantage to employ a relatively short offset number. One strategy is to apply peptides containing an offset of a single or two amino acids [10, 11]. This requires almost as many peptides as there are amino acids in the examined sequence or the immunogen. The advantage in applying a small offset is that terminal amino acid boundaries essential for antibody reactivity are readily determined. Employing a greater amino acid offset number provides lower resolution and may need to be followed up with a more defined study to determine the minimal epitope. Examples include peptides of 15–17 amino acids with an offset of five residues [15, 27]. In general, efficient epitope mapping has been reported when applying peptides of 8–20 amino acids in length, with an overlap of 1–10 amino acids, allowing for great variation between the design of the individual peptide sets [15, 17, 28].

Following screening of the overlapping peptides, a template peptide is identified for generation of truncated peptides. Dependent on the length of peptides and the number of overlapping amino acids, the peptide obtaining the highest reactivity is commonly selected as template for further analysis.

1.1.2 *Truncated Peptides*

Following screening of overlapping peptides, a template peptide is selected for generation of truncated peptides, which are used to identify the epitope and key interacting amino acid residues. Using the immunogen or the antigenic region identified as template, the peptides are truncated systematically from the N- or C-terminal end, where each peptide typically is truncated by one amino acid in the terminal ends. This type of peptide set may contain n peptides, where n represents the number of amino acid residues within the peptide used as template. However, as the antibodies typically do not recognize the terminal ends, which would require very short epitopes, the last two to five amino acids in the opposite terminals are often not truncated [3, 18, 29, 30]. Alternatively, it is possible to generate truncated peptide sets, where peptides are truncated with more than one amino acid at a time.

The advantage of using N-terminally truncated resin-bound peptides for epitope characterization is that the peptides can be synthesized from the same batch, where a small amount of resin is removed from the pool upon each amino acid coupling. For this approach to succeed, peptides are easier synthesized in syringes rather than in automated peptide synthesis machinery.

The N-terminal screening results can be used as template for generation of C-terminally truncated peptides, where identification of the N-terminal end essential for antibody binding may be used as template to design C-terminally truncated peptides, thereby reducing the number of peptides necessary [18, 21, 23, 30]. Reducing the number of C-terminal truncated peptides is an advantage as

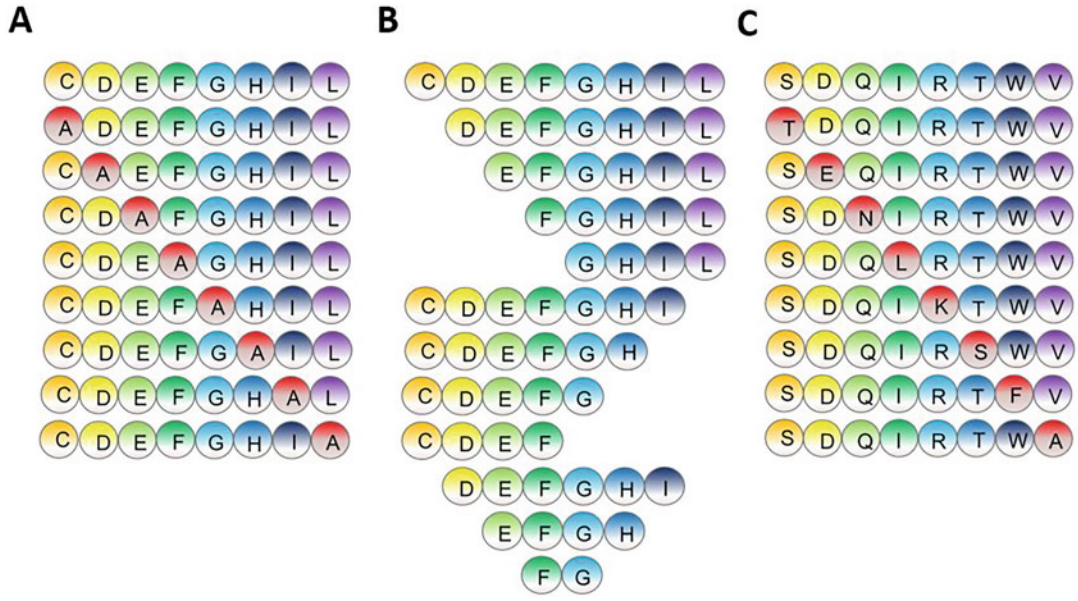

Fig. 2 Examples of truncated and substituted peptides. (**a**) Alanine scanning ensemble. Alanine is systematically substituted into each amino acid position. (**b**) Terminal truncation ensemble. Peptides are systematically truncated from the C- or N-terminal end. Alternatively, truncated peptide versions are generated using the minimum functional epitope as point of origin. (**c**) Functionality scanning ensemble. Selected amino acids are substituted with amino acids of similar functionality. A, C, D, E, F, G, H, I, K, L, N, Q, R, S, T, V, W: see table of standard amino acid abbreviations and properties for further information

C-terminally truncated peptides cannot be synthesized from the same batch and hence requires individual syntheses, corresponding to the number of truncated amino acids.

Using this approach, the most critical amino acids necessary for antibody reactivity are identified, also referred to as the minimum functional epitope, which may require testing of peptides of various lengths from 4- to 20-mers [20, 21, 23, 31, 32].

Alternatively, if the essential amino acids are known and a rather short epitope is used as immunogen, truncated peptides can be generated using the center of the immunogen as starting point for design of truncated peptides, as opposed to systematic truncation from both ends of the peptide sequence (Fig. 2).

1.1.3 Substituted Peptides

The final step in epitope mapping determines the amino acids essential for antibody reactivity using single modified peptides [3, 17–21, 23, 26, 29, 33, 34], which, however, first requires identification of the complete epitope, rather than only identification of the minimum functional epitope.

Identification of the complete epitope is straightforward when using the results obtained from the terminal truncations studies as template. Using the identified N- and C-terminal amino acids as terminal borders, the minimum functional epitope is used as

template, where one amino acid alternately is added to the N- or C-terminal end. Screening of these peptides will result in identification of the complete epitope. This approach is commonly conducted using peptides in solution to be tested in competitive inhibition assays.

Following identification, each of the amino acids in the epitope is substituted systematically with Ala in each position [17–21, 25, 26, 29, 30]. Ala scan determines the roles of individual amino acid side chains for antibody reactivity, as the substitution with Ala removes all side-chain atoms past the β-carbon. The amino acid Ala is normally used because of its non-bulky, chemically inert, methyl functional group, which nevertheless mimics the secondary structure preferences that many other amino acids possess. This type of peptide set consists of Ala single-site substitution analogs. Ala scan may be supported by functionality scans, where amino acids containing similar side-chain functionality are substituted in the identified epitope [20, 23, 34]. Here, each amino acid is substituted with an amino acid of similar functionality, generating a peptide set of single substitution analogs. This approach is applied to determine whether contributions from critical hot spot residues relate to the specific amino acid side chain or side-chain functionality itself. However, limitations exist when using this approach, as it can be difficult to find relevant substitutions for all amino acids. Alternatively, each amino acid can be substituted with all 19 amino acids, generating a library consisting of all possible single-site substitution analogs. However, this complete substitutional peptide analysis is rather time-consuming and requires many peptides [10, 11].

1.2 The Principle of Synthetic Resin-Bound Peptides for Rough Epitope Identification

Several approaches for peptide presentation have been described in the literature, for example, membranes, resins, biotin-streptavidin, and pins [10, 11, 26, 31, 35]. Peptides presented on resins have been used for epitope characterization with success [18, 20, 21, 23, 29, 30]. Resins or solid supports are a common term used to denote the matrix upon which peptide synthesis is conducted, which originally was introduced for use in peptide synthesis by Merrifield [36].

The majority of synthesized peptides are retained inside the porous resin matrix, whereas a small portion of peptides protrude from the surface of the resin, which is sufficient for the peptides to become recognized by antibodies [31].

The original resins used for peptide synthesis were composed of a porous matrix of a loosely cross-linked polystyrene polymer added 1–2% divinylbenzene for cross-linking of the matrix. A high level of cross-linking is necessary for the resin to remain stable and provides a larger area for reactions and easier removal of excess of reagents [37]. The TentaGel resin is commonly used for generation of peptides used in resin-bound characterization studies [19, 31],

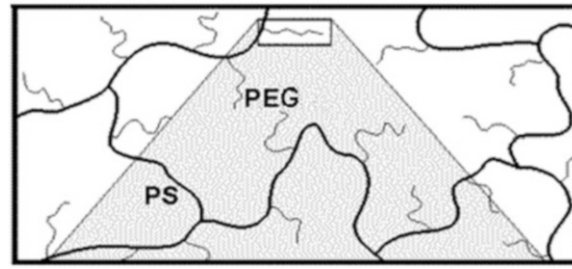

Fig. 3 Graphical illustration of the solid support TentaGel resin. The solid support is composed of a polystyrene (PS)-matrix, grafted with polyethylene glycol (PEG) chains, functioning as a spacer. At the end of the spacer, a linker may reside, which may contain several types of functions [38]

which contains a low cross-linked polystyrene core on which polyethylene glycol chains are grafted, as illustrated in Fig. 3. This has the advantage of a large internal volume, as the chains modify the character of the pore space so that the support-bound reactive moiety is compatible with a wider range of solvents and reagents [37]. Moreover, the polyethylene glycol chains increase the distance from the matrix to the peptide and minimizes the steric effects caused by the matrix.

Several resin complexes can be used for peptide presentation, which typically is composed of the actual resin matrix and eventually spacers and linkers as well, which originally was added to increase the distance from the resin to the peptide and ultimately the concentration of peptides near the matrix surface [23, 31]; however, this has later proved not to be necessary [20, 21, 30]. This approach requires careful knowledge of peptide chemistry, ensuring that the peptide is not cleaved from the linker upon removal of side chain protection groups. The advantage of employing linkers is that the peptide can be cleaved from the resin and hence can be used in a free form as well [23]. When synthesizing peptides directly on the resin, the peptide remains permanently attached.

The advantage of using resins as a solid support is that time-consuming steps, such as peptide cleavage, peptide purification, and coating steps, are avoided. A potential drawback of using resin-bound peptides is that the purity of the peptides is reduced with increasing amino acid number. However, screening of antibody reactivity using 20-mer resin-bound peptides has proved to be successful, in spite of the presence of potential impure peptides, which is circumvented by employing free peptides of high purify for final characterization [18, 21, 23].

When using synthetic peptides for epitope characterization, peptide presentation is of utmost importance. In relation to this, several advantages exist when using peptides coupled to resins in immunoassays, for example, potential poor peptide coating and

steric hindrance may be avoided and the initial coating step is avoided, as no pre-coating is necessary. In traditional peptide enzyme-linked immunosorbent assays (ELISA), the conformation of the free peptide may be altered when coated onto the surface of microtiter plates and potentially hiding essential amino acid side chains for reactivity. Similarly, the amino acids essential for antibody binding may interact with the microtiter plate during coating and become unable to interact with the antibody. Moreover, it may be difficult to ensure an efficient coating of small peptides, due to a limited number of possible interactions between the microtiter plate and the peptide. These issues are circumvented when using resin-bound peptides, where the peptides are screened for antibody reactivity directly on the solid support. When using resin-bound peptides, only the single C-terminal amino acid is linked to the solid support, making the remaining amino acids accessible for interaction. In addition, the peptides are capable of achieving a flexible structure, thereby aiding the peptides in adopting the conformations necessary for antibody binding.

In the present example, the resin-bound GAD67 peptides are screened for antibody reactivity, which allows cleavage of the side-chain protecting groups but leaves the peptide linked to the resin upon completion of peptide synthesis [23].

2 Materials

2.1 Design of Peptides

A total of five overlapping resin-bound peptides, covering amino acids 241–300, were synthesized based on the human GAD67 protein sequence, to cover the immunogenic peptide (amino acids 259–282, SIMAARYKYFPEVKTKGMAAVPKL) used for generation of the a monoclonal GAD67 peptide antibody generated in mice [3]. Every overlapping peptide was 20 amino acids long, containing ten amino acids overlap to the next peptide (Fig. 4).

Next, systematically N- and C-terminally truncated resin-bound peptides were generated using the immunogenic peptide as template, where amino acids were truncated one by one from the terminals in the individual peptide sets (Fig. 5). Using this approach, the minimum functional epitope was identified.

Following screening of terminally truncated peptides, truncated peptides free in solution were generated to be used for final epitope characterization. These peptides were generated using the minimum functional epitope as template, where amino acids were added to the terminals one by one, ultimately generating a small peptide library (Fig. 6).

Finally, substituted peptides free in solution were synthesized for identification of critical contract residues. For this, systematically Ala- and functionality-substituted peptides were generated using the recently identified epitope as template (Fig. 6).

```
241                                                                  300
SKDGDGIFSP GGAISNMYSI MAARYKYFPE VKTKGMAAVP KLVLFTSEQS HYSIKKAGAA

SKDGDGIFSP GGAISNMYSI (1)
           GGAISNMYSI MAARYKYFPE (2)
                      MAARYKYFPE VKTKGMAAVP (3)
                                 VKTKGMAAVP KLVLFTSEQS (4)
                                            KLVLFTSEQS HYSIKKAGAA (5)
```

Peptide	**(1)**	**(2)**	**(3)**	**(4)**	**(5)**
Reactivity in %	0	95	100	0	0

Fig. 4 Scan of overlapping GAD67 peptides analyzed by modified ELISA using resin-bound peptides. A section of the human GAD67 sequence containing the immunogenic peptide (SIMAARYKYFPEVKTKGMAAVPKL) used for production of the GAD67 monoclonal antibody is dissected into 20-mer overlapping peptides, containing ten amino acid residues overlap. Two overlapping peptides were recognized by the GAD67 mAb

C-terminal truncated peptides	*N-terminal truncated peptides*
SIMAARYKYFPEVKTKGMAAVPKL*	SIMAARYKYFPEVKTKGMAAVPKL*
SIMAARYKYFPEVKTKGMAAVPK-*	-IMAARYKYFPEVKTKGMAAVPKL*
SIMAARYKYFPEVKTKGMAAVP--*	--MAARYKYFPEVKTKGMAAVPKL*
SIMAARYKYFPEVKTKGMAAV---*	---AARYKYFPEVKTKGMAAVPKL*
SIMAARYKYFPEVKTKGMAA----*	----ARYKYFPEVKTKGMAAVPKL*
SIMAARYKYFPEVKTKGMA-----*	-----RYKYFPEVKTKGMAAVPKL*
SIMAARYKYFPEVKTKGM------*	------YKYFPEVKTKGMAAVPKL
SIMAARYKYFPEVKTKG-------*	-------KYFPEVKTKGMAAVPKL
SIMAARYKYFPEVKTK--------*	--------YFPEVKTKGMAAVPKL
SIMAARYKYFPEVKT---------*	---------FPEVKTKGMAAVPKL
SIMAARYKYFPEVK----------*	----------PEVKTKGMAAVPKL
SIMAARYKYFPEV-----------*	-----------EVKTKGMAAVPKL
SIMAARYKYFPE------------*	------------VKTKGMAAVPKL
SIMAARYKYFP-------------*	-------------KTKGMAAVPKL
SIMAARYKYF--------------*	--------------TKGMAAVPKL
SIMAARYKY----------------	---------------KGMAAVPKL
SIMAARYK-----------------	----------------GMAAVPKL
SIMAARY------------------	-----------------MAAVPKL
SIMAAR-------------------	------------------AAVPKL

Fig. 5 Design and screening of C- and N-terminally truncated peptides. GAD67 mAb reactivity was analyzed by modified ELISA using resin-bound peptides. Peptides recognized by the GAD67 mAb are marked with *

2.2 Additional Reagents

1. Free and resin-bound peptides. Resin-bound peptides are suspended in 10% ethanol, 100-mg resin per 1-mL solvent (*see* **Note 1**). The free peptides are supplied as lyophilized products, which are dissolved in a suitable solvent (following the manufacturer's instructions) to a concentration of 1 mg/mL (*see* **Notes 2** and **3**).
2. 96-Well multiscreen filterplate (e.g., Millipore, Copenhagen, Denmark) (*see* **Note 4**).
3. Maxisorp 96-well microtiter plate.

Truncated peptides	*Alanine-substituted peptides*	*Functionality-substituted peptides*
(1) -RYKYF---	(6) **A**YKYFP (R→A)[1]	(12) **K**YKYFP (R→K)[1]
(2) -RYKYFP--*	(7) R**A**KYFP (Y→A)[2]	(13) R**T**KYFP (Y→T)[2]
(3) -RYKYFPE-*	(8) RY**A**YFP (K→A)[3]	(14) RY**R**YFP (K→R)[3]
(4) ARYKYFPE-*	(9) RYK**A**FP (Y→A)[4]	(15) RYK**T**FP (Y→T)[4]
(5) ARYKYFPEV*	(10) RYKY**A**P (F→A)[5]	(16) RYKY**W**P (F→W)[5]
	(11) RYKYF**A** (P→A)[6]	

Peptides	**(1)**	**(2)**	**(3)**	**(4)**	**(5)**
Inhibition in %	0	95	100	100	100

Peptides (RYKYFP)	**(6)** (R→A)[1]	**(7)** (Y→A)[2]	**(8)** (K→A)[3]	**(9)** (Y→A)[4]	**(10)** (F→A)[5]	**(11)** (P→A)[6]
Inhibition in %	22	55	95	0	22	60

Peptides (RYKYFP)	**(12)** (R→K)[1]	**(13)** (Y→T)[2]	**(14)** (K→R)[3]	**(15)** (Y→T)[4]	**(16)** (F→W)[5]
Inhibition in %	40	70	96	0	0

Fig. 6 Design and screening of truncated and substituted GAD67 peptides analyzed by competitive inhibition ELISA using free peptides. To determine the level of inhibition by the designed peptides, the peptide used for immunization (SIMAARYKYFPEVKTKGMAAVPKL) was used for coating. An inhibitory concentration of 500 μg/mL was applied. Truncated peptides marked by * inhibited GAD67 reactivity to the coated peptide by 100%, indicating that the sequence RYKYFP constitutes the epitope recognized by the GAD67 mAb. For generation of substituted peptides, the identified RYKYFK epitope was used as template. As presented, the specific amino acid side chains of Tyr^4 and Phe^5 and a positively charged amino acid in position 3 were essential for antibody reactivity

4. Round bottom 96-well microwell reaction plate.
5. Tris-Tween-NaCl (TTN) buffer: 0.05-M Tris, 0.3 M NaCl, 1% Tween 20, pH 7.5 (*see* **Note 5**). Store at 4 °C.
6. Carbonate buffer: 15-mM Na_2CO_3, 35-mM $NaHCO_3$, 0.001% phenol red, pH 9.6. Store at 4 °C. Discard if changes in pH occur.
7. Alkaline phosphatase (AP)-substrate buffer: 1-M diethanolamine, 0.5-mM $MgCl_2$, pH 9.8. Store at 4 °C.
8. Primary antibody: In-house mouse anti-GAD67 IgG monoclonal antibody, directed to a synthetic peptide (amino acids, 259–282). Store at 4 °C (*see* **Note 6**).
9. Secondary antibody: AP-conjugated goat anti-mouse IgG antibody.
10. AP buffer: Dissolve one phosphatase substrate tablet (4-nitrophenyl phosphate) (5 mg) in 5-mL AP-substrate buffer to a final concentration of 1 mg/mL. Any remains should be discarded. The substrate is light-sensitive. Should be prepared immediately before use and kept in the dark or wrapped in foil.

3 Methods

3.1 Modified Enzyme-Linked Immunosorbent Assay Using Resin-Bound Peptides for Screening of Overlapping and N- and C-Terminally Truncated Peptides

It is strongly recommended to optimize concentrations of reagents such as peptides and antibodies for each ELISA system. The concentration mentioned does not apply to all antibodies examined.

1. Dilute resin-bound peptides (100 mg/mL resin) 1:30 in TTN buffer.
2. Add 100-μL resin-bound peptides to the necessary wells of a 96-well multiscreen filterplate (3.3 μg/μL), containing small membranes in the bottom, allowing fluids to be removed upon suction (*see* **Note 7**).
3. Excess buffer is removed by placing the 96-well filter plate on a vacuum suction manifold (e.g., Millipore, Copenhagen, Denmark) or on an equivalent device removing buffer from each well. Rinse the resins three times with TTN buffer (250 μL/well).
4. Add 200-μL TTN as blocking buffer to each well to block nonbinding sites and incubate for 30 min at room temperature (*see* **Note 8**).
5. Dilute the primary antibody (1:1000) in TTN just before use (*see* **Note 9**) and add 100 μL of the diluted antibody to each well.
6. Incubate the plate on a platform shaker at low speed for 1 h at room temperature.
7. After incubation, discard the solution and repeat the washing procedure described in **step 3**.
8. Immediately before use, dilute the secondary antibody (AP-conjugated goat anti-mouse IgG antibody) 1:1000 in TTN.
9. Dispense 100 μL of the diluted secondary antibody to each well and incubate as described in **step 6**.
10. Following incubation with secondary antibody repeat washing steps as described in **step 3**.
11. Detect the presence of bound antibodies by adding 100 μL of freshly prepared AP substrate buffer solution to each well. Place the plates on a platform shaker and inspect the plate when the solution within the wells turns yellow.
12. Tip the plate and transfer 90-μL AP-buffer from each well to a Maxisorp microtiter plate and measure the absorbance at 405 nm, with background subtraction at 650 nm, on a microtiter plate reader (*see* **Note 10**).

3.2 Competitive Inhibition Enzyme-Linked Immunosorbent Assay for Epitope Identification

1. Free peptides (truncated and substituted) (1 mg/mL) are diluted 1:1000 in carbonate buffer.
2. Coat 96-well microtiter plates with 100 μL of the free peptide to each well. Incubate overnight at 4 °C (*see* **Note 11**).
3. Incubate primary antibody (1:1000 dilution) with free peptides (final concentration 500 μg/mL) in a total volume of 100 μL in a round bottom 96-microwell plate for 1 h at room temperature on a platform shaker.
4. Remove the solution from the coated plate by slapping the plate (well side down) on a clean towel or absorbent paper. Wash the plates three times with TTN buffer (250 μL/well).
5. Add 200-μL TTN as blocking buffer to each well to block free nonbinding sites and incubate for 30 min at room temperature.
6. Remove the solution from the plate and repeat the washing procedure described in **step 4**.
7. Add 100 μL of peptide-antibody complex from the wells of the round bottom microwell plate to the wells of the coated microtiter plate. The microtiter plate is incubated on a platform shaker at low speed for 1 h at room temperature. The round-bottom microwell plate is discarded.
8. After incubation, remove the solution and repeat the washing procedure described in **step 4**.
9. Immediately before use, dilute the secondary antibody (goat anti-mouse IgG AP-conjugated antibody) 1:1000 in TTN.
10. Dispense 100 μL of the diluted secondary antibody to each well and incubate as described in **step 7**.
11. Following incubation with secondary antibody, repeat washing steps described in **step 4**.
12. Detect the presence of bound antibodies by adding 100 μL of freshly prepared AP buffer solution to each well. Place the plates on a platform shaker and read the plate when the solution within the wells turns yellow.
13. The absorbance is measured at 405 nm, with background subtraction at 650 nm, on a microtiter plate reader, using a wavelength of 405 nm and a reference wavelength of 650 nm.

4 Notes

1. Resin-bound peptides can be challenging to work with. The resins do not dissolve in solution and are found as a "precipitate" in the bottom of microtiters plates or tubes/vials. In order to obtain a sufficient amount of resin upon pipetting, vortex the microtubes carefully, and quickly collect the necessary volume of the center of the vial before the resins precipitate.

2. A common issue with synthetic peptides, especially those containing hydrophobic amino acid residues, is insolubility in aqueous solutions. Other solvents recommended for peptide solvation include 10% dimethyl sulfoxide, dimethylformamide, or acetonitrile in water.
3. After lyophilization, peptides retain significant amounts of water. Peptides are oxidized over time even at −20 °C and slowly degrade. Thus, the peptide stock solution should be stored in small aliquots upon arrival to prevent degradation caused by repeated freezing and thawing.
4. An alternative to membrane plates is to conduct the experiment in 1-mL tubes. After each washing and incubation step, the sample supernatant is removed, and the resin is retained in the tube. However, this is rather time-consuming and is not recommended.
5. Alternatively, TTN buffer can be replaced with phosphate-buffered saline.
6. It is strongly recommended to optimize concentration of reagents such as peptides and antibodies for each ELISA system.
7. The membranes in the filter plates can be very fragile. Avoid to touch the membrane with sharp instruments or pipettes. Similarly, it can be necessary to wipe the bottom of the membranes upon each incubation step if the membrane leaks.
8. Alternatively, use another or a similar blocking buffer, for example, phosphate-buffered saline containing 1% tween, or 1% skimmed milk, which reduces the possibility of nonspecific binding.
9. This dilution is not necessarily ideal if other types of antibodies or sera are being tested, because optimum dilution depends on the source and the antibody concentration in the sample.
10. Alternatively, transfer the buffer back for further development and read the plate as described.
11. Coat with a peptide containing the complete epitope, in this case the immunogenic peptide. Alternatively, coat with the complete protein.

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Chapter 15

Structural Characterization of Peptide Antibodies

Anna Chailyan and Paolo Marcatili

Abstract

The role of proteins as very effective immunogens for the generation of antibodies is indisputable. Nevertheless, cases in which protein usage for antibody production is not feasible or convenient compelled the creation of a powerful alternative consisting of synthetic peptides. Synthetic peptides can be modified to obtain desired properties or conformation, tagged for purification, isotopically labeled for protein quantitation or conjugated to immunogens for antibody production. The antibodies that bind to these peptides represent an invaluable tool for biological research and discovery. To better understand the underlying mechanisms of antibody-antigen interaction, here, we present a pipeline developed by us to structurally classify immunoglobulin antigen binding sites and to infer key sequence residues and other variables that have a prominent role in each structural class.

Key words Peptide antibody, Structure, Clustering, Linear epitope

1 Introduction

The usage of peptide antibodies in a plethora of analyses, for example, identification and purification of proteins, immunodiagnostics purposes, and epitope-based vaccine design, has been instrumental in the past and to date for drastic scientific and technological advancements. Nevertheless, a group of critical questions on the principles that govern the way in which an antibody recognizes a peptide remain unanswered. On the antigen side, the B-cell epitope prediction for peptides is currently far from perfection [1], whereas on the antibody side, the repertoire diversity of peptide specific B-cell paratopes still misses its full characterization.

To answer these questions, one needs to thoroughly analyze the antibody three-dimensional structure, since it ultimately determines its function. Antibodies are built from four polypeptide chains, two heavy and two light ones, joined by disulfide bonds so that each heavy chain is bound to a light chain and the two heavy chains are linked together. Each chain can be divided on a functional and structural basis in two different regions, called variable

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region (V_L and V_H) and constant region (C_L and C_H). Each variable region is in turn composed by four framework regions that surround three hypervariable regions, these latter bearing an extremely variable amino acid composition. Wu and Kabat [2] predicted them to assume a loop conformation arising from the relatively conserved framework. They were subsequently named "complementarity-determining regions" (CDRs) in contrast to the surrounding framework regions (FRs) and have been claimed to be responsible for the selective binding of the antigen.

To better understand the underlying mechanisms of antibody-antigen interaction, we developed a method to structurally classify immunoglobulin antigen binding sites and to infer key residues in sequence that have a prominent role in each structural class. It is an integrative approach of bioinformatics (structural superposition, sequence alignments, physiochemical analysis of structures), machine learning (clustering, random forests), and statistical (correlation tests, validation of predictions) techniques.

2 Materials

Prepare the antibody heavy and light chain sequences and structures together with the bound peptide antigens for the analysis. These data can be generated in house or retrieved by querying the publicly available IEDB database [3] (Immune Epitope database) with the key words: "Epitope = Linear peptide" or using any other available/private source of antibodies. In case of IEDB usage, you will get a comma-separated values (CSV) file that contains all of the data associated with the records. The CSV format can be easily manipulated using a spreadsheet program, such as Microsoft Excel, or edited with a word processing program, such as Microsoft Word or Notepad.

2.1 Sequence

You will find PDB [4] (Protein Data Bank) codes of the immunoglobulins in the abovementioned CSV file. For each PDB code, retrieve antibody heavy and light chain sequences together with their cognate peptide sequence in FASTA format from Protein Data Bank.

2.2 Structure

Similarly, for each PDB code, retrieve the structure of the antibody-peptide complex in PDB format.

3 Methods

3.1 Structure Renumbering

1. The unambiguous identification of structurally equivalent residues of each immunoglobulin is of crucial importance. Fortunately, the architecture of antibodies has made possible the development of several unified numbering schemes. Such

numberings define portions or specific residues of immunoglobulins that have a similar position in their three-dimensional structure. Currently, there are several numbering schemes [5–10]. You can obtain renumbered structures by uploading the PDB files to http://www.bioinf.org.uk/abs/abnum/ or writing a script to perform this step according to the numbering scheme of preference.

2. The resulting file that contains the renumbered antibody structure needs to be saved in PDB format for later analyses.

3.2 ABS Superposition

1. Once all the structures have the same numbering scheme, you need to select the residues that are part of the antigen binding site (ABS) (*see* **Note 1**). There is a recent article describing the existing ABS definitions [11].
2. After choosing the suitable ABS definition, you need to perform one-against-all ABS comparison. To this aim, you can use any algorithm that does comparative analysis of two selected 3D protein structures or fragments of 3D protein structures (*see* **Note 2**).
3. A number of different possible subsets of the atoms that make up a protein macromolecule can be used as a reference in a structural alignment. When aligning structures bearing very different sequences, the side-chain atoms generally are not taken into account because their identities differ between the aligned residues. For this reason, the structural alignment methods commonly use only the protein backbone atoms or often, for efficiency, only the C_α atoms, since the peptide bond has a minimally variant planar conformation. When the structures to be aligned are highly similar or identical, it's meaningful to align side-chain atoms that will reflect not only the backbone conformation similarity but also the side-chain rotameric states. Thus, it is advisable to use C_α atoms as reference in structural alignment, unless the user has a specific case of highly similar antibodies and would like, for example, to monitor side chains changes correlated to the antigen properties, etc.
4. There are numerous measures of model similarity. The most popular one is the root mean square deviation (RMSD), the measure that is calculated after the best superimposition to show the divergence of one structure from another. Other more sophisticated measures have been developed, such as the global distance test (GDT) [12] and the template modeling score (TM-Score) [13]. Each of these measures has its own qualities and drawbacks, and the user should select them according to the specific case (*see* **Notes 3** and **4**).
5. After choosing an appropriate superimposition algorithm, the user is advised to create a simple script to perform one-against-

all superimposition of ABSs of all the structures of the dataset. This can be done using any of the existing programming/scripting languages (*see* pseudocode in Subheading 4). The resulting distances should be saved in matrix form to facilitate further analysis. The distance between ABSs of antibody i and j will be stored in the cell at position (i, j) of such a matrix.

3.3 ABS Clustering

1. All further analyses will be performed using R, an open-source, free statistical package. R uses a command-line syntax, meaning that you will have to type commands for R. There are several projects that add a graphical user interface (GUI) to R, such as RStudio and R Commander, but currently, only limited point-and-click functionality like opening files and viewing charts are available. Nonetheless, the user can decide to use one or more tools to replace R, such as SPSS, Q, Julia, or Python.
2. Clustering methods are used to group together samples based on their similarity. Even though in our procedure we use hierarchical clustering, it is important to underline that any clustering method that accepts a distance matrix as input can be adopted.
3. Hierarchical clustering is a method of cluster analysis that builds a hierarchy of clusters showing relations between the individual members based on similarity. There are two strategies for hierarchical clustering: agglomerative (bottom-up) or divisive (top-down). In the agglomerative approach, each observation starts as its own cluster, and pairs of clusters are merged as one moves up the hierarchy, whereas in the divisive approach, all observations start in one cluster, and splits are performed recursively as one moves down the hierarchy. The results of hierarchical clustering are usually presented in a dendrogram.
4. You are advised to obtain several antibody clustering by applying different methods. R cluster package (http://cran.r-project.org/web/packages/cluster/) contains diana (divisive) and hclust (agglomerative) methods. The agglomerative clustering can be performed using average, Ward, single, and complete joining functions. These functions differ in how the distance between each cluster is measured. To select the method and the distance function that are giving the most compact and well-separated cluster definition, you might want to use the silhouette function [14]. The highest value of silhouette is used to identify the optimal cut level for each clustering and the best clustering in general.

3.4 Correlation Analysis

1. Random forest [15] is one of the most powerful "black-box" supervised learning methods. It's an ensemble classifier that uses many decision tree models and can be used for classification (categorical variables) or regression (continuous variables) applications.
2. You must use the sequences of immunoglobulins together with the structural clustering as training data for the random forest analysis, to see whether a particular sequence position have a prominent role in each structural class. The user is advised to create a unique CSV file containing the name of PDB structure followed by its sequence and by a label that identify the structural cluster it belongs to. For example, if you obtained four structural classes while clustering the ABS of immunoglobulins, you might want to name them a, b, c, and d.
3. The sequences must be aligned according to a single numbering scheme, so that they all will have the same length (including insertions and deletions) and that conserved residues, insertion, and deletion sites will be in the same columns of the CSV file for all the sample sequences.
4. Apart from antibody sequences, additional data can be checked for correlation with the structural clustering. It has to be represented by a small set of labels or by numbers and added to your training data. For example, if you have done a mutation analysis, you may want to divide the dataset into a discrete number of groups according to the level of mutation and label each sample in the dataset with a label representing its level of mutation. Similarly, you can perform the labeling of any data that needs to be correlated: peptide size, antigen amino acid residues that are in contact with the antibody, antibody germline, etc. Add these labels to each immunoglobulin in the unique CSV file created earlier.
5. You are advised to perform random forest analysis using the R package "randomForest." In our protocol, the random forest is trained to predict the structural cluster using the sequence data and the additional variables alone.
6. Random forests are tuned and trained on the data described above, and the Gini Impurity Index [16] is computed to select the most significant sequence positions and variables that display the best correlation with the structural data. The Gini Impurity Index is a measure of the importance of each variable to correctly identify the structural cluster of the corresponding sample and therefore is a strong hint that the given residue or variable is a key element for the ABS to adopt one of the different conformations identified by the structural clusters.
7. The variable importance plot is a critical output of the random forest algorithm. The plot shows each variable on the *y*-axis and

their importance on the *x*-axis. They are ordered from top to bottom as most to least important, and an estimate of their importance is given by the position of the dot on the *x*-axis. You should use the most important variables, as determined from the variable importance plot, to conduct the further analyses or as a support for your hypothesis. In order to decide how many variables to analyze, you are advised to look for a major difference in the values between one variable and the next one ("elbow") in the importance plot to decide how many variables to choose. This is an important step for reducing the number of variables for further data analysis techniques, but you should be careful to have neither too few variables (that won't suffice to explain the structural data) nor too many (having the risk of introducing noise and second-order correlations in the analysis) (*see* **Note 5**).

4 Notes

1. Structure renumbering needs to be done with caution to prevent errors in the ABS selection and superposition. Since the antibody loops are hypervariable in sequence, special care needs to be taken to correctly align conserved residues that serve as an additional help in aligning the insertions and deletions.
2. The following pseudocode might help in creating a one-against-all ABS comparison script.

```
FOREACH PDB structure Xi in the dataset A:
      create a directory DXi;
      FOREACH PDB structure Xj≠Xi in the dataset A:
            create a directory DXi/DXj;
            run the superposition software on the selected
            residues and save its output in the DXi/DXj folder;
 END OF FOREACH;
END OF FOREACH;
```

3. In order to have a more robust superposition, you might want to superimpose the loop regions (following the chosen definition) plus two/three flanking residues at the N- and C- termini of loop.
4. Depending on the similarity measure of choice, you might want to convert the calculated pairwise distances into the new distance matrices using the corresponding formulae (*see* GDT, TM-score, RMSD definitions).
5. An example of application: The protocol described here has been applied to a small set of peptide antibodies to identify whether it is possible to structurally classify their antigen binding sites and subsequently to infer key residues in

immunoglobulin sequence that have a prominent role in each structural class. For demonstration purposes, we added also the antigen contact area as an additional variable to check its effectiveness in differentiating each structural class.

(a) Querying IEDB database for antibodies binding to linear peptides has retrieved the dataset consisting of 29 peptide antibodies.

(b) The antibodies have been renumbered according to Kabat-Chothia numbering scheme.

(c) We used Local Global Alignment (LGA) [17] for structural superposition of the antigen binding site. The residues used in this analysis were the following:

Chain L: 24 : 34, 48 : 54, 89 : 98

Chain H: 24 : 34, 51 : 57, 93 : 104

The abovementioned residues have been used altogether in the superposition, and average C_α RMSD value has been saved in a 29×29 matrix.

(d) Package "cluster" of R has been used to conduct diana and hclust clustering methods. Average, ward, single, and complete joining functions were applied to the agglomerative clustering. The best clustering has been defined by silhouette analysis. The highest silhouette value 0.35 has been observed for hclust method with the "Ward" function and total number of seven clusters.

(e) The structural clustering with the highest silhouette value is shown in Fig. 1. The seven identified clusters are outlined by red boxes in the dendrogram (clustering tree).

(f) A unique file containing aligned sequences of heavy and light chains of the 29-peptide antibodies has been created (Fig. 2). The contact area for antigen has been retrieved from IEDB query, and its distribution is shown in Fig. 3.

(g) In order to create suitable labels for the random forest analysis, we divided the contact area (ConA) information in four groups as following:

Group A: ConA $\leq$ mean $-$ 1sd

Group B: (mean $-$ 1sd) $<$ Con A $\leq$ mean

Group C: mean $<$ Con A $\leq$ (mean + 1sd)

Group D: (mean + 1sd) $<$ Con A

Each peptide antibody has been labeled according to the group of contact area it belongs to, and this information has been added to the unique file containing aligned sequences (data RF).

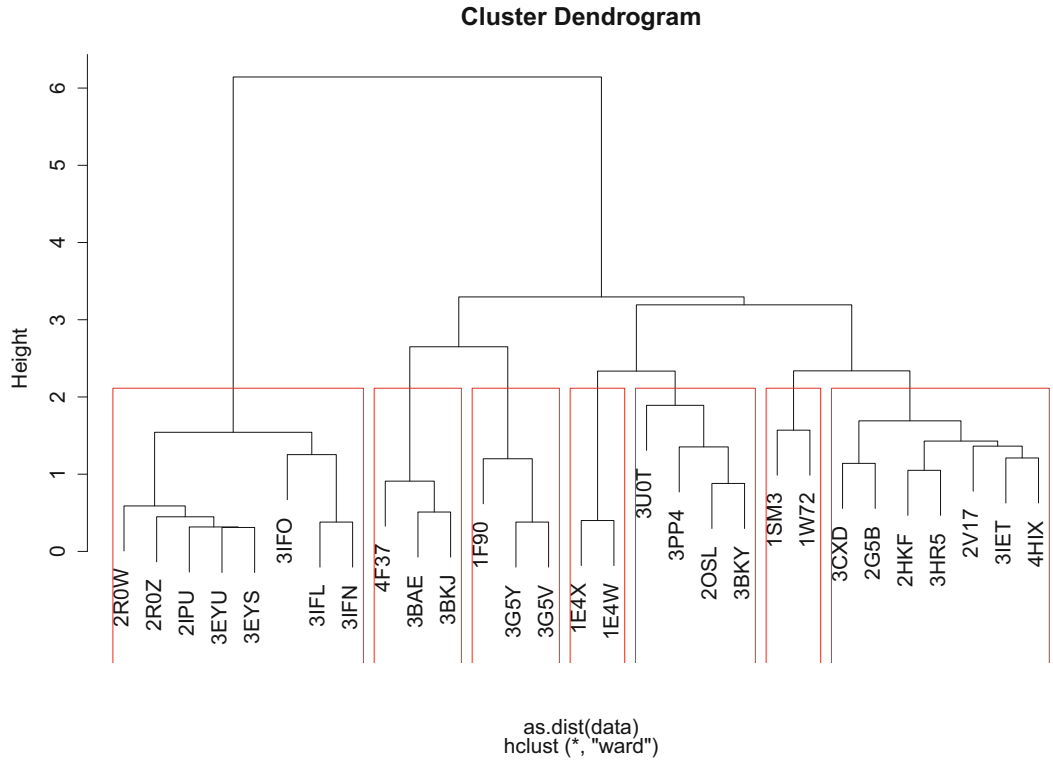

Fig. 1 The structural clustering with the highest silhouette value

Fig. 2 Data RF file containing antibody aligned sequences with structural clustering (column 2) and antigen contact area (last column) labels

(h) Random forests have been trained on the data RF. The most informative variables have been identified and shown in Fig. 4.

(i) The residues 96, 97, and 58 of chain H and the residue 93 of chain L appear to be the most important ones and thus have a prominent role in the structural clustering, whereas the antigen contact area is not in the list of important variables and thus doesn't have significant impact.

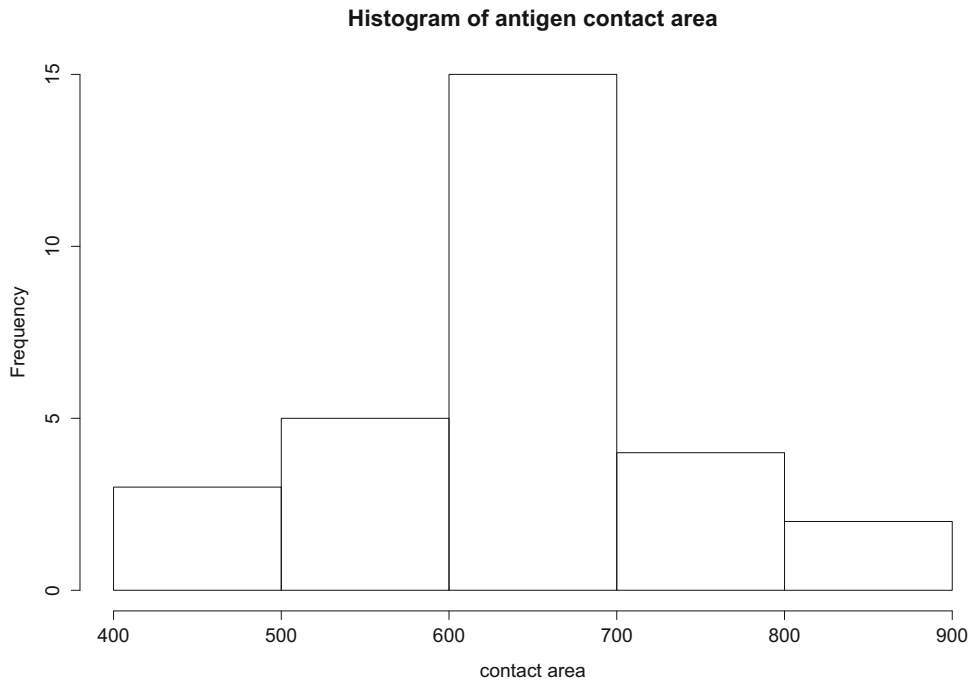

Fig. 3 Antigen contact area distribution

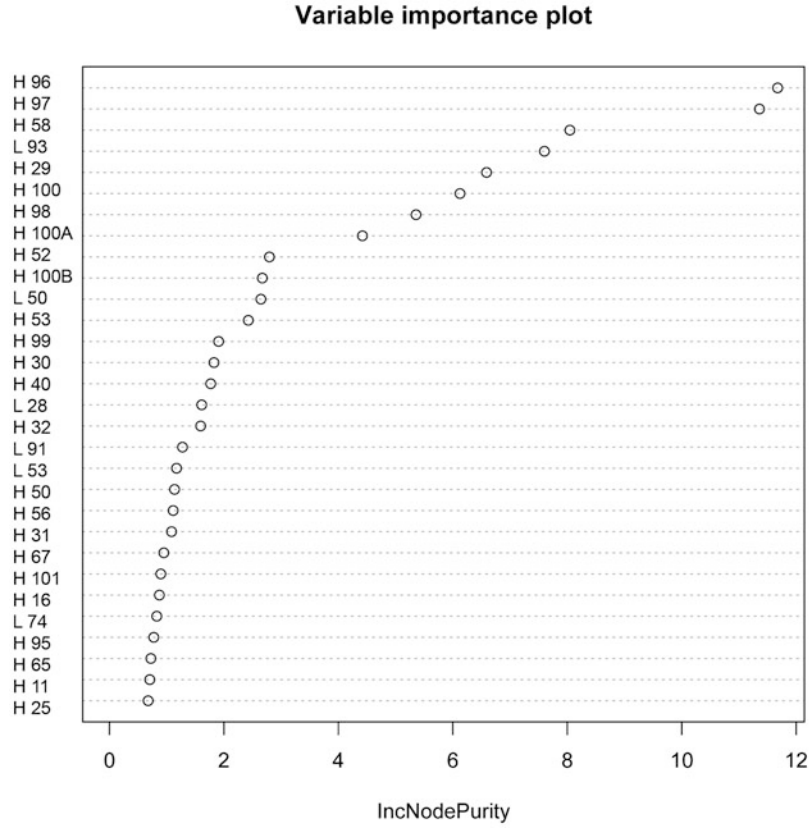

Fig. 4 The random forests variable importance plot

(j) A thorough analysis of the variable importance plot by means of structural superposition with in deep physiochemical analysis of structures will permit you to support or reject your hypothesis and thus finalize the antibody characterization.

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Chapter 16

An Analytical Protocol for Detecting Antibody Titer Levels in Serum/Saliva by Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

Jingwen Wang, Sahra Bashiri, Istvan Toth, and Mariusz Skwarczynski

Abstract

Enzyme-linked immunosorbent assay (ELISA) detects qualitatively and quantitatively the presence of antibodies or antigens in a sample. Due to its simplicity, high sensitivity, and user-friendliness, the test is widely used in laboratory research, clinical diagnoses, and food testing. This chapter describes the indirect semiquantitative ELISA protocol used to monitor antibody levels in animals and analyze the titer levels of specific antibodies against a target antigen in serum and saliva.

Key words ELISA, Antibody titers, Semiquantitative analysis, Enzyme-linked immunosorbent assay, Antigens

1 Introduction

The enzyme-linked immunosorbent assay (ELISA) was first described by Eva Engvall and Peter Perlmann in 1971 [1]. ELISA is a highly specific and sensitive technique that detects proteins or antibodies in very low concentrations (0.1–1 femtomole or 0.01–0.1 ng of a protein). In addition, it is relatively quick and economical compared to other conventional methods. ELISA has been widely used to detect and track (1) the presence of proteins, hormones, antigens, and haptens in a sample; (2) the concentration of antibodies in the serum, saliva, etc.; (3) allergens in food; and (4) pathophoresis in the epidemic area [2, 3].

Typically, in the ELISA assay, a solid surface of the ELISA plates is coated with an antigen. Unoccupied sites on the plate are then blocked by a solution containing a protein that binds to any remaining unoccupied sites. Subsequently, the sample containing the antibody of interest is added to bind to the antigen. Finally, a secondary antibody specific to the primary antibody is added. The secondary antibody is specifically tagged to enable its detection by

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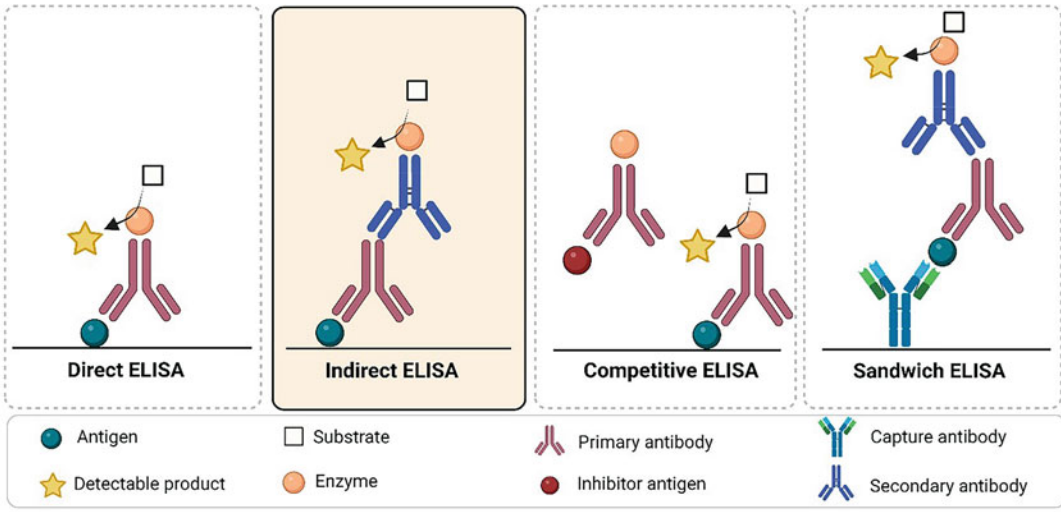

Fig. 1 Principles of four different kinds of ELISA

colorimetric, fluorescent, or chemiluminescent methods, thereby permitting the quantification of the primary antibody. A similar ELISA protocol can also be used to quantify the amount of the antigen of interest.

The four primary ELISA types are direct, indirect, sandwich, and competitive [4]. In direct ELISA, only an enzyme-conjugated primary antibody is used (Fig. 1). In indirect ELISA, an enzyme-conjugated secondary antibody is used along with a primary antibody of interest. In competitive ELISA, enzyme-conjugated antibody form complexes with a selected antigen (inhibitor antigen) at first, and then complexes are transferred to the antigen of interest that is bound on the plate; thus, the antigen on the plate competes for antibody binding. In sandwich ELISA, the antigen is trapped on the surface of the plate with the help of an immobilized antibody, and then it captures the target antibody, followed by the addition of an enzyme-conjugated secondary antibody that binds to the primary antibody (Fig. 1). Each of these methods has its own specific advantages and disadvantages, which have been summarized in Table 1 [5, 6].

This chapter provides a detailed protocol for indirect ELISA used to determine antibody titers against a model antigen in the sera and saliva (Fig. 1). In this method, the primary antibodies from the serum of immunized mice bind to the specific antigens attached to the solid phase of the microplate, which is made of rigid polystyrene, polyvinyl, or polypropylene [4]. Afterward, the secondary antibodies, conjugated with horseradish peroxidase (HRP), are added. The secondary antibody recognizes and binds to the primary antibodies that are bound to the antigen. The presence of HRP in the conjugate enables colorimetric detection of the

Table 1
Advantages and disadvantages of four types of ELISA

ELISA	Advantages	Disadvantages
Direct	Simple and fewer steps than other ELISA	Higher background than other ELISA as all proteins in the samples bind to the solid surface; less flexibility; no signal amplification
Indirect	High sensitivity and flexibility-one secondary antibody can be used for several different primary antibodies	The secondary antibody may be cross-reactive; longer procedure when compared with direct ELISA
Competitive	Crude or impure compounds can be used; suitable for small antigens	Too complex. Inhibition antigen is needed
Sandwich	High sensitivity than direct and indirect ELISA; high specificity involves two antibodies	Require designing; difficult antibody optimization; cross-reactivity; not suitable for very small antigens (e.g., epitopes)

antigen-bound antibody via cleavage of o-phenyl-diamine-dihydrochloride. Specifically, the HRP transforms a noncolored substrate into a colored product. Sulfuric acid is then added to the plate to stop the reaction [7]. The intensity of the produced color is proportional to the amount of antigen in the sample and can be quantified using a spectrophotometer or plate reader [5, 8].

Overall, data produced by ELISA assay can be qualitative, semiquantitative, or quantitative. The qualitative assay is used to detect the presence of a particular antigen/antibody in a sample; the semiquantitative assay compares the level of the titers, that is, compare relative antigen/antibody levels in various samples, and the quantitative determines the precise antigen/antibody concentration by comparing it to a standard concentration curve [9].

2 Materials (*See* Note 1)

2.1 *ELISA*

1. Glass bottles (2 L, 500 mL, 200 mL).
2. Cylinders (100 mL, 500 mL).
3. Stir bars.
4. Pipette (20–200 μL, 2–20 μL).
5. Pipette tips (1–200 μL, non-sterile).
6. Syringe without needle (1 mL).
7. ELISA-Plate, high binding (96-well).
8. Microtube (1.5 mL).
9. Centrifugal machine.

10. Multichannel pipette (50–1200 μL).
11. Stir plate.
12. Microbalance.
13. Incubator.
14. Balance.
15. ELISA plate scanner.
16. pH-meter.
17. PBS solution: dissolve four PBS tablets (5.0 g) in 2-L milli-Q water (*see* **Note 2**).
18. Wash buffer: add 1-mL Tween 20 into the 2-L PBS solution using the 1-mL syringe (*see* **Note 3**).
19. 5% skim milk solution: weigh out 10-g skim milk powder and dissolve it in 200-mL wash buffer.
20. 0.5% skim milk solution: measure 50-mL 5% skim milk and 450-mL wash buffer in cylinders and mix them together (*see* **Note 4**).
21. Carbonate coating buffer (CCB) solution: weigh out 1.93-g sodium carbonate and 3.81-g sodium hydrogen carbonate and dissolve them into 1-L milli-Q water. Adjust the pH of CCB to 9.6 using sodium hydroxide (*see* **Note 5**).
22. Model antigen (J8 peptide— QAEDKVKQSREAKKQVE KALKQLEDKVQ [10, 11]) stock solution: dissolve 0.5-mg antigen in 50-μL milli-Q water (1 mg/100 μL). Then, transfer 100 mL of the CCB solution to 200-mL glass bottle and add 50-μL antigen stock into the CCB solution (50-μg antigen/ 10 mL CCB solution) (*see* **Note 6**).
23. Anti-mouse IgG horseradish peroxidase (HRP)-linked solution: add 33.33-μL anti-mouse IgG-HRP to 100-mL 0.5% skim milk (3.33-μL IgG-HRP/10-mL 0.5% skim milk) (*see* **Note 7**).
24. OPD solution: dissolve 5 OPD tablets in 100-mL milli-Q water and add five buffer tablets as per instruction of a manufacturer (*see* **Note 8**).
25. 1 N sulfuric acid: add slowly 13.5-mL 95–98% sulfuric acid into 486.5-mL milli-Q water (*see* **Note 9**).
26. Blood sera, centrifuge blood at 3600 rpm for 10 min, and then separate supernatant in the sterile tubes for ELISA (*see* **Note 10**).
27. Saliva: transfer 2-μL phenylmethylsulfonyl fluoride (PMSF) solution (17.4-mg PMSF in 1-mL ethanol) to the bottom of 1.5-mL sterile tube and collect the saliva in the tube (*see* **Note 11**).

2.2 Calculation and Drawing

1. Microsoft (Microsoft Excel version 16.0.13901.20336., Redmond, WA, USA).

2.3 Statistical Analysis

1. GraphPad Prism (GraphPad Prism version 9.0., San Diego, CA, USA).

3 Methods

3.1 ELISA

1. Label the high-binding plate as shown in Fig. 2: A–B (naïve serum – control group), C (blank), and D–H (immunized mice serum) (*see* **Note 12**).
2. Add antigen stock in CCB (100 μL/well) in ELISA plate by using multichannel pipette and incubate the plate in incubator for 90 min at 37 °C to coat the antigen on solid surface.
3. Remove the plate contents by quickly pouring out the liquid (*see* **Note 13**). Tap the plate (wells face down) strongly several times on a paper towel and wipe any remaining liquid from the bottom of the plate gently; do not touch wells.

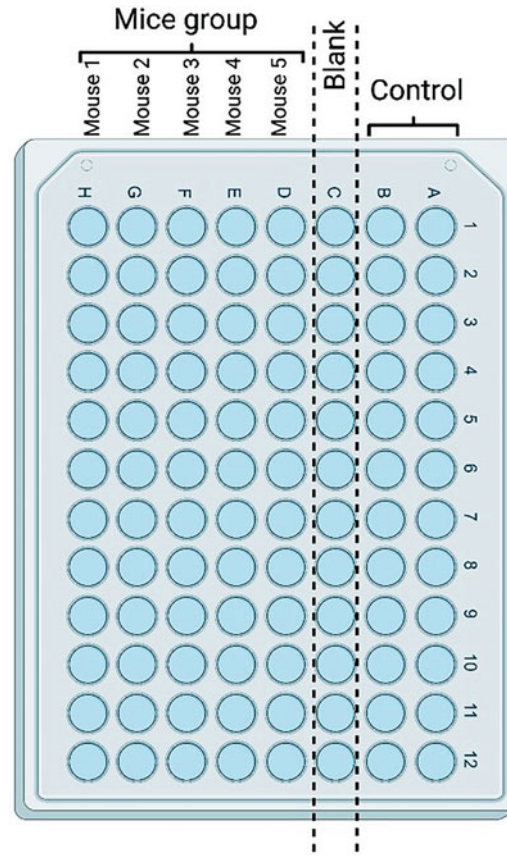

Fig. 2 The way to label the plate (*see* **Note 12**)

4. Add 150 μL of 5% skim milk per well and then store the plate in 4 °C fridge overnight to block the antigen unoccupied sites of the plate wells (*see* **Notes 14** and **15**).
5. Remove the contents from the plate by quickly pouring out the liquid. Add and remove water to the ELISA plate wells three times and then wash buffer four times by using multichannel pipette to wash the ELISA plate. Tap the plate (wells face down) strongly several times on a paper towel and wipe any spilled liquid from the plate after each wash.
6. To produce serum IgG-antigen complex in serial dilutions, add 180-μL 0.5% skim milk to the first top row of the plate (Fig. 3). Then, add 100 μL/well 0.5% skim milk to the rest of rows (column 2–12). Add 20-μL sera samples (ten times diluted in PBS) to well 1D-1H. Add 20-μL naïve sera (ten times diluted in PBS) to well 1A–1B as negative control. Add 20 μL of PBS to well 1C as a blank.

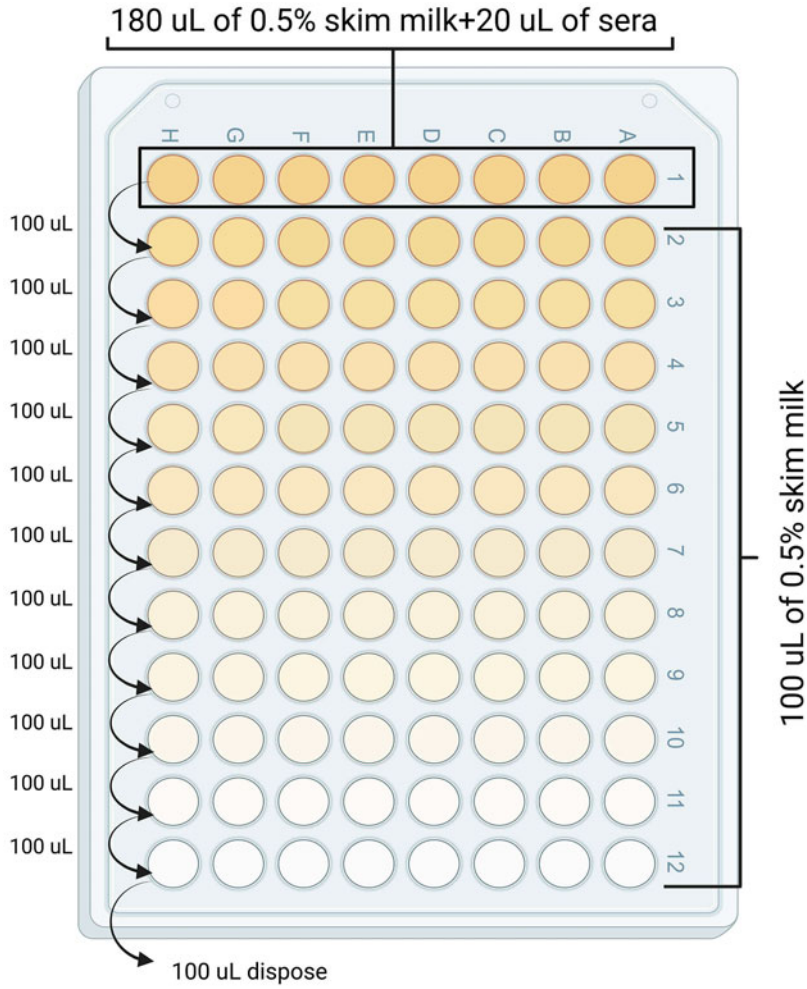

Fig. 3 Titration of the sera into the 0.5% skim milk

7. Mix the produced solution in the first row using a multichannel pipette five times, then transfer 100 μL of the mixture to the next row for each column. Then again mix the produced solution in the second row using a multichannel pipette five times, and then transfer 100 μL of the mixture to the next row for each column. Repeat mixing and transferring of solutions until the last row. Dispose of remaining last 100 μL of the mixture (*see* **Note 16**). After diluting all the sera, incubate the plate in the incubator for 90 min at 37 °C. Saliva IgA-antigen complex can be prepared in the similar manner (*see* **Note 17**).
8. Repeat **step 5**.
9. Add secondary antibody and 0.5% skim milk mixture to plate (100 μL/well) using multichannel pipette. Incubate the plate in incubator for 90 min at 37 °C.
10. Repeat **step 5**.
11. Add 10-mL OPD solution to plate (100 μL/well) and incubate in the dark for 20 min at room temperature.
12. Add 5-mL 1 N sulfuric acid to plate (50 μL/well) to stop the color change reaction.
13. Read the plate at A450 nm by using ELISA plate scanner (*see* **Note 18**).

3.2 Calculation and Drawing

1. Absorbance values determined in Subheading 3.1, **step 12** insert in Excel sheet as shown on Fig. 4.
2. Calculate the average absorbance of control groups (column A and column B, e.g., calculate the average of 1A and 1B for column 1) and the STDEV.S (A/B) of absorbance (column A and column B) of two naïve groups (Fig. 4).
3. Calculate average (A/B) plus three times STDEV.S (A/B).
4. Calculate $\log_{10}$ of dilution from column 1–12.
5. Highlight the absorbance values higher than AVE +(3*STDEV) and higher or equal 0.07 (*see* **Note 19**). The highest highlighted dilution values read as $\log_{10}$ (dilution) represent titters.

3.3 Statistical Analysis

1. Transfer corresponding $\log_{10}$ (dilution)/titers into GraphPad software.
2. Calculated statistical significance using one-way ANOVA with Tukey's multiple comparison test (*see* **Note 20**).
3. Draw the graph and show statistically significant differences as on Fig. 5.

a)

Negative group	1	2	3	4	5	6	7	8	9	10	11	12
A	0.053	0.05	0.049	0.047	0.049	0.051	0.048	0.05	0.049	0.05	0.051	0.058
B	0.049	0.047	0.045	0.047	0.047	0.048	0.045	0.046	0.048	0.049	0.049	0.056
C	0.046	0.046	0.05	0.046	0.046	0.048	0.046	0.046	0.054	0.063	0.114	0.061
D	0.063	0.052	0.053	0.048	0.048	0.047	0.05	0.059	0.046	0.048	0.07	0.053
E	0.06	0.053	0.05	0.05	0.048	0.047	0.047	0.047	0.047	0.065	0.05	0.053
F	0.063	0.058	0.055	0.055	0.052	0.051	0.052	0.052	0.054	0.053	0.058	0.057
G	0.073	0.058	0.066	0.053	0.058	0.051	0.051	0.053	0.058	0.063	0.059	0.056
H	0.075	0.061	0.055	0.052	0.051	0.05	0.049	0.049	0.049	0.05	0.054	0.058
Average (A/B)	0.051	0.049	0.047	0.047	0.048	0.050	0.047	0.048	0.049	0.050	0.050	0.057
STDEV.S (A/B)	0.003	0.002	0.003	0.000	0.001	0.002	0.002	0.003	0.001	0.001	0.001	0.001
AVE+3*STDE	0.059	0.055	0.055	0.047	0.052	0.056	0.053	0.056	0.051	0.052	0.054	0.061
Dilution	100	200	400	800	1600	3200	6400	12800	25600	51200	102400	204800
$\log_{10}$(Dilution)	2.000	2.301	2.602	2.903	3.204	3.505	3.806	4.107	4.408	4.709	5.010	5.311

b)

Positive group	1	2	3	4	5	6	7	8	9	10	11	12
A	0.052	0.049	0.048	0.048	0.048	0.048	0.048	0.048	0.048	0.049	0.048	0.050
B	0.049	0.047	0.046	0.044	0.046	0.044	0.044	0.045	0.045	0.045	0.046	0.049
C	0.050	0.048	0.048	0.045	0.045	0.044	0.045	0.045	0.045	0.046	0.047	0.051
D	1.390	0.967	0.653	0.403	0.247	0.159	0.106	0.082	0.065	0.057	0.055	0.056
E	1.518	1.212	0.898	0.610	0.374	0.224	0.140	0.097	0.076	0.063	0.056	0.056
F	1.585	1.353	1.055	0.747	0.478	0.283	0.181	0.122	0.087	0.073	0.064	0.062
G	0.683	0.243	0.158	0.111	0.087	0.072	0.063	0.061	0.060	0.054	0.054	0.057
H	1.226	0.595	0.379	0.265	0.157	0.105	0.079	0.064	0.059	0.055	0.056	0.065
Average (A/B)	0.051	0.048	0.047	0.046	0.047	0.046	0.046	0.047	0.047	0.047	0.047	0.050
STDEV.S (A/B)	0.002	0.001	0.001	0.003	0.001	0.003	0.003	0.002	0.002	0.003	0.001	0.001
AVE+3*STDE	0.057	0.052	0.051	0.054	0.051	0.054	0.054	0.053	0.053	0.055	0.051	0.052
Dilution	100	200	400	800	1600	3200	6400	12800	25600	51200	102400	204800
$\log_{10}$(Dilution)	2.000	2.301	2.602	2.903	3.204	3.505	3.806	4.107	4.408	4.709	5.010	5.311

c)

Sample group	1	2	3	4	5	6	7	8	9	10	11	12
A	0.049	0.048	0.048	0.048	0.049	0.048	0.046	0.048	0.049	0.049	0.05	0.052
B	0.047	0.046	0.046	0.045	0.045	0.046	0.045	0.046	0.045	0.046	0.046	0.049
C	0.045	0.046	0.046	0.045	0.046	0.045	0.045	0.046	0.046	0.046	0.047	0.05
D	0.24	0.099	0.078	0.063	0.056	0.051	0.049	0.047	0.047	0.047	0.048	0.053
E	0.143	0.067	0.058	0.054	0.05	0.048	0.047	0.047	0.047	0.048	0.048	0.053
F	0.207	0.083	0.069	0.062	0.057	0.053	0.048	0.053	0.052	0.052	0.053	0.057
G	0.073	0.059	0.07	0.052	0.055	0.051	0.052	0.052	0.053	0.053	0.053	0.055
H	1.224	0.742	0.489	0.295	0.189	0.121	0.088	0.07	0.063	0.058	0.058	0.067
Average (A/B)	0.048	0.047	0.047	0.047	0.047	0.047	0.046	0.047	0.047	0.048	0.048	0.051
STDEV.S (A/B)	0.001	0.001	0.001	0.002	0.003	0.001	0.001	0.001	0.003	0.002	0.003	0.002
AVE+3*STDE	0.052	0.051	0.051	0.053	0.055	0.051	0.048	0.051	0.055	0.054	0.056	0.057
Dilution	100	200	400	800	1600	3200	6400	12800	25600	51200	102400	204800
$\log_{10}$(Dilution)	2.000	2.301	2.602	2.903	3.204	3.505	3.806	4.107	4.408	4.709	5.010	5.311

Fig. 4 Example of absorbance of wells in ELISA plates (**a**) negative group, mice immunized with negative control; (**b**) positive group, mice immunized with positive control; and (**c**) sample group, mice immunized with the vaccine candidate and antibody titers calculation. Sera of control groups: column A–B; experiment groups: column D–H; blank: column C. Average (A/B): average of column A and column B; STDEV.S (A/B): estimates standard deviation of column A and column B. AVE+3* STDE: Average (A/B) plus 3 times of STDEV.S (A/B). Dilution: dilution times of serum. Log_{10} (dilution): calculated the $\log_{10}$ of dilution equal to sera titers/$\log_{10}$

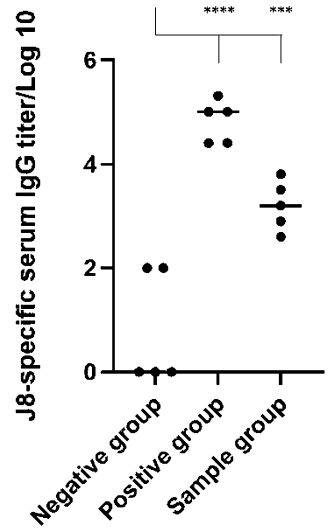

Fig. 5 J8-specific IgG antibody titers of as determined by ELISA. Antibody titers elicited by negative, positive, and sample group. Statistical analysis was analyzed by one-way ANOVA with Tukey's multiple comparison test in comparison to negative control ((*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$) (*see* **Note 21**). The *p*-value shown in the graph are positive and sample groups compared with negative group

4 Notes

1. All aqueous solutions should be prepared using endotoxin-free Milli-Q water (resistivity 18.2 MΩ·cm at 25 °C). Prepare at least 20% more of the solutions than required.
2. The PBS solution can be prepared using individual ingredients instead of tablets. Dissolve 8.000-g sodium chloride, 0.200-g potassium chloride, 1.440-g disodium hydrogen phosphate, and 0.245-g potassium dihydrogen phosphate in 1 L of milli-Q water. In addition, the PBS solution can be prepared by dilution of 100-mL 10× PBS in 900-mL water. The pH of PBS solution usually needs to be adjusted to 7.4 with hydrochloric acid and sodium hydroxide using pH-meter.
3. Put the glass bottle with PBS solution and Tween 20 on stir plate with a stir bar until the PBS tablets and Tween 20 fully dissolved. Wash buffer can be left in room temperature for 1 week.
4. The 5% and 0.5% skim milk can be kept in 4 °C fridge up to 3 weeks.
5. CCB solution can be stored in 4 °C fridge up to 3 months.

6. To analyze IgA antibody titers, dissolve 0.5-mg antigen in 25-μL milli-Q water (1 mg/50 μL). Then, transfer 50 mL of the CCB solution to 200-mL glass bottle and add 25-μL antigen stock into the CCB solution (50 μg antigen/5 mL CCB).
7. Secondary antibody should be prepared just before being added to ELISA plate. Dilution of IgG and IgA-specific secondary antibodies is 1:3000 (secondary antibody:0.5% skim milk). Instead of HRP, other enzymes, such as alkaline phosphatase, can be used.
8. Put the glass bottle of OPD mixture with a stir bar on the stir plate until the tablets dissolved fully. OPD is light sensitive, and it must be prepared just prior to use and used in the dark throughout the entire process. Instead of OPD, other reagents such as tetramethylbenzidine can be used to detect the HRP activity.
9. *Safety precaution*. The concentrated sulfuric acid should be added very slowly into the water while stirring constantly. Adding water to concentrated sulfuric acid can cause it to boil and spit.
10. Transfer serum carefully avoiding sucking the plasma from the bottom of tubes.
11. PMSF is added to the saliva to protect saliva against the protease digestion.
12. The plates can be labeled in alternative orientation (Fig. 6); thus, one ELISA plate can also be used to analyze ten different serum samples.
13. The washing of ELISA plates on this stage is not required. Remove the contents from the plate by pouring it out quickly.
14. To speed up the process, the plates containing blocking solution can be incubated in incubator at 37 °C for 90 min before proceeding to the next step (Subheading 3.1, **step 5**).
15. If the assay could not be continuing, the plates can be washed and stored in 4 °C fridge up to 3 days.
16. The serial dilution of serum should be performed at room temperature and relatively quickly to prevent extensive antibody binding prior to dilution and incubation steps.
17. To produce saliva IgA-antigen complex in serial dilutions, add 50-μL 0.5% skim milk to the first top row of the plate. Then, add 50 μL/well 0.5% skim milk to the rest of rows (column 2–12). Add 50 μL of saliva sample to well 1D–1H. Add 50-μL naïve saliva to well 1A–1B as negative control. Add 50-μL PBS to well 1C as a blank. Mix the produced solution in the first row using the multichannel pipette five times, and then transfer

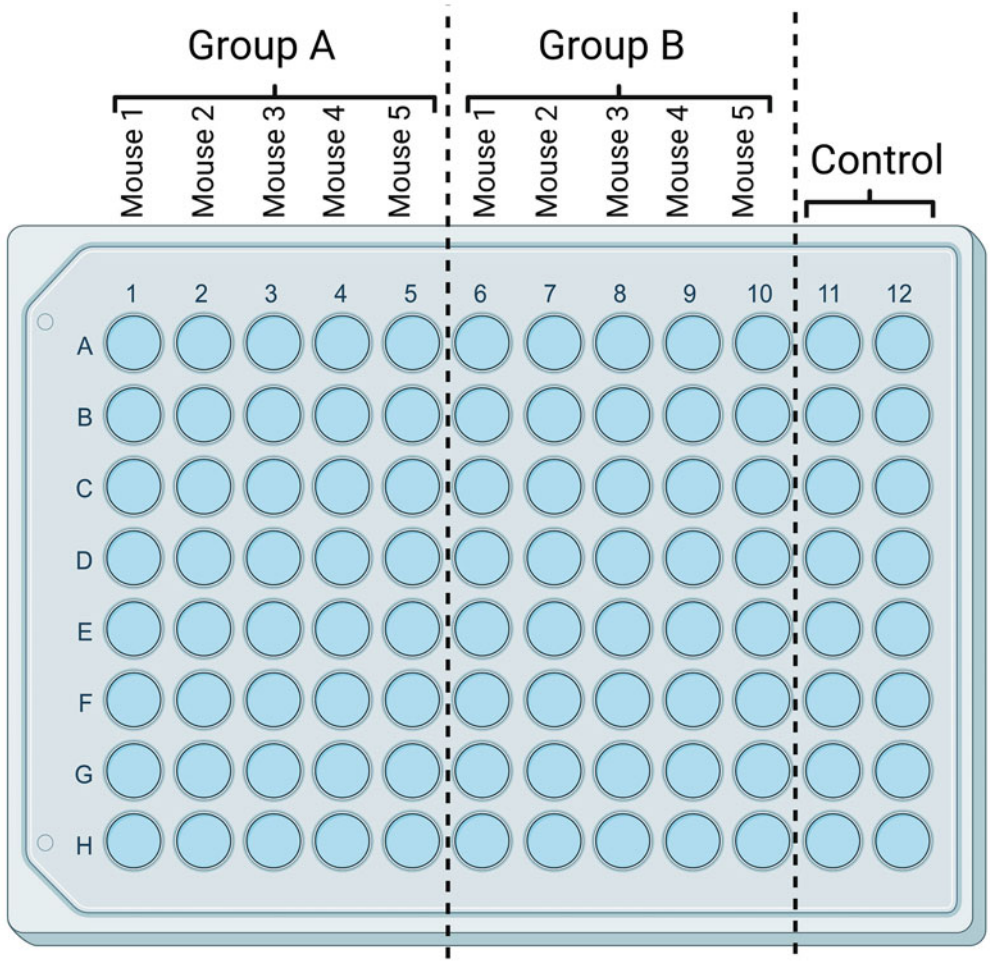

Fig. 6 Another method to label the plate

50 μL of the mixture to the next row for each column. Then, again mix the produced solution in the second row using the multichannel pipette five times, and then transfer 50 μL of the mixture to the next row for each column. Repeat mixing and transferring of solutions until the last row. At the end, discard the remaining 50 μL of mixture. After diluting all the sera, incubate the plate in incubator for 90 min at 37 °C.

18. The plate should be kept in a dark, and it should be read as soon as possible (within 10 min preferably); otherwise, the intensity of the color will be changed.
19. The limit of detection/quantification value in this method is typically around 0.07, and the absorbance values below 0.07 are not reliable (Fig. 4).
20. Instead of one-way ANOVA with Tukey's multiple comparison test other multiple comparison test can be applied.

21. In Fig. 5, statical differences were presented in comparison to a negative control. *p*-Value is used to quantify significance of the difference of data sets (group). The confidence level is calculated from the *p*-value as 100 × (1 − *p*-value). For example, $p < 0.05$ means that there is 95% confidence that the difference between data sets is significant. In Fig. 5, confidence level 95%, $p < 0.05$ is represented by one star (*); confidence level 99%, $p < 0.01$ is represented by two stars (**); confidence level 99.9%, $p < 0.001$ is represented by three stars (***); and confidence level 99.99%, $p < 0.0001$ is represented by four stars (****).

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Chapter 17

Enzyme-Linked Immunosorbent Assay for Antibodies Against the Tumor-Associated Antigen-Derived Cytotoxic T-Lymphocyte Epitope

Yusuke Oji

Abstract

Antibodies serve as crucial indicators of the immune system in clinical tests. In therapeutic cancer vaccines, IgG antibodies against target antigens are vital for immune monitoring. Additionally, assessing baseline antigen-specific immune responses before cancer vaccine administration is possible by measuring IgM and IgG antibodies against the target antigen. To this end, we have developed an enzyme-linked immunosorbent assay (ELISA) system that detects and quantifies serum levels of IgG and IgM antibodies against the WT1 cytotoxic T-lymphocyte epitope peptide. The assay immobilizes the epitope peptide in a microplate to capture antigen-specific antibodies. Here, this article presents the details of our ELISA system to detect and measure antibodies against a tumor-associated antigen-derived cytotoxic T-lymphocyte epitope with high reproducibility. Detecting these antibodies has novel significance in the context of emerging critical roles of B lineage-cells in tumor immunity.

Key words Enzyme-linked immunosorbent assay, Tumor-associated antigen, Cytotoxic T-lymphocyte epitope, WT1, WT1 peptide-based cancer immunotherapy

1 Introduction

Antibodies are widely used as "reporters of the immune system" in clinical tests, as they provide insights into systemic immune responses using just a small volume of blood samples. Traditionally, T lymphocytes have been the focus of research on tumor immunity. However, recent groundbreaking reports have highlighted the crucial roles of B cells in tumoral tertiary lymphoid structures, including their production of antibodies, antigen presentation, and helper functions [1–3]. As a result, antibodies are increasingly recognized as valuable markers for assessing the activities of the antigen-specific B-cell lineage, reflecting a novel understanding of B-cell functions.

Gunnar Houen (ed.), *Peptide Antibodies: Methods and Protocols*, Methods in Molecular Biology, vol. 2821,
https://doi.org/10.1007/978-1-0716-3914-6_17,

The *WT1* gene is reported to be overexpressed in leukemia and various solid tumors [4–6]. To harness the potential of the WT1 gene product as a therapeutic target, we have developed a WT1 peptide-based cancer vaccine that targets the WT1 CTL epitopes (WT1–235 and WT1–126) and have demonstrated its clinical efficacy [7–9]. Vaccines targeting tumor-associated antigens can induce immune responses against target antigens, thereby controlling tumors. Thus, it is crucial to monitor the induction of immune responses against target antigens in the context of therapeutic cancer vaccines. We have established that the production of IgG antibodies and the development of skin delayed-type hypersensitivity to the administered WT1 peptides serve as surrogate markers for WT1 peptide vaccines and correlate with a favorable patient prognosis [10, 11]. Additionally, we have demonstrated that co-administration of a WT1 helper peptide significantly enhances the production of IgG antibodies against the WT1–235 peptide, the targeted CTL epitope of the WT1 peptide vaccine [12]. Therefore, IgG antibodies specific to target antigens are expected to play essential roles as markers for immune monitoring during therapeutic cancer vaccine administration.

Production of epitope-specific IgG antibodies requires help from Th lymphocytes, making their presence a reflection of the activation of antigen-specific helper T lymphocytes. In contrast, IgM production does not require Th lymphocyte help and occurs earlier than IgG production. In a study analyzing IgM antibody production against WT1 before WT1 peptide vaccine immunotherapy for soft tissue sarcoma, we found that all WT1–235 IgM antibody-positive cases were WT1–235 IgG-negative [13]. Furthermore, the production of WT1–235 IgM antibodies before vaccination tended to correlate with a poorer prognosis, indicating that cases where class switching to IgG antibodies does not occur despite the production of IgM antibodies before the start of vaccination may have insufficient Th helper activity. These findings suggest that we can evaluate the potential of baseline antigen-specific immune responses before cancer vaccine administration by combining IgM and IgG antibodies against the target antigen.

In a recent study, we observed that healthy young individuals, who are considered to have optimal immunocompetence, exhibit humoral and cellular immune responses against multiple tumor-associated antigens [14]. Notably, in response to WT1–235, the production of IgM and IgG antibodies showed a correlation with IFN-γ production, but not TNF-α or IL-10, in terms of antigen-specific cytokine production by PBMCs, analyzed via an ELISPOT assay. This observation is consistent with a previous report of antibody production against CTL epitopes in healthy individuals [15]. Immune responses against tumor-associated antigen CTL epitopes could function as an immune surveillance system for tumors in healthy individuals. In this context, we describe an

ELISA system to detect and quantify serum levels of IgG and IgM antibodies against the WT1 cytotoxic T-lymphocyte epitope, which could serve as a valuable tool for evaluating immune responses to antigenic epitopes of tumor-associated antigens.

2 Materials

1. Peptide coating kit (e.g., Peptide Coating Kit, Takara, Shiga, Japan): An antigen epitope peptide is used as a capture antigen to detect an antigen epitope-specific antibody. We use the kit because synthetic peptides hardly adsorb spontaneously to 96-well microplates. The reaction buffer and blocking solution mentioned in the methods section are provided as accessories of the kit (*see* **Notes 1** and **2**).
2. Synthetic peptide: A peptide with the following sequence is used to detect IgG and IgM antibodies against the target antigen epitope WT1–235: CMTWNQMNLGAPKK (*see* **Notes 3–5**).
3. Blocking agent: For example, Blocking One (Nacalai Tesque, Kyoto, Japan).
4. 0.05% TBST buffer: 40-mM Tris–HCl, pH 8.0, 133-mM NaCl, 0.05% Tween20.
5. Antibodies: For IgG detection, rabbit antihuman IgG-HRP and goat anti-rabbit IgG-HRP are used as secondary and tertiary antibodies, respectively. For IgM detection, goat antihuman IgM and anti-goat IgG (H+L chain)-HRP rabbit IgG/Fab are used as a secondary and tertiary antibody, respectively.
6. Coloring substrate: As the coloring substrate of tertiary antibody-conjugated horseradish peroxidase (HRP), we use tetramethylbenzidine (TMB) (e.g., 1-Component Microwell Peroxidase Substrate, SureBlue, KPL, Gaithersburg, MD).
7. Citrate buffer: 68.5-mM citrate, 31.5-mM sodium citrate, pH 3.6 (*see* **Note 6**).

3 Methods

3.1 Immobilization of Capture Antigen Peptides

1. Add 50 μL of WT1 peptide solution (4 μg/mL) in the reaction buffer (provided in the peptide-coating kit) into each well. Add 50 μL of sodium citrate (5 mM) into the negative control well (*see* **Note 5**).
2. Add 30 μL of coupling agent (provided in the peptide-coating kit; dissolved in dH_2O) into each well.

3. Incubate at 30 °C for 2 h (*see* **Note 7**).
4. Wash the ELISA plate three times with 200 μL per well of pure water (*see* **Note 8**).

3.2 Blocking

1. Add 200 μL per well of 1 × Blocking One and incubate at 30 °C for 2 h.
2. Wash the ELISA plate three times with 200 μL per well of 0.05% TBST (*see* **Note 8**).

3.3 Capture of Antibodies of Interest

1. Add 100 μL per well of sample sera diluted 1:100 in Blocking Solution (provided in the peptide-coating kit). Sample wells are set in duplicate (*see* **Note 9**).
2. Incubate at 4 °C overnight.
3. Wash the ELISA plate three times with 200 μL per well of 0.05% TBST (*see* **Note 8**).

3.4 Secondary Antibody Reaction

1. Add 100 μL per well of secondary antibody in Blocking Solution (provided in the peptide-coating kit). For IgG and IgM detection, rabbit anti-human IgG-HRP diluted at 1:2000 and Goat anti-Human IgM are used, respectively.
2. Incubate at 30 °C for 2 h (*see* **Note 7**).
3. Wash the ELISA plate three times with 200 μL per well of 0.05% TBST (*see* **Note 8**).

3.5 Tertiary Antibody Reaction

1. Add 100 μL per well of tertiary antibody in 0.05% TBST. For IgG and IgM detection, goat anti-rabbit IgG-HRP diluted at 1:5000 and anti-goat IgG (H+L chain)-HRP rabbit IgG/Fab diluted at 1:5000 are used, respectively.
2. Incubate at 30 °C for 2 h (*see* **Note 7**).
3. Wash the ELISA plate four times with 200 μL per well of 0.05% TBST (*see* **Note 8**).

3.6 Color Development and Absorbance Reading

1. Add 100 μL per well of TMB diluted 1:4 in citrate buffer (*see* **Notes 7** and **9**).
2. Incubate at 30 °C for 3 min.
3. Terminate reactions by adding 160 μL per well of 1 N HCl.
4. Read absorbance at 450 nm within 15 min.
5. The antibody titer for each serum sample is determined as the average absorbance value of duplicate wells, after subtracting the absorbance value of the negative control well.
6. In our ELISA system, the cutoff levels for the positivity of WT1–235 IgG and WT1–235 IgM are set at 0.15 and 0.10, respectively, based on the absorbance values of the mean +3SD from five independent assays in negative control serum samples.

4 Notes

1. In this method, the free carboxyl group (−COOH) of the synthetic peptide reacts with the coupling reagent (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; EDC), and then the amino group (−NH2) exposed on the bottom of the microplate well (−NH2) reacts with the EDC-activated peptide. Through these processes, the target peptide is immobilized directly onto the bottom of the plate. While this article describes an ELISA method using the Takara Peptide Coating kit, this protocol can be applied to other kits that immobilize peptides based on the same principle with minor modifications.
2. It is important to note that there may be lot-to-lot differences in peptide-coating kits that can have an impact on the assay results, particularly the background signal. Therefore, it is recommended to use the same lot of peptide-coating kit throughout the experiment or to compare assay results using the same sample to check for any lot-to-lot differences.
3. This article describes a method using CTL epitope peptides, but other types of peptides can also be used [12–14].
4. The solubility of peptides varies depending on their sequence. In this kit, the carboxyl group of the peptide is cross-linked with the amino group exposed on the bottom of the microplate well using a coupling reagent. Peptide solubility can affect the coupling reaction. Therefore, we add a proline-lysine-lysine (PKK) sequence to the C-terminus of sparingly soluble peptides to improve their solubility.
5. In this ELISA, an antigenic peptide is used as the capture antigen. It is important to avoid long-term storage of antigenic peptides in their diluted form. Peptides should only be stored in a diluted form for a maximum of 2–3 days.
6. It is recommended to routinely check the pH of the buffer.
7. Accurate temperature control is critical for the success of this ELISA. Please note that steps performed at "room temperature" are susceptible to seasonal temperature changes. For example, in the summer, cool air from air conditioning can impact the assays. Temperature control is especially crucial in the final coloring step. To ensure consistent results, we recommend (1) maintaining the temperature of the coloring reagent at a constant 22 °C, (2) working on a Styrofoam board as a heat insulator to prevent the ELISA plate from cooling on the bench, and (3) performing the color development step in an incubator set at 30 °C. Additionally, we recommend setting positive control wells at different locations on the ELISA plate to ensure reproducibility.

8. Sufficient time must be allocated for the washing step. We recommend a 3-min first wash and subsequent washes of 10 min each. This sufficient time of wash critically decreases the background of the assays.
9. We recommend using the blocking agent from the peptide-coating kit as the diluent for both serum and the secondary antibody. The blocking agent inactivates the amino group on the bottom of the microplate wells, which were activated during the peptide-coupling reaction step. The use of the blocking agent significantly reduces the background of the assays, resulting in more reliable and reproducible results.

Acknowledgments

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Chapter 18

Assessment of the Antigen-Binding Capacity and Separation of Extracellular Vesicles Coated with Antigen-Specific Antibody Light Chains

Katarzyna Nazimek and Krzysztof Bryniarski

Abstract

Many researchers are interested in the possibility of manipulating the targeting specificity of extracellular vesicles (EVs) for their use as physiological delivery vehicles for drugs and bioactive molecules. Our studies demonstrated the possibility of directing EVs toward the desired acceptor cell by coating them with antigen-specific antibody light chains. Here, we describe the methods for detection of the presence of antibody light chains on the EV surface, proving their ability to specifically bind the antigen and for separating the antigen-binding EV subpopulation.

Key words Antibody light chains, Antigen-affinity chromatography, Antigen binding, Cell targeting by extracellular vesicles, Cytometry, ELISA, Exosomes, Extracellular vesicles, Separation of extracellular vesicles, Specificity of antigen binding

1 Introduction

The growing demand for new, personalized therapies has prompted researchers to look for unconventional, selective, safe, and well-tolerated carriers for biological drugs that could enhance the expected biological effects and reduce adverse reactions. Due to their biocompatibility and ability to cross barriers, extracellular vesicles (EVs) are promising candidates for such applications [1, 2]. However, the specificity of targeting EVs to cells is a major challenge for their therapeutic use [3]. Our observations so far showed that the binding specificity of target cells by EVs could be ensured by B1 cell-derived antibody *kappa* light chains (LCs). These LCs naturally bind to the surface of the vesicle subpopulation released by suppressor T cells [4–10]. Furthermore, we found that EVs released by suppressor T cells of $JH^{-/-}$ mice that do not produce LCs can be supplemented with antigen-specific LCs to restore their cell-binding capacity [6, 11]. The exact mechanism

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of LC binding to the EV membrane needs to be investigated. However, our preliminary observations suggest the involvement of membrane lipids [11, 12]. Identification of the binding sites will enable the standardization of the EV coating procedure with selected LCs in biomedical applications. It can be speculated that similar effects could be achieved by supplementing EVs with antigen-specific antibodies, analogously to synthetic liposomes [13]. Therefore, here, we present experimental protocols that allow to: (i) detect the presence of LCs on the EV surface by flow cytometry, (ii) prove the ability of EV-bound LCs to specifically bind the antigen by a modified ELISA, and (iii) separate the antigen-binding EV subpopulations by affinity chromatography. Adapting these methods to the detection of whole antibody molecules (primarily by using antibodies specific for immunoglobulin H + L chains in cytometry) will allow their use in the assessment of the same parameters but mediated by whole immunoglobulin molecules.

2 Materials

2.1 Mouse EV Suspension for Cytometric and ELISA Testing

1. The "working" suspension of mouse EVs should be prepared by resuspending the ultracentrifuged EV pellet in Dulbecco's phosphate-buffered saline (DPBS, prepared by dissolving 880 mg of NaCl, 300.4 mg of $Na_2HPO_4 \times 12H_2O$, and 25.5 mg of $NaH_2PO_4 \times 1H_2O$ in 100 mL of deionized water) to dilute the suspension to a final concentration of approximately 1×10^{11} EVs per mL (as estimated by nanoparticle tracking analysis, NTA; *see* **Note 1**). It is recommended to filter EV-containing starting material (e.g., cell culture supernatant) through 0.45 and 0.22-μm molecular filters before ultracentrifugation.

2.2 Mouse EV Suspension for Affinity Chromatography

1. In affinity chromatography, it is recommended to use full material containing EVs (e.g., cell culture supernatant or blood serum), which should be deprived of cellular debris by 10-min centrifugation at 2000 *g* and subsequent filtration through 0.45 and 0.22-μm molecular filters. In the case of blood serum, dilute it 1:10 with DPBS at the beginning of the procedure. For 7 mL of gel matrix, it is recommended to use no more than 15 mL of the prepared material.

2.3 Flow Cytometry Analysis

1. Stock suspension (4% w/v) of aldehyde/sulphate latex beads (4 μm) stored at 4 °C.
2. Blocking buffer: 100-mM glycine solution in Dulbecco's phosphate-buffered saline without calcium chloride and magnesium chloride (DPBS): weigh out 750.7 mg of glycine in powder and dissolve in 100 mL of DPBS. Store at 4 °C.

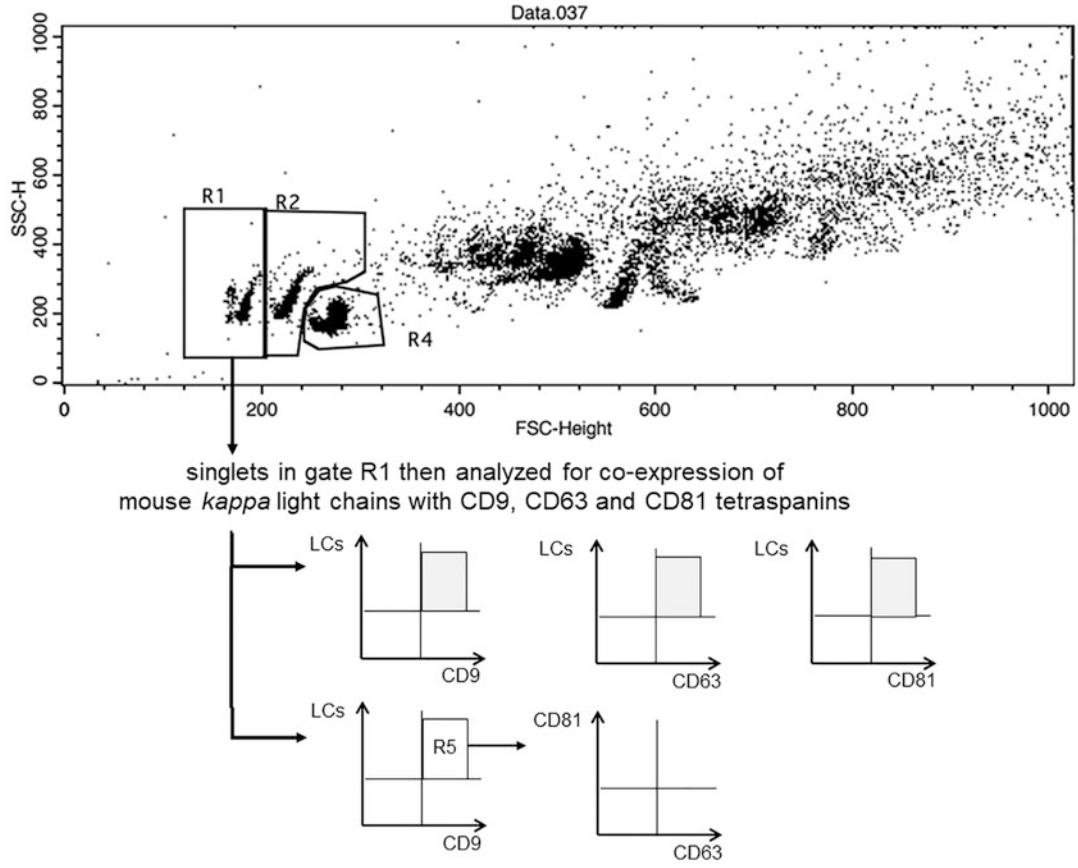

Fig. 1 Protocol design for cytometric analysis to demonstrate the presence of antibody light chains on the surface of EVs expressing selected tetraspanins, analyzed individually or after gating the events co-expressing LCs and CD9 tetraspanin. Fluorochrome conjugates of detection antibodies should be selected depending on the cytometer used for measurement

3. Washing buffer: 0.1% (w/v) bovine serum albumin (BSA) in DPBS: weigh out 100 mg of BSA in powder and dissolve in 100 mL of DPBS. Store at 4 °C.
4. Monoclonal antibodies: Rat anti-mouse *kappa* LCs (e.g., clone 187.1), rat anti-mouse CD9 (e.g., clone KMC8), rat anti-mouse CD63 (e.g., clone NVG-2), hamster anti-mouse CD81 (e.g., clone Eat2), and their respective isotype controls [14]. Fluorochrome conjugates should be individually selected depending on the measurement capabilities of the cytometer available in the laboratory and the staining design (Fig. 1). "Working" solutions of antibodies for staining (25 µL of each antibody solution per sample) should be prepared ex tempore by mixing 1 µL of antibody stock solution with 200 µL of 100-mM glycine solution in DPBS (*see* **Note 2**).

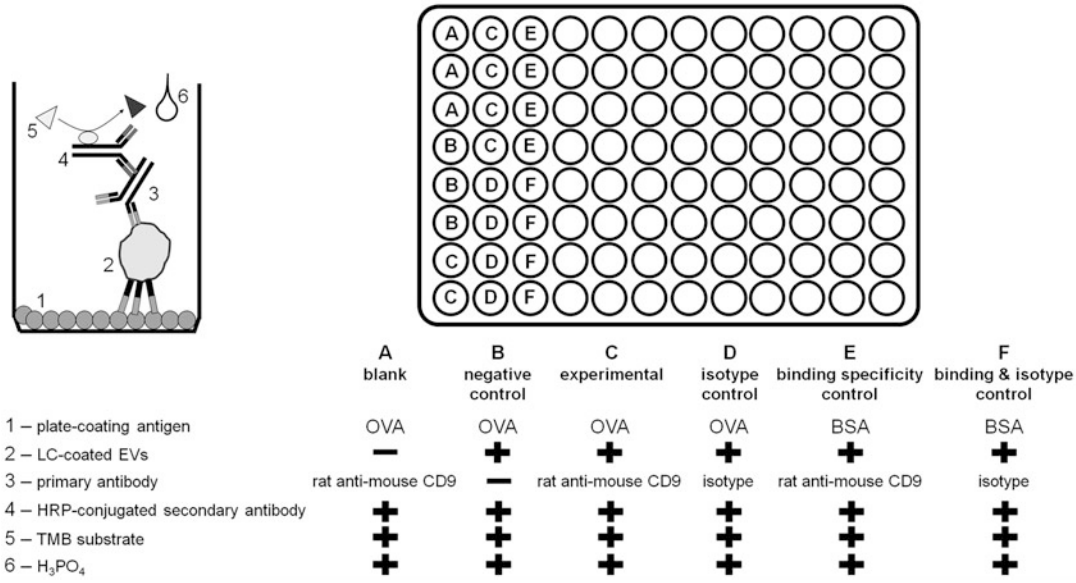

Fig. 2 Protocol design for a modified ELISA to demonstrate the ability of EVs to specifically bind the antigen (ovalbumin, OVA). This example considers one population of EVs (one sample). If you have more samples to analyze, multiply the number of relevant groups accordingly

2.4 Modified ELISA

1. Ninety six wells clear flat bottom polystyrene highly binding ELISA microplate (maximal well volume 360 μL). If a plate with a smaller maximum volume (and therefore smaller surface area) is selected, the reagent volumes used must be reduced accordingly.
2. For the preparation of an antigen solution in phosphate-buffered saline (PBS, 100 μg/mL) for coating the ELISA plate wells as proposed in designed protocol (Fig. 2), weigh out 200 μg of ovalbumin (OVA) lyophilized powder and dissolve in 2 mL of PBS. For the preparation of an antigen solution in PBS for coating all 96 wells of one ELISA plate, weigh out 1 mg of OVA lyophilized powder and dissolve in 10 mL of PBS. Store at 4 °C.
3. Binding control buffer for coating the ELISA plate wells as proposed in designed protocol (Fig. 2). BSA solution in PBS (100 μg/mL): weigh out 100 μg of BSA lyophilized powder and dissolve in 1 mL of PBS. Store at 4 °C.
4. Blocking buffer for 24 wells of the ELISA plate (Fig. 2): 2% (w/v) BSA solution in PBS: weigh out 100 mg of BSA-lyophilized powder and dissolve in 5 mL of PBS. Store at 4 °C.
5. Washing and diluting buffer for 24 wells of the ELISA plate (Fig. 2): 0.1% (w/v) BSA in PBS: weigh out 100 mg of BSA in powder and dissolve in 100 mL of PBS. Store at 4 °C.

6. Primary antibody for 13 wells of the ELISA plate (Fig. 2): purified rat IgG antibody anti-mouse CD9 (e.g., clone KMC8), add the volume of antibody stock solution that contains 6 μg of antibody to 1.5 mL of 0.1% BSA in PBS. Store at 4 °C.
7. Purified rat IgG isotype control antibody for 8 wells of the ELISA plate (Fig. 2): add the volume of antibody stock solution that contains 4 μg of antibody to 1 mL of 0.1% BSA in PBS. Store at 4 °C.
8. Secondary antibody for 24 wells of the ELISA plate (Fig. 2): horseradish peroxidase (HRP)-conjugated rabbit IgG polyclonal antibody anti-rat IgG, add the volume of antibody stock solution that contains 5 μg of antibody to 2.5 mL of 0.1% BSA in PBS. Store at 4 °C.
9. 3,3′,5,5′-tetramethylbenzidine (TMB) substrate prepared ex tempore according to the manufacturer's instructions in a volume of 2.5 mL (Fig. 2).
10. Stopping reagent for one ELISA plate: 1 M phosphoric acid (V) (H_3PO_4), add 342 μL of 85% acid solution to 4.658 mL of deionized water.

2.5 Affinity Chromatography

1. Agarose gel filtration base matrix (recommended: CNBr-activated Sepharose 4B), activated according to manufacturer's instructions just before conjugating with an antigen (*see* Subheading 3.3, **step 1**).
2. Coupling buffer: weigh out 8.4 g $NaHCO_3$ and 29 g NaCl, dissolve both in 1 L of deionized water in a laboratory beaker and adjust the pH to 8.3.
3. Blocking buffer: mix 1.5 mL 1 M ethanolamine (pH 9.0) with 23.5 mL of coupling buffer. Store at 4 °C.
4. Acetate buffer (pH 4.0): add 820 μL 80% CH_3COOH to 100 mL of deionized water, then weigh out 720 mg CH_3COONa and dissolve it in the diluted CH_3COOH. Adjust the final buffer volume to 200 mL and check the pH.
5. Eluting buffer: 5 M guanidine hydrochloride, weigh out 6.6871 g of guanidine hydrochloride (ultrapure for molecular biology) and dissolve in 14 mL of deionized water to obtain a pH 4.5 solution. Store at 4 °C up to 2 weeks.

3 Methods

3.1 Cytometric Analysis of the Presence of LCs on the Surface of EVs

In addition to **step 1**, the following procedure steps allow to prepare the volume of suspension of bead-coated EVs needed for ten samples for cytometric staining. If more samples are planned, increase the proportions accordingly.

1. To wash and prepare aldehyde/sulfate latex beads, mix 4 μL of their stock suspension with 1 mL of DPBS and centrifuge at 3000 *g* for 10 min at 20 °C. Then completely remove the supernatant from above and resuspend the pelleted latex beads in 1 mL of DPBS.
2. Add 100 μL of latex bead suspension in DPBS to 15-mL polypropylene conical tube. Then add 50 μL of mouse EV suspension in DPBS (*see* **Note 1**) to the tube, close it tightly, and incubate on a hematological roller mixer for 10 min at room temperature (*see* **Note 3**).
3. Then add 1350 μL of DPBS to the tube and continue incubation on a hematological roller mixer for 2 h at room temperature.
4. Afterward, add 1 mL of 100-mM glycine solution in DPBS to the tube and continue incubation on a hematological roller mixer for 30 min at room temperature to block remaining binding sites on the latex beads.
5. Centrifuge the tube at 600 *g* for 10 min at 4 °C and completely aspirate the supernatant from above. To wash the bead-coated EVs, resuspend the pellet in 1 mL of 0.1% BSA in DPBS and centrifuge at 600 *g* for 10 min at 4 °C. Repeat the washing step one more time.
6. Completely aspirate the supernatant from above and resuspend bead-coated EVs in 4 mL of DPBS.
7. Prepare a number of 5-mL round bottom polystyrene FACS tubes equal to the number of control and experimental samples for EV cytometric analysis (up to ten samples). For processing each individual sample, add 400 μL of bead-coated EVs in DPBS to one FACS tube, and then add 25 μL of each antibody solution, as designed in the staining protocol (Fig. 1), especially considering the tetraspanin and LC co-expression analysis. Gently vortex and incubate in the dark at room temperature for 40 min with gentle agitation.
8. Then centrifuge the FACS tubes at 600 *g* for 10 min at 4 °C and completely aspirate the supernatant from above. To wash the stained bead-coated EVs, resuspend the pellet in 500 μL of 0.1% BSA in DPBS and centrifuge at 600 *g* for 10 min at 4 °C.
9. Completely aspirate the supernatant from above and resuspend stained bead-coated EVs in 400 μL of DPBS.
10. Analyze samples by flow cytometry under standard conditions and settings (*see* **Note 2**), and, as recommended (*see* **Note 4**), analyze only singlets; that is, EVs coupled to a single bead (Fig. 1).

3.2 Modified ELISA to Evaluate the Ability of EV-Bound LCs to Specifically Bind an Antigen (For Example, Native Ovalbumin, OVA)

1. To coat the 96-well clear flat bottom polystyrene ELISA microplate with an antigen (e.g., OVA, *see* **Note 5**), add 100 µL of antigen (OVA) solution in PBS (100 µg/mL) to the first 16 wells (Fig. 2). Then, add 100 µL of BSA solution in PBS (100 µg/mL), used as binding control, to the next 8 wells (Fig. 2). Seal the plate tightly with the adhesive strip and incubate overnight at 4 °C (in a refrigerator).
2. Uncover the plate, aspirate the content of each well and add 200 µL of PBS per well to all 24 wells. Shake off the PBS by vigorously inverting the plate and taping it on absorbent paper.
3. To block the plate, add 200 µL of 2% BSA solution in PBS to all 24 wells, seal the plate with the adhesive strip and incubate for 2 h at room temperature.
4. Repeat the washing step described in **step 2** twice.
5. Add 50 µL of mouse EV suspension in DPBS (*see* **Note 1**) to selected wells (Fig. 2, groups B–F), and add 50 µL of DPBS alone to blank wells (Fig. 2, group A). Seal the plate tightly with the adhesive strip and incubate overnight at 4 °C (in a refrigerator).
6. Uncover the plate, aspirate the content of each well and add 200 µL of 0.1% BSA in PBS per well to all 24 wells. Shake off the wash buffer by vigorously inverting the plate and taping it on absorbent paper. Repeat the washing step two more times.
7. Add 100 µL of rat IgG antibody anti-mouse CD9 (4 µg/mL in PBS with 0.1% BSA) or an equal volume of the appropriate antibody isotype at the same concentration or 100 µL of PBS to the designated wells (Fig. 2). Seal the plate with the adhesive strip and incubate for 2 h at room temperature.
8. Repeat the washing step described in **step 6** thrice.
9. Add 100 µL of HRP-conjugated rabbit IgG antibody anti-rat IgG (2 µg/mL in PBS with 0.1% BSA) to all 24 wells. Seal the plate with the adhesive strip and incubate for 1 h at room temperature.
10. Just before the end of the incubation, prepare the TMB substrate solution by mixing equal volumes of reagents A and B according to the manufacturer's instructions.
11. Wash the plate five times by repeating the washing procedure described in **step 6**.
12. Add 100 µL of TMB substrate to each well and incubate at room temperature in darkness from 8 to 10 min (until the color will develop).

13. Then stop the reaction immediately by adding 50 μL of 1 M H_3PO_4 to each well.
14. Read the plate at 450 nm with a reference wavelength of 570 nm.

3.3 Affinity Chromatography to Separate the Antigen-Binding Subpopulation of EVs

All reagent quantities and volumes are based on a 7-mL volume of activated gel matrix (Sepharose) that allows the separation of EVs from no more than 15 mL of the EV suspension. Be careful not to dry the OVA-conjugated gel matrix during the entire procedure.

1. The following procedure should be performed under a chemical fume hood due to the formation of a small amount of volatile compounds. To activate and prepare ex tempore the final volume of 7 mL of the CNBr-activated Sepharose 4B gel matrix weigh out 2 g of the dried powder and suspend it in an excess volume of 1-mM HCl (about 30 mL) in a 50-mL polypropylene conical tube. Transfer the whole content of the tube on the sintered glass filter placed in the vacuum pump and wash the gel with 350 mL of gradually pipetted 1-mM HCl (washing ratio 50 mL of HCl per 1 mL of gel matrix). Then drain off the remaining 1-mM HCl, turn off the vacuum pump, and resuspend the 7-mL gel matrix in 14 mL of coupling buffer to transfer the entire activated gel matrix volume to 50-mL polypropylene conical tube. Allow the gel matrix to settle to the bottom of the tube.
2. Weigh out 70 mg of OVA lyophilized powder and dissolve in 7 mL of coupling buffer in 15-mL polypropylene conical tube.
3. To conjugate the activated Sepharose 4B with its ligand (e.g., OVA antigen), take a 50-mL polypropylene conical tube with the gel matrix settled on the bottom (prepared ex tempore as described in Subheading 3.3, **step 1**), carefully pipet the buffer over the gel and replace immediately with the 7 mL of OVA solution (10 mg/mL) in coupling buffer. Close the tube tightly and incubate on a hematological roller mixer for 2 h at room temperature (*see* **Note 3**). Be careful not to foam the mixture. Otherwise, the tube can be incubated on a hematological roller mixer overnight at 4 °C (in a refrigerator).
4. To block the remaining binding sites, add 25 mL of blocking buffer (1 M ethanolamine in coupling buffer) to the tube with OVA-conjugated gel matrix, close it tightly, and incubate on a hematological roller mixer for 2 h at room temperature (*see* **Note 3**).
5. Transfer the whole content of the tube on the sintered glass filter placed in the vacuum pump and wash the OVA-conjugated gel matrix by gradual pipetting of 50 mL of coupling buffer, followed by gradual pipetting of 50 mL of acetate buffer. Repeat the washing step with 50 mL of coupling

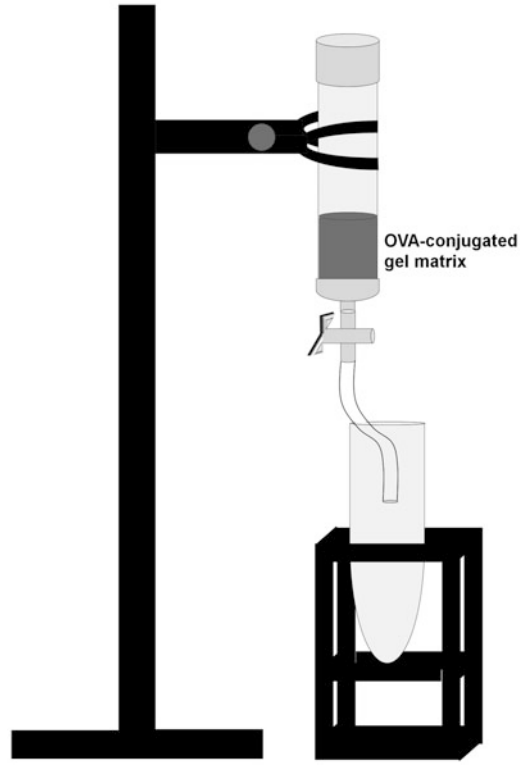

Fig. 3 Scheme of an affinity column chromatography set. Whenever the valve is open, care must be taken not to dry the OVA-conjugated gel matrix

buffer followed by 50 mL of acetate buffer twice. Then wash the OVA-conjugated gel matrix by gradual pipetting of 40 mL of eluting buffer. Be careful not to dry the OVA-conjugated gel matrix.

6. Add 50 mL of DPBS to the sintered glass filter placed in the vacuum pump to replace the washing and eluting buffers. Stop the vacuum pump. Add 15 mL of DPBS and transfer the entire volume of the OVA-conjugated gel matrix to a stoppered glass affinity column. Wait for the gel packing to settle tightly to the bottom of the column (*see* **Note 6**).
7. To prepare the column affinity chromatography, firmly fix the glass column in the tube holder, connect valve, and drain (Fig. 3). Place the end of the drain in the washing vessel. Whenever the valve is open, care must be taken not to dry the OVA-conjugated gel matrix. Open the valve and allow the DPBS level to drop almost to the top of the OVA-conjugated gel matrix. Immediately close the valve and gradually and carefully pipet the entire volume of EV suspension (prepared as described in Subheading 2.2) on the top of the

OVA-conjugated gel matrix. Keep all buffers, reagents, EV suspension, and column packing cooled to 4 °C. It is recommended to carry out the procedures 6–10 in a cold room.

8. Place the end of the drain in the flow-through (negative) collection tube and open the valve. Allow the fluid level to drop almost to the top of the OVA-conjugated gel matrix and immediately close the valve.
9. Add 10 mL of DPBS on the top of the OVA-conjugated gel matrix, open the valve, and allow the DPBS level to drop almost to the top of the OVA-conjugated gel matrix. Then immediately close the valve.
10. Place the end of the drain in the washing vessel. Add 10 mL of DPBS on the top of the OVA-conjugated gel matrix, open the valve, and allow the DPBS level to drop almost to the top of the OVA-conjugated gel matrix. Then immediately close the valve, and repeat the washing step thrice. Allow the DPBS level to drop almost to the top of the OVA-conjugated gel matrix and immediately close the valve.
11. Place the end of the drain in the eluate (positive) collection tube. Add 2 mL of eluting buffer on the top of the OVA-conjugated gel matrix, open the valve, and gradually and carefully pipet 12 mL of eluting buffer, taking care not to dry out the column packing. Then gradually and carefully pipet 140 mL of DPBS, all the volume should be collected to eluate tube to dilute acidic guanidine. Allow the DPBS level to drop almost to the top of the OVA-conjugated gel matrix and immediately close the valve.
12. Transfer all eluate to ultracentrifuge tubes, carefully balance their weight and ultracentrifuge the eluate at 100,000 *g* for 70 min at 4 °C. Then gently aspirate the supernatant from above and resuspend the eluted EV-containing pellet in DPBS to repeat ultracentrifugation in the same conditions. Then, gently aspirate 90% of the volume of supernatant from above and carefully decant the remaining supernatant by slowly turning the tube upside down and placing it on an absorbent towel to reduce the residual volume as much as possible.
13. Resuspend the washed antigen-binding EVs in DPBS by adding no more than 300 μL of DPBS to the bottom of the tube and gently but vigorously stirring it with a pipette, scraping the bottom of the tube with the tip and turning the tube in your fingers. Then transfer the entire volume to an Eppendorf tube. EVs collected in this way can be immediately used in analyzes or stored at −80 °C for up to 6 months.

4 Notes

1. In our experimental conditions, we have validated the described protocols for EVs isolated from supernatant from the culture of mouse spleen and lymph node cells (T lymphocytes) and from mouse serum. However, analogous protocols can also be used to analyze EVs from humans and other animal sources. In this case, it is important to select the appropriate antibody clones against human or other animal markers. Furthermore, some researchers estimate the amount of EVs in samples by referring it to the protein content determined, among others, by Bradford or bicinchoninic acid protein (BCA) assays. In this case, it is recommended to prepare an EV suspension with a protein concentration in the range of 50–100 μg/mL in DPBS. However, this recommendation has not been tested by us under any conditions.
2. In our experimental conditions, we have used monoclonal antibodies conjugated with either fluorescein isothiocyanate (FITC, anti-LC antibody) or phycoerythrin (PE, antibodies against tetraspanins), and the recommended dilution 1:200 worked best. However, it is good laboratory practice to validate dilutions of antibodies conjugated with given fluorochromes in each laboratory prior to experimental use. Moreover, it is not recommended to use the high-resolution flow cytometer for the analysis of bead-coated EVs. Otherwise, possible aggregates should be removed from monoclonal antibody preparations prior to use and appropriate controls for antibody aggregation should be applied (*see* ref. 14).
3. The hematological roller mixer can be replaced with a laboratory tube shaker, but the shaking force should then be set to the lowest setting. Never use a magnetic stirrer.
4. In general, cytometric analysis of singlets, considered to be a single latex bead individually coated with EVs, gated on SSC to FSC dot plots is recommended. However, in another study, we have demonstrated the possibility to aggregate EVs that express MHC class II and antigenic determinant with the use of antigen-specific IgG antibodies, which can be detected by flow cytometry (*see* ref. 9).
5. In our experimental conditions, to detect the ability of EV-coating LCs to bind the antigen, we have used ELISA plates coated with native OVA, OVA tryptic peptides and OVA-323-339 antigenic determinant, and BSA as the control of binding specificity. If required, other soluble protein antigens (dissolved in PBS at an analogous concentration) could be used for coating the ELISA plate for evaluation of the ability of EV-coating LCs to bind the antigen.

6. If the OVA-conjugated gel matrix is prepared in advance, replace DPBS with 20% ethanol and fill the entire glass column with 20% ethanol under the stopper. Store at 4 °C in a refrigerator up to 1 month. Furthermore, in the case when the material, from which EVs are separated, is biologically safe, the column can be reused after extensive washing with eluting buffer, followed by DPBS, and its replacement with 20% ethanol. The washed column can be stored as described before.

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Chapter 19

Sequential Double Immunoblotting with Peptide Antibodies

Nicole Hartwig Trier and Gunnar Houen

Abstract

Immunoblotting, also termed western blotting, is a powerful method for detection and characterization of proteins separated by various electrophoretic techniques. The combination of sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE), having high separating power, immunoblotting to synthetic membranes, and detection with highly specific peptide antibodies, is especially useful for studying individual proteins in relation to cellular processes, disease mechanisms, etc. Here, we describe a protocol for the sequential detection of various forms of an individual protein using peptide antibodies, exemplified by the characterization of antibody specificity for different forms of the protein calreticulin by double SDS-PAGE immunoblotting.

Key words Peptide antibodies, Double immunoblotting, Calreticulin

1 Introduction

Western immunoblotting is commonly used in research in combination with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate native or denatured proteins in a given sample, and application of this technique has made it possible for researchers to identify specific proteins in a complex protein mixture and obtain information about the relative amount of protein present in a sample [1–3]. In addition, western blotting provides important knowledge about the molecular mass of a protein, which gives this technique an advantage over other antibody-based techniques such as enzyme-linked immunosorbent assay and immunohistochemistry. Based on this, western blotting has become a highly valuable tool [1, 2]. The western blotting technique consists of several major steps: separation of proteins, transfer of separated proteins to a membrane, interaction between the target protein and specific primary antibodies, and finally visualization of the target protein [3, 4]. Each step should be carefully conducted and potentially optimized to obtain a satisfying result.

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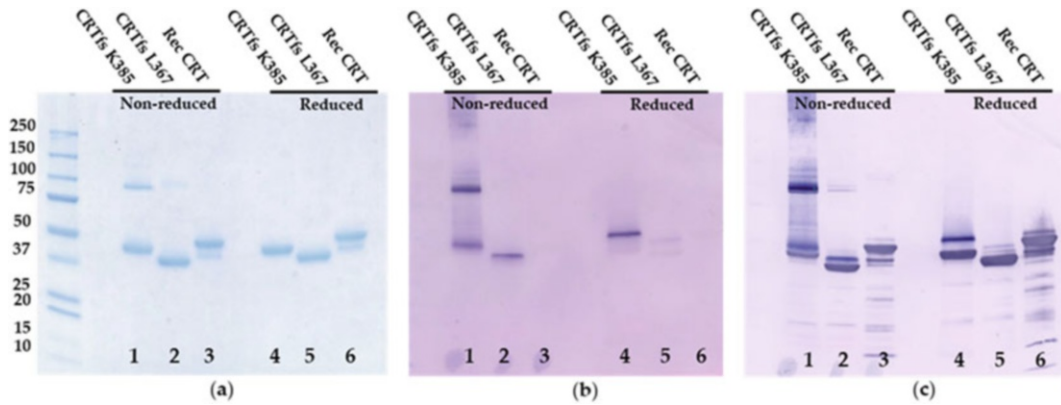

Fig. 1 Immunostaining of frameshifted calreticulin (CRTfs) and wild-type calreticulin (CRT*wt*). (**a**) Coomassie Brilliant Blue staining of recombinant CRT proteins. (**b**) Western blotting of recombinant CRT proteins incubated with a specific mouse monoclonal peptide CRTfs IgG recognizing the mutated C-terminal of CRTfs (**c**). Western blotting of membrane from figure (**b**) developed with a CRT antibody, recognizing both the N-terminal domain of CRT*wt* and CRFfs [6, 12]. Lane 1: non-reduced CRTfs K385, lane 2: non-reduced CRTfs L367, Lane 3: non-reduced recombinant CRT*wt*, Lane 4: reduced CRTfs K385, Lane 5: reduced CRTfs L367, Lane 6: reduced recombinant CRT

Peptide antibodies are valuable tools in western blotting, where peptide antibodies have been used for protein identification, epitope characterization, and as diagnostic tools [5–10]. They are at present time mainly used as primary antibodies; however, they may function as secondary antibodies as well [5, 6].

Traditionally, "simple" immunoblotting is most often applied; however, occasionally, double immunoblotting can be performed, where several protein bands are identified within a single sample [1, 6, 11]. Double blotting was originally developed to overcome problems with nonspecific binding caused by secondary antibodies; however, the principle can be used in other contexts as well, for example, for detection of various protein forms as illustrated in this chapter (Fig. 1) [1, 6, 11].

1.1 Electrophoresis

Initial steps of immunoblotting include sample preparation followed by electrophoresis, where proteins are separated according to their molecular weight and net charge [13, 14]. Cell lysates or protein solutions are most often used in western blotting, where it is important to have a rough estimate of the protein concentration, to ensure that samples are compared on an equivalent level [1, 3, 15]. In general, 1–10 μg of a purified protein mixture/cell lysate, containing the relevant protein is often recommended to obtain linear signals, which can be quantified [3]. Protein samples are usually diluted in a loading buffer or sample buffer, which contains glycerol, making the samples sink into the wells of the gel [3]. Moreover, a tracking dye, such as bromophenol blue, is

included in the buffer, which allows for visualization of the separation process [1, 3]. Protein samples are typically heated in the presence of SDS (0.1%) to denature the higher-order structure of proteins prior to loading, which however retains disulfide bonds, which may be broken by the addition of reducing agents such as dithiothreitol (DTT) [1, 3]. As a result of protein denaturation, the protein-SDS complex acquires a net negative charge and consequently moves toward the positive electrode, when a voltage is applied during electrophoresis [16, 17]. If determination of native proteins is required, preheating should be omitted, and no detergent should be used for initial sample preparations to preserve the native structure of proteins to be loaded [1, 3].

Gel electrophoresis can be conducted using various matrices, for example, agarose, acrylamide, or even starch [13, 14, 16, 17]. Poly-acrylamide is the most common used matrix for gel electrophoresis, which include native PAGE, two-dimensional (2D)-PAGE, and SDS-PAGE [18, 19]. While 2D-PAGE separates proteins by native net charge in the first dimension and by molecular weight in the second dimension, native PAGE and SDS-PAGE primarily separate proteins by molecular weight (and net charge) in one dimension [1, 2, 13, 18]. Moreover, native PAGE retains the higher-order structure of proteins, whereas SDS-PAGE often is conducted in combination with reducing agents for examination of denatured protein structures [3, 20].

As acrylamide is toxic, pre-cast gels purchased from suppliers are often preferred for electrophoresis, which also is more convenient [3, 21]. The gels applied are divided into two parts, a stacking gel and a resolving gel [3]. The stacking gel is slightly acidic (pH 6.8) and has a lower acrylamide concentration creating a more porous gel, which separates protein poorly but allows for them to "stack" into distinct bands [1, 3]. The lower phase, referred to as the separating or resolving gel, is more basic (pH 8.8) and has a higher polyacrylamide content, making the gel pores narrower [3]. As a result, the proteins are separated according to size, as the smaller proteins migrate more rapidly than larger proteins. The percentage of the resolving gel should correspond to the size of the protein examined; the smaller the protein size, the higher the percentage of the resolving gel [3, 17].

Following loading of samples in the wells of the gels, running buffer is added to the electrophoresis apparatus. The most commonly used running buffers are Tris–glycine buffers, but other running buffers such as acetate, 2-(N-morpholino)-ethanesulfonic acid (MES), 3-(N-morpholino)-propanesulfonic acid (MOPS), Tris–acetate, and Tricine may be used as running buffers as well [1, 22, 23].

1.2 Immunoblotting

Separated proteins are transferred to a membrane, which often is performed by electroblotting due to its highly effective protein transfer, although other techniques such as capillary transfer, vacuum blotting, and diffusion can be used [24–27]. In electroblotting, an electric field is applied, which is oriented perpendicular to the surface of the gel, resulting in that negatively charged proteins migrate from the gel to the membrane, when the membrane is located between the gel surface and the positive electrode [26]. In this process, sponges and filter paper are added at each end of the gel-membrane ensample to generate a sandwich [3]. Soaking of the sandwich (wet-transfer) in transfer buffer or the addition of buffer-soaked filter paper placed in the sandwich (semidry transfer) are the most common types of transfer, although "dry" transfer of proteins can be achieved as well [1, 3, 26]. Semidry transfer is in general much faster and only requires minutes compared to wet transfer, which may take an hour or more; however, semidry transfer may result in a less efficient transfer compared to wet transfer and is mainly recommended for transfer of smaller proteins, whereas wet transfer is recommended for proteins larger than 100 kD [3].

Membranes used for protein transfer are primarily made of nitrocellulose or polyvinylidene difluoride (PVDF) [28]. Nitrocellulose membranes have good protein binding affinity and are compatible with several detection methods [29, 30]. PVDF membranes typically have a higher capacity for protein binding and are especially good for binding of hydrophobic proteins [1, 3, 28]. In addition, the application of PVDF membranes in principle allows for reuse, as the membrane can be stripped, reprobed, or stored [28]. However, PVDF membranes may sometimes yield higher background staining compared to nitrocellulose membranes; thus, attention is needed to reduce potential background signals [3]. Moreover, PVDF membranes should be preactivated prior to use, where the membrane is soaked in methanol for 30 s followed by washing in deionized water for 1 min [1, 3].

Transfer buffers used are primarily based a tris–glycine buffer, which may be supplemented with methanol (10–20%) or sometimes SDS (0.1%), although other transfer buffers occasionally have been applied as well [1, 26, 31, 32]. Methanol is added to prevent the gel from swelling and may improve the efficiency of protein binding to especially nitrocellulose membranes [1]. SDS is normally omitted from the transfer buffer; however, if proteins have a tendency to aggregate or precipitate or large proteins are to be transferred, it may be necessary to add SDS to the transfer buffer [31].

Transfer time and transfer current should not be neglected, as these significantly may influence the transfer process as well, as too low current/and or transfer time may lead to incomplete transfer. Similarly, if the current is too high and the transfer time is too long,

the proteins may migrate through the membrane too fast without binding to the membrane, which especially relates to proteins with a small molecular weight [1, 3]. 1–2 h, alternatively overnight transfer, is often recommended for wet transfer, whereas semi-wet transfer takes 7–10 min [1, 3].

1.3 Membrane Blocking

Following protein transfer, the membrane is blocked using various blocking solutions, which reduces nonspecific binding to the membrane during incubations [3, 9, 26]. Blocking is most often achieved by incubation with blocking solution for 1 h or more at 4 °C by agitation, using solutions such as Tris–buffered saline containing 0.1% Tween 20 (TBST) or PBS + 0.1% Tween 20 (PBST) combined with 1–5% nonfat milk or 1–5% bovine serum albumin (BSA), or a Tris–Tween 20 (0.1–1%) or sodium chloride (TTN) buffer [1, 3, 6, 27]. Critical is the addition of Tween 20 to the blocking buffer, as Tween blocks hydrophobic interactions, thereby reducing nonspecific binding to the membrane [26].

Blocking buffers should be carefully selected, as these may interfere with the final development. For example, buffers containing BSA are often preferred with biotin- and AP-labelled antibodies, as casein present in milk powder may interfere, and as milk powder contains biotin, hence blocking solutions containing nonfat milk should be avoided when using avidin-biotin detection systems [1, 3]. Clearer results may be obtained when using BSA blocking solutions, as the number of different proteins in the buffer is significantly reduced compared to nonfat milk powder buffers, which in theory should result in a reduced probability of nonspecific binding [1, 3].

1.4 Incubation with Primary Antibodies

For detection of specific proteins transferred to the membrane, the membrane is incubated with a primary antibody specific for the protein to be identified [1, 3, 6, 26]. Unbound antibodies are washed away leaving only the primary antibody bound to the protein of interest. Monoclonal and polyclonal antibodies may be applied; however, monoclonal antibodies are often preferred due to high specificity and purity, which may result in a lower background. Alternatively, serum or ascites fluids or crude antibody preparations can be used, which, however, may increase nonspecific binding [1, 3, 6, 26]. It is suggested to incubate with the primary antibody in the selected blocking buffer, using the antibody concentration recommended by the manufacturers.

The membranes are usually incubated with primary antibody for 1 h at room temperature or at 4 °C overnight with agitation. Upon incubation with primary antibodies, the membranes are rinsed and washed using buffers such as PBST, TBST, or TTN with agitation [1, 3, 6, 26]. Washing steps are critical to ensure

minimization of background noise and removal of unbound antibodies; however, extended washing should be avoided as it may reduce the specific signal during development [1, 3, 6, 26].

Alternatively, incubation can be conducted using a primary antibody labeled with a fluorescent dye or an enzyme, thereby omitting the need for incubation with a secondary antibody [1]. This method is faster, as only one incubation with antibody is needed; however, as only a limited number of conjugated antibodies is available and since labeling of individual primary antibodies is time-consuming, an additional incubation step with secondary antibodies is most commonly used [1].

1.5 Visualization of Protein Staining

Next after incubation with a primary antibody followed by washing, the membrane is to be incubated with a labeled secondary antibody, usually 1–2 h at room temperature or overnight at 4 °C with agitation, whereafter the membrane is washed with TBST, PBST, or TTN buffer, allowing for excess of unbound secondary antibody to be removed [1, 26]. Final protein identification involves the detection of the probes on labeled antibodies, which can be achieved using several approaches such as enhanced chemiluminescence (ECL), fluorescence, infrared, or colorimetric methods, where secondary antibodies most often are labeled with biotin, fluorescent probes (rhodamine or fluorescein), radio isotopes, or enzyme conjugates (horseradish peroxidase (HRP) and alkaline phosphatase (AP)), where identified proteins are visualized by the addition of a substrate (cloro-1-napthol, 3,3′-diaminobenzidine, 3,3′,5,5′-tetramethylbenzidine (4CN, DAB, TMB) for HRP and 5-bromo-4-chloro-3-indolyl-phosphate, nitro blue tetrazolium (BCIP, NBT) for AP) [1, 6, 33]. ECL is one of the most applied techniques, where a HRP-labeled secondary antibody catalyzes the oxidation of a luminol substrate, leading to the emission of light (428 nm), which is detected using X-ray films or a charged-coupled devise camera and which allows for high sensitivity and high resolution [15, 26]. The main difference between ECL and the application of fluorescent dye-conjugated secondary antibodies is that in ECL the light emitted is based on a chemical reaction, whereas in fluorescence, the light emitted is a result of absorption of light, detected by a fluorescent digital system [1]. Similar to ECL, fluorescence detection provides high sensitivity and resolution and may even be combined to detect multiple targets using fluorophores with nonoverlapping excitation-emission spectra [1, 15].

Colorimetric detection is primarily conducted using an enzyme-conjugated secondary antibody (AP or HRP), which converts a substrate to a colored precipitate, whereby protein bands are visualized [34, 35]. Removal of the substrate buffer stops the development, where protein bands are visible to the eye; hence, no further equipment is necessary for detection, but occasionally

optimization may be required to improve signal-to-background noise [36]. When using enzyme-conjugated detection, it can be difficult to quantify the exact protein concentration in a sample, which is more readily done by ECL and fluorescence [37].

2 Materials

1. Protein sample for analysis; here, recombinant calreticulin (CRT)wt and frameshifted CRT (CRTfs) L367 and K385 (*see* **Note 1**).
2. Reducing and nonreducing sample preparation buffer (*see* **Note 2**).
3. Size marker panel.
4. SDS-PAGE gels (4–20% Tris–glycine gels) and matching electrophoresis equipment (*see* **Note 3**).
5. Electrophoresis running buffer/SDS running buffer (*see* **Note 4**).
6. Transfer buffer (*see* **Note 5**).
7. PVDF blotting membranes (*see* **Note 6**).
8. Methanol 20%.
9. Blocking, incubation, and washing buffers, here exemplified with TTN buffer (0.3-M NaCl, 20-mM Tris, 1% Tween-20 (w/v), pH 7.5) (*see* **Note 7**).
10. Primary monoclonal or polyclonal antibodies, here exemplified with peptide antibodies to CRTwt and CRTfs (*see* **Note 8**).
11. Relevant secondary antibodies (conjugates), for example, goat-anti mouse IgG-AP or goat-anti rabbit IgG-AP (*see* **Note 9**).
12. Coomassie Brilliant Blue (CBB) staining solution (*see* **Note 10**).
13. Deonized milliQ water/CBB destaining solution (*see* **Note 11**).
14. Substrate buffer for visualization of identified bands, here exemplified with AP-color-developing substrate (BCIP 0.5 mg/mL, NTB 0.3 mg/mL) (*see* **Note 12**).
15. BCIP Substrate tablets.
16. Shaking table.
17. Power supply.
18. Electrotransfer apparatus.
19. Heating block.
20. Deonized water.

3 Methods

3.1 Sample Preparation

1. Prepare a suitable amount of reducing and/or nonreducing sample buffer and protein.
2. Dilute proteins (10 μg) 1:2 in (non)-reducing sample buffer in a small tube and mix.
3. Incubate samples for 2 min in a dry heating block at 95 °C.

3.2 Electrophoresis

1. Place the gel cassettes in the electrophoresis apparatus, and carefully remove the combs by pushing directly upward with your thumbs at the end of the ridge.
2. Pour running buffer outside in the holder (and inside the holder if two gels are applied) and make sure the running buffer covers the gels (*see* **Note 13**).
3. Optionally, rinse the wells in the gel with running buffer before samples are loaded.
4. Load an appropriate size exclusion marker (2 μL) (e.g., lane 1) followed by protein samples (10 μL) in the remaining wells (*see* **Note 14**).
5. Connect the electrophoresis unit to the power pack and run for 2 h at 50 V and 250 mA followed by 30 min at 100 V and 250 mA or until the dye almost reaches the bottom of the gel (*see* **Note 15**).
6. Once the electrophoresis is finished, carefully remove the gels from the cassettes (*see* **Note 16**).

3.3 Gel Staining

1. Prepare the CBB staining solution by mixing 0.1% CBB dye diluted in 40% ethanol and 10% acetic acid to a total volume of 1 L in deionized water.
2. Incubate gels in a staining container with staining solution (100 mL).
3. Incubate overnight at 4 °C with staining solution and gentle agitation on a rocking table (*see* **Note 17**).
4. Decant the staining solution and rinse the gel with deionized water.
5. Destain the gel using deionized water. Replace the water up to five times.
6. Gently shake the gel at room temperature on an orbital shaker until the desired background is achieved (*see* **Note 18**).

3.4 Immunoblotting

1. Pre-activate the PVDF membrane by soaking the membrane in methanol for 30 s (*see* **Note 19**).
2. Wash the membrane in deionized water for 1 min under gentle agitation.

3. Place the membrane in ice cold transfer buffer for 5 min.
4. Transfer proteins from the gel to a membrane using an electroblotting device (semidry blotting) and follow blotting instructions provided by the manufacturer (*see* **Note 20**).
5. Following transfer, remove the membrane from the blotting device and mark the orientation of the gel with a waterproof pen.

3.5 Immunostaining

1. Place the membrane in a suitable container and add enough blocking solution (TTN) to cover the membrane. Make sure not to pour the solution directly on the membrane.
2. Block for nonspecific binding on the membrane in TTN buffer overnight at room temperature (*see* **Note 21**).
3. Apply washing buffer (TTN) and wash the membrane for 2 × 5 min by gentle agitation.
4. Incubate the membrane with appropriate dilutions of primary antibody (mouse anti-CRT IgG, 1 μg/mL) diluted in blocking buffer (TTN) for 1 h at room temperature.
5. Wash the membrane with TTN for 2 × 5 min.
6. Add secondary antibody (conjugate) (e.g., goat-anti mouse IgG-AP (specific for mouse IgG)) diluted in TTN (1 μg/mL) and incubate for 1 h at room temperature (*see* **Note 22**).
7. Wash the membrane with TTN for 2 × 5 min.
8. Dissolve AP-substrate tablets in AP-substrate buffer to a final concentration of 1 mg/mL.
9. Visualize the presence of specific proteins by adding AP color developing substrate (BCIP 0.5 mg/mL, NTB 0.3 mg/mL) and incubate until visible protein staining is obtained.
10. Stop the reaction by washing the membrane with deionized water followed by drying on filter paper.
11. Repeat **steps 4–9** by replacing the primary antibody with a second "primary antibody" (here CRT FMC 75 mAb) diluted 1:10,000 in TTN buffer [12].

3.6 Documentation

1. Photography/scanning of membranes at high resolution.

4 Notes

1. Cell lysates and other protein mixtures can be used as well. 1–10 μg of protein is recommended.
2. Reducing sample buffer typically contains: 10% w/v SDS, 10-mM DTT or β-mercaptoethanol, 20% v/v glycerol, 0.2-M Tris–HCL, pH 6.8, 0.05% bromophenol blue.

DTT/β-mercaptoethanol is only added for preparation of reducing sample buffer. For native PAGE gels SDS, DTT/β-mercaptoethanol are omitted.

3. Pre-cast or in-house gels can be used. SDS-PAGE gels are often applied for separation of denatured proteins, whereas native PAGE are gels used for separation of native proteins. The polyacrylamide percentage should correspond to the size of proteins to become separated.
4. SDS running buffer typically contains 192-mM glycine, 25-mM Tris–base, 0.1% SDS, pH 8.3. For native PAGE, running buffer omit SDS from the solution. Alternatively, other running buffers such as acetate, MES, MOPS, Tris–acetate, and Tricine buffers can be applied.
5. Tris–glycine buffer (25-mM Tris–Base, 192-mM glycine), containing methanol (10–20% (v/v)) and alternatively SDS (0.1–0.25%), pH 8.3. Methanol can be omitted if preactivated PVDF membranes are used. Alternatively, other buffers can be applied.
6. Alternatively, nitrocellulose membranes can be used.
7. Numerous buffers can be used for blocking, incubation, and washing such as PBST (137-mM NaCl, 2.7-mM KCl, 10-mM phosphate, 0.05% Tween 20 (w/v), 20 pH 7.4) or TBST (20-mM Tris, 150-mM NaCl, 0.1% Tween-20 (w/v), pH 7.4) supplemented with 1–5% BSA or 1–5% nonfat milk powder.
8. The primary antibody should specifically recognize the target protein.
9. Secondary antibodies applied must recognize the IgGs from the animal used for generation of the primary antibodies and must be labelled, for proteins to be visualized.
10. CBB gel stain contains 0.1% CCB dye diluted in 40% ethanol and 10% acetic acid. Alternatively, other staining solutions can be applied, for example, silver staining, fluorescent dye staining, zinc staining, or functional group-specific staining [38].
11. Alternatively, use a destaining solution containing 10% ethanol and 7.5% acetic acid.
12. The substrate buffer must correspond to the approach used for development. For example, for HRP labeling, a suitable HRP substrate developing solution should be used.
13. Select a running buffer compatible with the membrane. For native PAGE and SDS-PAGE, native and SDS running buffer is applied, respectively.
14. Alternatively, loading controls can be used to ensure equal loading in the wells. Common loading controls include β-actin, glyceraldehyde-3-phosphate dehydrogenase, histone H3, α-tubulin, and β-tubulin.

15. Alternatively, run at 60–100 V for 60 min. Be careful to stop before any protein passes through the gel.
16. The gel is easily removed by separating the casing in the sides. Be careful when handling the gel; it is very fragile.
17. Alternatively, incubate for 1 h at room temperature until bands are visible or in a microwave oven for 40 s to 1 min at high power, until the staining solution boils.
18. Destaining can be accelerated by placing the gel in a microwave oven for 40 s to 1 min at high power, followed by 10 min of incubation.
19. When applying premade sandwiches, activation of PVDF membranes is omitted.
20. Alternatively, transfer the proteins by wet or dry transfer applying appropriate buffers, current, and transfer time [1–3].
21. Alternatively, block for 1 h. Blocking for longer than 1 h at room temperature may result in lower band intensities.
22. Use a secondary antibody directed against the immunoglobulins of the animal used for generation of the primary antibody, which is compatible with the technique used for visualization.

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Chapter 20

Cytochemical and Histochemical Staining with Peptide Antibodies

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Abstract

Peptide antibodies are particularly useful for immunocytochemistry (ICC) and immunohistochemistry (IHC), where antigens may denature due to fixation of tissues and cells. Peptide antibodies can be made to any defined sequence, including unknown putative proteins and posttranslationally modified sequences. Moreover, the availability of large amounts of the antigen (peptide) allows inhibition/absorption controls, which are important in ICC/IHC, due to the many possibilities for false-positive reactions caused by immunoglobulin Fc receptors, nonspecific reactions and cross-reactivity of primary and secondary antibodies with other antigens and endogenous immunoglobulins, respectively. Here, simple protocols for ICC and IHC are described together with recommendations for appropriate controls.

Key words Absorption control, Immunocytochemistry, Immunohistochemistry, Peptide antibodies, Specific staining

1 Introduction

Antibodies allow precise and specific cellular and tissue localization of antigens by immunocytochemistry (ICC) and immunohistochemistry (IHC) [1, 2]. Peptide antibodies, in particular, are useful for localization of many peptides and proteins including unknown/putative peptides/proteins predicted to exist from DNA sequences. Another advantage of peptide antibodies is that they can be made to posttranslationally modified sequences, for example, phosphorylated sequences or citrullinated sequences. Also, in ICC and IHC, denaturing conditions are often used (e.g., heat treatment or ethanol fixation), and the epitopes of peptide antibodies will generally be less sensitive to such treatments, compared to three-dimensional epitopes. Finally, since peptides usually can be produced in large quantities, they allow control experiments in the form of inhibition/absorption controls (Fig. 1).

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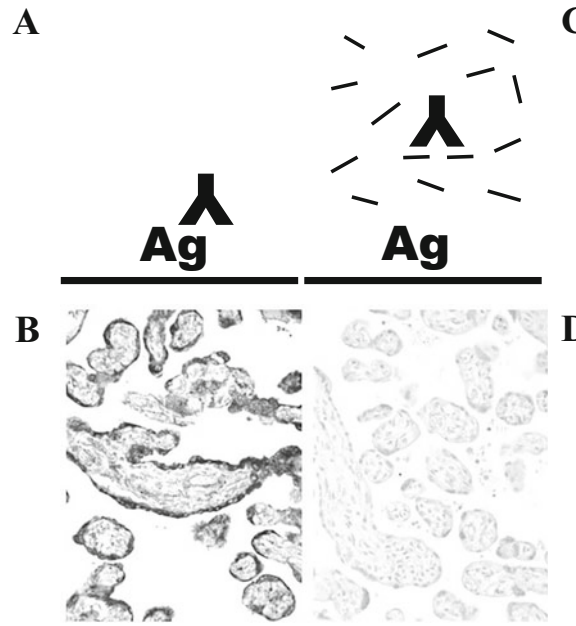

Fig. 1 Principle of ICC/IHC and absorption control with peptide antibody (Ab). (**a**) Binding of peptide Ab to epitope in cell/tissue antigen (Ag). (**c**) Absorption control with excess peptide containing the epitope for the Ab. (**b**) Localization of calreticulin in human placenta by IHC with enzyme activity staining (peroxidase) and light microscopy. The tissue section was incubated with a sequence-specific rabbit antiserum against a synthetic peptide from the C-terminus of calreticulin [3] followed by incubation with horseradish peroxidase-conjugated goat immunoglobulins against rabbit IgG. Note the strong staining of the syncytiotrophoblast layer. (**d**) Absorption control with excess peptide. Note the essentially complete absence of staining

Peptide antibodies may be produced following procedures described in this volume. Incubation of cells and tissues with peptide antibodies is not different from the use of other antibodies, and bound antibodies may be visualized by a number of well-known techniques (i.e., enzyme activity staining or fluorescence) [1, 2].

For ICC and IHC, cells or tissues may be obtained commercially, by cell culture or from clinical departments and animal facilities, taking appropriate ethical precautions.

Cells may be stained directly with antibodies or prepared by fixation with crosslinking agents (e.g., formaldehyde) or precipitating agents (e.g., ethanol). In the case of cell surface antigens, living cells can be stained directly. With intracellular antigens, cells have to be fixated and permeabilized to make the antigen accessible. Depending on the antigen and the method of fixation, various methods for permeabilization (i.e., detergent treatment) and epitope/antigen retrieval may have to be attempted (e.g., heat treatment, protease treatment, incubation with different detergents and

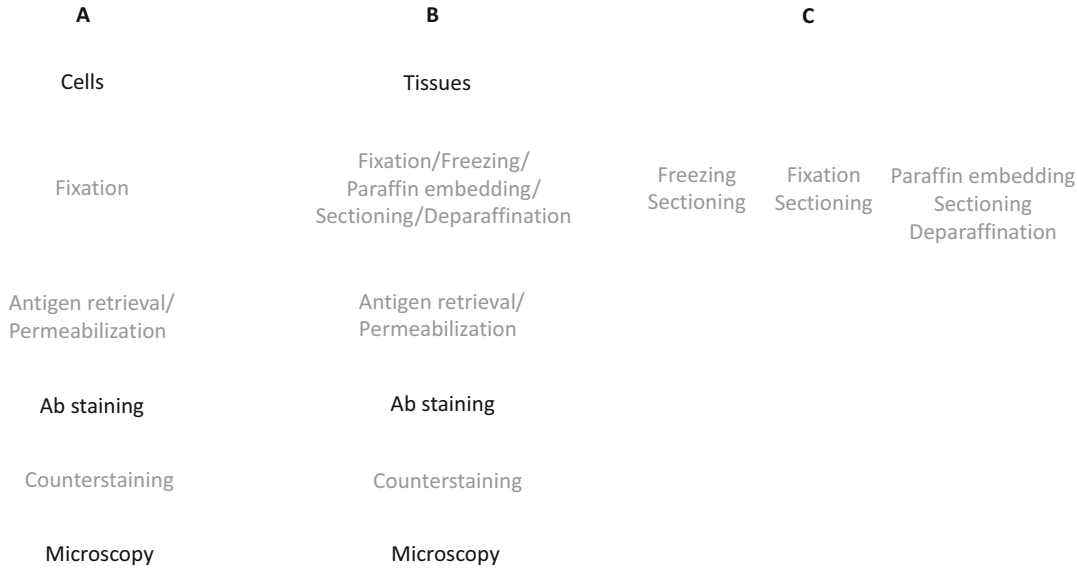

Fig. 2 (**a**) Workflow for immunocytochemical staining with antibodies. (**b**) Workflow for immunohistochemical staining with antibodies. (**c**) Options for tissue processing before Ab staining. Black: obligatory. Gray: optional

buffers at low or high pH, etc.) [2]. Tissues may also be sectioned directly for subsequent staining, but usually, tissue samples are frozen before sectioning/fixation/staining or fixated before freezing/sectioning/staining (Fig. 2).

Visualization of bound antibodies is achieved by labelling of antibodies (primary or secondary) with fluorophores or enzymes. Staining patterns can then be inspected by fluorescence microscopy or by light microscopy after enzyme activity staining (e.g., phosphatase or peroxidase). Staining of abundant components of cells and tissues (e.g., nuclei) with conventional dyes (contrast staining) may be used to facilitate microscopical analysis [1, 2, 4].

Validation of specific antibody staining in ICC/IHC requires performance of a number of positive and negative control experiments, and a staining pattern is only specific if all controls exclude nonspecific staining [2, 4–6]. Special for peptide antibodies is the usually high specificity and the availability of large amounts of the antigen (peptide) at a reasonable cost, thus providing the possibility to perform well-defined inhibition/absorption controls (Fig. 1). These are particularly important when using newly developed or uncharacterized antibodies in ICC/IHC due to the heterogenous nature of the substrate and the possibility of false-positive reactions caused by endogenous enzyme activity or autofluorescence, Fc receptors binding primary or secondary antibodies, or cross-reactivity of primary or secondary antibodies with other antigens or endogenous immunoglobulins, respectively. For ideal absorption controls, staining is abolished by inhibition/preabsorption of the primary antibody with purified antigen but not with carrier

protein or irrelevant antigen. Unfortunately, simple preincubation of antibody with antigen does not always eliminate antibody staining completely, since antigen may dissociate from antibody during incubations, thus allowing unbound antibody to bind antigens in the section. To circumvent this, it may be advisable to use antigens coupled to beads for preabsorption of specific antibodies and to use more rounds of absorption.

Reagent substitution controls may be used to identify false-positive staining reactions (e.g., by omitting primary or secondary antibodies or by replacing the primary antibody with an irrelevant species- and isotype-matched antibody known not to react with the cells/tissues under study).

Positive and negative controls may be performed on other relevant cells/sections prepared as those being tested. A negative control with tissue known not to contain the antigen of interest may exclude false-positive staining, and a positive tissue control may exclude the possibility of false-negative staining. The positive tissue control is most valuable when performed on a section containing low concentrations of the antigen of interest, thus displaying weak staining, since it will be more sensitive to impacts that influence the antibody staining than a section containing high antigen levels.

This chapter describes simple protocols for ICC/IHC together with suggestions for appropriate controls to verify the specificity of the observed reactions (*see* **Note 1**).

2 Materials

2.1 Chemicals

1. Tween 20.
2. Triton X-100.
3. EtOH.
4. Glycerol.
5. Xylene.
6. Hexane.
7. Paraffin.
8. Tissue Tek.
9. Hematoxylin.
10. Eosin.
11. 4′,6-diamidino-2-phenylindole (DAPI).
12. Hoechst 33342.
13. Propidium iodide (PI).
14. 3,3′-Diaminobenzidine (DAB).
15. 5-Bromo-4-chloro-3′-indolyl-phosphate (BCIP).

16. Nitroblue tetrazolium (NBT).
17. Proteinase K.
18. Peptides may be obtained commercially or can be synthesized and characterized according to procedures described in this volume.
19. Peptides may be coupled to various matrices (e.g., CNBr-Sepharose) or beads (e.g., paramagnetic beads or beads for immunoassays) following the instructions of the manufacturer for preabsorption controls.

2.2 Equipment

1. Scalpels, forceps, scissors.
2. Plate for cutting.
3. Metal plate for freezing of tissue samples.
4. Metal tissue "holders".
5. Paper towels.
6. Gloves.
7. Plastic (polystyrene) tubes/containers.
8. Syringes.
9. Disposable 0.45-μm filters.
10. Glass slides.
11. Glass cover slips.
12. Microtome.
13. Microscope (light, fluorescence).
14. Beakers.
15. Plastic (polystyrene) ware.
16. Pipettes and tips.
17. Boxes for incubation of slides (humidity chambers).

2.3 Buffers and Fixatives

1. PBS (50-mM NaPi, 0.15-M NaCl).
2. TBS (50-mM Tris, pH 7.5, 0.15-M NaCl).
3. TTN (50-mM Tris, pH 7.5, 1% Tween 20, 0.3-M NaCl).
4. Saline (0.15-M NaCl).
5. Formaldehyde (HCHO) (37%).

2.4 Antibodies

Primary antibodies are obtained commercially or produced following procedures described in this volume. The species of origin and the isotype must be known. Optimally, control antibodies from the same species and of the same isotype, but another specificity should also be obtained.

Secondary antibodies are obtained commercially, usually as horseradish peroxidase- or alkaline phosphatase-conjugated rabbit or goat immunoglobulins against mouse immunoglobulins or

rabbit immunoglobulins, respectively (for light microscopy), or as FITC- or rhodamine-conjugated rabbit or goat immunoglobulins against mouse immunoglobulins or rabbit immunoglobulins, respectively (for fluorescence microscopy) (*see* **Note 2**).

Animal sera should be available from the same species as the secondary antibodies and optionally also for the primary antibodies, in the case of polyclonal antibodies.

2.5 Cells

Cells are obtained commercially, grown in culture or are obtained from blood samples or other biological fluids following current ethical guidelines.

Cells may be stored at −80, −135, −150, or −180 °C, or in liquid N_2, with or without prior fixation.

2.6 Tissues

Tissue samples are obtained from clinical departments, animal departments, or slaughterhouses following current ethical guidelines and for animal tissue and current guidelines for animal welfare (e.g., for sedation and sacrifice of animals).

Tissues may be stored at −80, −135, −150, or −180 °C, or in liquid N_2, with or without prior fixation, or at room temperature for paraffin-embedded tissues.

3 Methods

3.1 Immunocytochemistry

3.1.1 Cells

Premade cell preparations may be obtained from a number of commercial suppliers (e.g., HEp-2 cells for immunofluorescence cytochemistry).

(a) *Leukocytes*

Hemopoietic/lymphopoietic cells and mature leukocytes are prepared from heparinized blood samples or other biological fluids using differential (gradient) centrifugation or Ab-coupled (paramagnetic) beads (*see* **Note 3**). Cells from such samples may be stained directly or prepared for ICC by centrifugation (cytospins) or smeared on glass slides, where they may be stained directly, be air-dried, and/or fixated before staining.

(b) *Cell Lines*

Cell lines may be cultured in suspension or on glass slides, coverslips, or plastic ware designed for ICC (*see* **Note 4**).

When cells have reached an appropriate density and have been treated according to experimental protocols (e.g., stimulation with growth factors or treatment with chemicals), they are washed briefly with medium/PBS/TBS/saline on the support or in suspension with intermittent centrifugations (1000 *g*) followed by resuspension to 10^6 cells/mL.

Cells can then either be stained directly with antibodies (cell surface antigens), fixated in suspension or on the support (adherent cells), or smeared on a glass slide (suspension cells) and then air-dried and/or treated with an appropriate fixative before further processing.

3.1.2 Direct Immunofluorescent Staining of Living Cells

Wash cells briefly with medium at 5–37 °C depending on the antigen under study (5 °C for cell surface localization, 37 °C for internalization studies) (*see* **Note 5**).

Incubate 20–30 min (in a humidity chamber) with fluorescence-labeled primary antibody diluted 1:100, 1:1000, and 1:10,000 in cell growth medium or an appropriate buffer (with or without 1% serum albumin) at the chosen temperature (*see* **Note 6**).

If the primary antibody is unlabeled or biotinylated, wash cells briefly two to three times with medium/PBS/saline and incubate 20–30 min (in a humidity chamber) with fluorescence-labeled secondary antibody or streptavidin diluted 1:1000, 1:2000, or 1:5000.

Wash cells briefly with medium or buffer at 5–37 °C and proceed with fluorescence microscopy (and/or flow cytometry for suspension cells).

3.1.3 Fixation of Cells

Cells may be fixated to varying degrees depending on the antigen (s) under study (*see* **Note 7**).

(a) *Formaldehyde Fixation*

Incubate the cells in 0.1–3.7% formaldehyde in PBS for 10–30 min (or overnight) at 5 °C or for 10–30 min at room temperature.

Wash three times with PBS, TBS, or saline.

(b) *Ethanol Fixation*

Incubate the cells with cold EtOH (96–99%, 5 °C) for 10–15 min. Then wash briefly with buffer and proceed with antibody staining.

3.1.4 Permeabilization of Cells (Optional)

If cells have been fixated with EtOH, this may be sufficient to allow access to the antigen(s). For aldehyde fixation, cells are treated with a detergent to dissolve membrane lipids and permit access to internal structures.

Wash two to three times with TTN buffer for 10–30 min at 5 °C or 5–10 min at room temperature (*see* **Note 8**).

The degree of permeabilization may be tested with a nucleic acid stain (*see* **Note 9**).

3.1.5 Antigen Retrieval (Optional)

This is mainly used in case of prolonged formaldehyde fixation. *See* Subheading 3.2.6 for methods.

3.1.6 Pretreatment (Optional)

If necessary (depending on the cells and the antibodies), cells are incubated with buffers containing blocking agents (e.g., H_2O_2 for blocking of endogenous peroxidase, levamisole for blocking of endogenous alkaline phosphatase, and avidin for blocking of endogenous biotin).

Autofluorescence due to endogenous compounds or aldehyde groups may be a problem and is most easily relieved by switching to another fluorophore (*see* **Note 10**).

(a) *Irreversible Inhibition of Endogenous Peroxidase Activity*

Incubate 15 min with 0.5% H_2O_2 in methanol or PBS, depending on the cells and antigens.

Wash 3 × 5 – 10 min in medium/saline/PBS/TBS.

(b) *Competitive Inhibition of Endogenous Phosphatase Activity*

Include 1-mM levamisole in washing buffer before activity staining and in buffer for activity staining.

(c) *Inhibition of Endogenous Biotin*

Incubate 30 min with streptavidin (1 μM) in TTN/PBS/TBS.
Wash three times 10 min in TTN/PBS/TBS.
Incubate 30 min with 1-mM biotin in TTN/PBS/TBS.
Wash three times 10 min in TTN/PBS/TBS.

3.1.7 Antibody Staining and Inhibition/Absorption Controls

(a) *Primary Antibody*

Wash three times 10 min in buffer (PBS/TBS for non-permeabilized cells, TTN, or PBS/TBS with 0.1–1% detergent/Triton X-100, Tween 20, or others). Optionally, add 1% BSA or another abundant protein for bocking of nonspecific binding. Another means of reducing nonspecific binding is to include immunoglobulins from the species in which the secondary antibody is produced (e.g., inclusion of 0.1–1% rabbit serum).

Incubate with primary antibody diluted 1:1000 or another predetermined dilution (*see* **Note 11**) in buffer.

NB. Perform all incubations with antibodies in a humidity chamber.

(b) *Inhibition/Absorption Controls*

Inhibition control: Include at least a 1000-fold molar excess of peptide together with primary antibody (*see* **Note 12**) and preincubate 1 h at room temperature or overnight at 5 °C before incubation on the cells. Absorption control: preincubate the primary antibody with peptide coupled to a solid support (*see* **Note 13**) before incubation on the cells.

Reagent control: Substitute the primary antibody with a species- and isotype-matched antibody of irrelevant specificity (*see* **Note 14**).

Wash three times 10 min in buffer.

(c) *Secondary Antibody*

Incubate with secondary antibody (conjugate) diluted 1:1000, 1:2000, or 1:5000 (as determined by preliminary titration experiments) in the same buffer as used for the primary antibody.

Wash three times 10 min in buffer.

3.1.8 Development (Only for Light Microscopy)

For these procedures, it is optimal to use commercially available substrate tablets.

(a) *Peroxidase*

Incubate with 0.01–0.03% H_2O_2 (3–9 mM) and a suitable chromogenic substrate, for example, DAB, 0.05% (1 mM) in TBS/PBS for 10–30 min at room temperature.

Wash three times with buffer or water.

(b) *Alkaline Phosphatase*

Incubate with 0.02% BCIP (0.5 mM) and 0.03% NBT (0.04 mM) in TBS for 30–60 min (or overnight if required).

Wash three times with buffer or water.

3.1.9 Contrast Staining (Optional)

Double staining with different sets of primary and secondary antibodies may be used for localization of other antigens (*see* **Note 15**). Otherwise, staining of, for example, DNA or protein can aid in microscopical analysis.

(a) *Fluorescence ICC*

Incubate with, for example, 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/mL), PBS/TBS for 5 min (*see* **Note 16**).

Wash three times with PBS/TBS for 1–2 min.

(b) *Enzyme Activity ICC (Light Microscopy)*

Stain for a few min with a suitable cytochemical stain, for example, Wright-Giemsa stain, May-Grünwald stain, or Papanicolaou stain (*see* **Note 17**).

Wash three times with PBS/TBS for 1–2 min.

3.1.10 Microscopy

Mount slides with coverslips using a drop of buffer (e.g., PBS) or buffer with glycerol (e.g., PBS with 90% glycerol). A commercial fainting-reducing mounting medium may also be used.

Inspect with fluorescence microscope or light microscope using magnifications from 10 to 100. Reactions may be semiquantified using a scale from 0 (no staining) to 3 (strong staining). Slides with positive staining verified by stringent controls may be studied using more advanced techniques (*see* **Note 18**).

3.2 Immunohistochemistry

IHC procedures are generally equivalent to ICC procedures, but longer times and higher concentrations of Abs may be required for some applications.

3.2.1 Tissue Samples

Tissue slides may be obtained commercially as single tissues or as tissue arrays. Alternatively, tissues are obtained from clinical departments and animal facilities with appropriate ethical consent and following current ethical and animal welfare guidelines.

3.2.2 Tissue Preparation for Immunohistochemical Staining

As shown in Fig. 2, tissues may be processed in various ways before staining with antibodies, depending on the antigens in question and the purpose of the studies. Tissues may be frozen first, then sectioned, fixated, and prepared for immunohistochemical staining. Alternatively, tissues may be fixated and sectioned directly or frozen and then sectioned. Finally, fixated tissue samples may be dehydrated, embedded in paraffin, sectioned, deparaffinated, and then prepared for immunochemical staining. Each procedure has advantages and disadvantages, particularly with respect to antigen preservation and storage of specimens (*see* **Note 19**).

In general, more modifications are introduced to antigens the more, and the harsher treatments are used in sample processing following the order: fresh, frozen, alcohol-, aldehyde-, and paraffin-exposed tissue. However, some treatments may diminish or abolish some forms of nonspecific binding (e.g., FcRs), thus improving detection of specific antigens.

3.2.3 Freezing, Sectioning, and Fixation of Tissue Samples

Cut tissue in appropriate pieces of approximately 1–10 mm^3 or a height of a few mm for larger pieces.

Place the pieces on a precooled (dry ice) metal tissue holder for subsequent sectioning or on a metal plate cooled with dry ice and allow to freeze (*see* **Note 20**).

Record the samples in a logbook and store in plastic containers at −80 °C or lower (*see* **Note 21**).

Disinfect equipment and workplace with EtOH after use (*see* **Note 22**).

3.2.4 Sectioning

For mounting, tissue pieces may be firmly attached to the tissue holders using drops of PBS or with, for example, Tissue Tek.

Place the holders with tissue in the (cryo)microtome and cut sections of 2–4 μm. Gently transfer the sections to (optionally precoated, e.g., with aminoalkylsilane) glass slides optionally with the aid of a fine brush and allow to attach.

The sections may be dried voluntarily or in a stream of cold air.

The location of the tissue can be marked with a waterproof pen for ease of subsequent incubations.

3.2.5 Fixation, Paraffin Embedding, Sectioning, and Deparaffination of Tissue Samples

Fix the tissue sample for 12–48 h in 3.5% paraformaldehyde in 0.1-M phosphate buffer.

The tissue may now be sectioned directly, frozen for later sectioning (*see* above), stored in 70% EtOH at 5 °C, or embedded in paraffin or a suitable polymer.

Before embedding in paraffin, dehydrate the tissues ((1) 30 min in 70% ethanol, (2) 30 min in 95% ethanol, (3) 30 min in 99.9% ethanol, (4) 1 h in 99.9% ethanol, (5) 1 h in 99.9% ethanol, (6) 1 h in 99.9% ethanol, (7) 30 min in 99.9% ethanol/xylene, (8) 1 h in xylene, (9) 2 h in xylene, (10) 2 h 30 min in paraffin wax, (11) 4 h or longer in paraffin wax).

Slice the tissue in 4–5 μm sections and transfer sections to a water bath (~45 °C).

Transfer sections to (optionally precoated) glass slides and allow to attach freely (*see* **Note 23**).

Dry slides freely in air (or in a stream of cold air, or for 20 min at 60 °C).

For paraffin-embedded sections, melt paraffin in oven to firmly adhere tissue sections to glass slides and subsequently deparaffinate and rehydrate by four washes in xylene, three washes in 99.9% ethanol, and three washes in 96% ethanol (and then buffer).

3.2.6 Pretreatment/ Epitope Retrieval

Tissue sections may be treated with detergents, proteases, or other enzymes to facilitate tissue penetration and allow access to individual antigens (*see* Subheading 3.1.4). Moreover, several methods for epitope/antigen retrieval may be used (*see* **Note 24**). Most often, heat treatment, which reverses some of the chemical modifications from fixation, is used.

(a) *Detergent Treatment*

Incubate in TTN or PBS/TBS with 1% Tween-20/Triton X-100 for 30–60 min.

(b) *Protease Treatment*

Incubate with proteinase K (10–20 μg/mL) in PBS/TBS/TTN for 10–20 min at room temperature.

(c) *Heat-Induced Epitope Retrieval*

Treat sections in buffer (e.g., TTN/PBS/TBS/0.5 M Tris buffer, pH 10.0 or other buffer, e.g., 10-mM sodium citrate, pH 6.0) for 10 min in a microwave oven at 800 W.

Cool for approximately 20 min.

Wash 5 min in buffer or water.

Dry the sections by using a cold air blower.

Use a Pap Pen to draw a circle around the tissue.

Dry the sections by using a cold air blower.

Rehydrate the sections for 5 min in TBS.

(d) *Blocking of Endogenous Activity*

Block endogenous enzyme activity (peroxidase, alkaline phosphatase) or biotin as described under Subheading 3.1.6.

Wash shortly in buffer or water, followed by washing for 2 × 5 min in TBS.

3.2.7 Antibody Staining and Inhibition/Absorption Controls

Incubate sections for 1 h at room temperature or overnight at 4 °C with primary antibody diluted appropriately in TBS + 1% BSA.

Inhibition/absorption control: Primary antibody preincubated with excess antigen (*see* Subheading 3.1.7).

Reagent control: isotype-matched antibody from the same species.

Negative control: TBS + 1% BSA.

Tissue controls: Positive and negative tissue samples with known concentration of antigen of interest (e.g., using tissue arrays).

Wash the slides 3 × 5 min in TBS.

Incubate all sections (except control without secondary antibody) for 30 min with secondary antibody at appropriate dilution (*see* **Note 25**).

Wash the slides 3 × 5 min in TBS.

3.2.8 Enzyme Activity Staining (Only Light Microscopy)

(a) *Peroxidase*

Incubate with 0.01–0.03% H_2O_2 (3–9 mM) and a suitable chromogenic substrate, for example, DAB, 0.05% (1 mM) in TBS/PBS for 10–30 min at room temperature.

(b) *Alkaline Phosphatase*

Incubate with 0.02% BCIP (0.5 mM) and 0.03% NBT (0.04 mM) in TBS for 30–60 min (or overnight if required).

Wash the slides for 3–5 min in buffer or running water.

3.2.9 Contrast Staining

(a) *Fluorescence Histochemistry*

Incubate with DAPI or another fluorescent dye (*see* Subheading 3.1.9).

(b) *Enzyme Histochemistry*

Incubate slides with a suitable histochemical stain (*see* **Note 26**).

Wash the slides for 5 min in running water.

3.2.10 Microscopy

Mount cover glass with buffer (optionally add glycerol to 90%) or an aqueous mountain medium. A commercial fainting-reducing mounting medium may also be used.

Inspect with fluorescence microscope or light microscope using magnifications from 10 to 100. Reactions may be semiquantified

using a scale from 0 (no staining) to 3 (strong staining). Slides with positive staining verified by stringent controls may be studied using more advanced techniques (*see* **Note 18**).

4 Notes

1. The protocols mainly relate to initial use and characterization of peptide antibodies for research purposes. For diagnostic and routine uses, consult recent textbooks, reviews, and articles [2, 4, 7–9].
2. A variety of secondary antibodies with other fluorescence labels are available, for example, Alexa fluor, CyDye, and quantum dots.
3. *See*, for example, [10–12].
4. *See*, for example, [12, 13].
5. With intermittent centrifugation (e.g., 1000 *g*, 10 min) for suspension cells.
6. Depending on the antibody, other dilutions may have to be used. In this case, perform a twofold titration from the concentration yielding a satisfactory staining.
7. Mild non-permeabilizing fixation can be done with 0.1% formaldehyde for 10–30 min at 5 °C. Higher concentrations, longer times, and higher temperatures will result in stronger fixation. Ethanol fixation at 5–37 °C will produce an almost instantaneous membrane-disrupting fixation.
8. The optimal detergent and buffer may have to be determined individually for each antigen, especially pH, ionic strength, and presence/absence of Ca^{2+}; other ions or cofactors may have to be taken into consideration. Triton X-100 (0.1–1%), saponin, or another detergent in PBS 5–10 min at room temperature is often used.
9. Several DNA intercalating stains are useful (e.g., DAPI, propidium iodide, Hoechst 33342).
10. Autofluorescence due to residual aldehyde groups may be reduced by incubation with amine-containing buffers (e.g., 50-mM Tris/hydroxylamine/glycine, pH 7–8) or by $NaBH_4$ treatment (1% in PBS).
11. A titration experiment with primary antibodies diluted 1:100, 1:1000, and 1:10,000 (and later on a two-fold titration from one of these) will determine a suitable dilution.
12. Since the concentration of specific antibodies can only be accurately determined for monoclonal antibodies, a molar excess cannot be calculated for polyclonal antibodies. In this case, use peptide concentrations of 0.1–1 mg/mL.

13. Use CNBr Sepharose, magnetic beads, or other matrices following the instructions of the manufacturer.
14. For sera obtained by immunization, a pre-immunization bleed may also be included as an extra control.
15. Double staining with different sets of primary and secondary antibodies.
16. Other dyes may be used, for example, Hoechst 33342 (10 μg/mL for 10–20 min).
17. *See*, for example, [4, 14].
18. Confocal laser scanning microscopy or more advances techniques (e.g., photon correlation microscopy) [12, 15, 16].
19. Since a number of antibodies are not suitable for use on both frozen and paraffin-embedded tissue, it might be valuable to divide the tissue samples in two parts and freeze one part and use the other part for paraffin-embedding.
20. Alternatively, embed the tissue in Tissue Tek and snap freeze in n-hexane.
21. The tissue should be kept at −80 °C for at least 24 h before use.
22. Disposal of tissues must follow local guidelines for disposal of biological waste.
23. Coating of slides with aminoalkylsilane or polylysine may facilitate attachment.
24. The optimal antigen retrieval protocol depends on the antigen and must be determined individually. Other suitable treatments for antigen retrieval include incubation for 10 min in a microwave oven in 0.01-M citrate buffer pH 6.0 or by enzymatic treatment, for example, treatment for 15 min at 37 °C with 0.1% trypsin type III in 0.1% $CaCl_2$, pH 7.8 (dissolve the trypsin in 37 °C warm $CaCl_2$ solution) [2].
25. For biotinylated Abs, incubate sections for 30 min with preincubated StreptABComplex/HRP prepared according to the instructions of the manufacturer.
26. For example, hematoxylin (HE for DNA/nuclei and eosin for protein/cytoplasm) [4, 14].

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