Mass spectrometric approaches for elucidation of antigen–antibody recognition structures in molecular immunology

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Mass spectrometric approaches have recently gained increasing access to molecular immunology and several methods have been developed that enable detailed chemical structure identification of antigen–antibody interactions. Selective proteolytic digestion and MS-peptide mapping (*epitope excision*) has been successfully employed for epitope identification of protein antigens. In addition, "*affinity proteomics*" using partial epitope excision has been developed as an approach with unprecedented selectivity for direct protein identification from biological material. The potential of these methods is illustrated by the elucidation of a ß-amyloid plaque-specific epitope recognized by therapeutic antibodies from transgenic mouse models of Alzheimer's disease. Using an immobilized antigen and antibody-proteolytic digestion and analysis by high resolution Fourier transform ion cyclotron resonance mass spectrometry has lead to a new approach for the identification of antibody paratope structures (paratope-excision; *"parexprot*"). In this method, high resolution MS-peptide data at the low ppm level are required for direct identification of paratopes using protein databases. Mass spectrometric epitope mapping and determination of "*molecular antibody-recognition signatures*" offer high potential, especially for the development of new molecular diagnostics and the evaluation of new vaccine lead structures.

Keywords: affinity mass spectrometry, proteolytic epitope excision, antibody paratope, Alzheimer's disease, FT-ICR-MS

Introduction

An increasing number of pathogens have recently been identified which are capable of eluding our body's natural immune defense, for which no prophylactic vaccines exist, although a number of viral and bacterial diseases have been eradicated.[1] Recent advances in immunology and molecular biology have lead to the development of therapeutic vaccines which are of potential use in chronic diseases such as cancer, cardiovascular disorders and neurodegenerative diseases, where efficacies of available therapies are poor.[2,3] Clearly, future advances in vaccine development will rely substantially on a more complete understanding of the structural basis of immune response.

In the past decade, a variety of methods, including mass spectrometry-based (MS) approaches, have been developed to provide structural information about antigen–antibody complexes, particularly antigen epitopes.[4–6] These include X-ray crystallography and nuclear magnetic resonance of antigen and antibody structures, identification of specific antigens from complex biological mixtures, immunoanalytical characterization of antigen–antibody interactions, identification of linear or discontinuous epitopes and the design of peptide–epitope mimotopes as potential vaccine components. Recent work in our laboratory has focused on the development of high resolution and high selectivity MS approaches and applications to the identification of antibody recognition structures, as a key pre-requisite for vaccine design and targeting. Selective

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proteolytic digestion and MS-peptide mapping (*epitope excision MS*) has been developed and successfully used for the identification of peptide and protein epitopes.[7–9]

Furthermore, an "*affinity-proteomics*" approach using *partial* epitope excision has been developed that provides unprecedented selectivity for direct protein identification from biological material.[10–12] The potential of these methods has been shown in applications by many laboratories and is illustrated by the elucidation of a ß-amyloid plaque-specific epitope recognized by therapeutic antibodies from transgenic mouse models of Alzheimer's disease. Most recently, a "*retro*"-approach to epitope excision has been developed using an antigenepitope affinity column and proteolytic digestion of a partially reduced, affinity-bound antibody; high resolution proteome analysis using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) with mass accuracy at the low ppm level enabled the direct identification of antibody- paratope peptides (*paratope-excision; parexprot*).[13,14] In this article, applications of epitope excision MS to the identification a ß-amyloid plaque-specific epitope recognized by therapeutic antibodies are described first, followed by a proteome analysis-type application of the epitope excision method for direct protein identification from biological material. In a third section, the development and first applications of the parexprot method are illustrated by the identification of functional, high-affinity paratope peptides from an anti-lysozyme antibody, using high-resolution FT-ICR-MS for the analysis and determination of proteolytic antibody fragments.

Experimental methods

Antibodies

The polyclonal anti-Aß(1-42) antibody was raised in TgCRND8 transgenic Alzheimer's disease (AD) mice in response to immunization with ß-amyloid (1-42) oligomers; immunization protocol and purification of the antibody were as previously described.[15] The anti-troponin T antibody (clone IgG1-M7) used in affinity-proteomics from heart cell lysates was produced by Roche Diagnostics, Penzberg, Germany.[11] The polyclonal rabbit anti-lysozyme antibody used for paratope excision was purchased from Chemicon, Temecula, CA, USA.

Antibody immobilization and antigen epitope excision

For preparation of the antibody column, the lyophilized anti-Aß(1-42) antibody was dissolved in 0.2 mM $NaHCO_3$, 0.5 mM NaCl coupling buffer (pH 8.3) to a final concentration of 0.5 µg µL^{-1} and the solution added to dry NHS-activated 6-aminohexanoic acid-coupled Sepharose (Sigma–Aldrich, Steinheim, Germany). The final Sepharose-coupled antibody was loaded into a 0.8 mL microcolumn (Mobitec, Göttingen, Germany) and washed sequentially with blocking and washing buffers. The microcolumn was stored at 4°C using specific buffers for long-term storage.[11]

Epitope excision was performed with 5 µg of Aß antigen bound to the affinity matrix. The microcolumn was gently shaken for 2 h to provide binding equilibrium and non-bound peptide was removed by washing with 10 mL PBS buffer. The affinity-bound antigen was digested for 2 h at 37°C with proteases (for example, trypsin, α-chymotrypsin) as described.[15] Supernatant non-epitope material was removed by washing with PBS buffer and the immune complex then dissociated with 0.1% trifluoroacetic acid. The released epitope peptides were collected in a microreaction cup and the column regenerated by washing with dilute trifluoroacetic acid followed by 20 mL PBS buffer.[11] Epitope extraction was performed by applying the proteolytic mixture directly to the column.

Heart cell lysate

Mammalian heart cell lysate was prepared by cutting pieces of fat-free bovine heart tissue and tissue material washed extensively with phosphate-buffer saline, pH 7.3 at 4°C and then homogenized in PBS buffer.[11] The lysate was centrifuged for 15 min at 500 g for removal of membrane fragments and then stored at –20°C.

Preparation of antigen column

An antigen affinity column was prepared with 150 µg of lysozyme dissolved in 400 µL coupling buffer and the solution added to NHS-activated 6-amino-hexanoic acid-coupled Sepharose. Two different solutions were used to remove unbound material and block the remaining active carboxyl groups of the Sepharose matrix: (a) 0.5 M amino ethanol, 0.5 M NaCl (pH 8.3) and (b) 0.1 M ammonium acetate, 0.5 M NaCl (pH 4.0). The immobilized antigen column was washed with 10 mL each of solutions (a) and (b) and the column was stored in a PBS buffer at 4°C.

Antibody cleavage and parexprot

Reductive partial cleavage of the antibody was performed with DTT added in a 1000-fold molar excess. The mixture was incubated for 2 h at 56°C and the reduced antibody molecules alkylated with a 2.5 molar excess of iodacetamide. Paratope excision was performed with 50 µg of the DTT-reduced antibody for 2 h. Unbound antibody was removed by washing with PBS-buffer and paratope excision was performed with 2.5 µg Trypsin (enzyme to substrate ratio 1 : 20) for 16 h at 37°C. Elution of the paratope peptides was carried out with 10 mL 0.1% TFA. Both supernatant and elution fractions were collected and lyophilized for analysis by matrix-assisted laser desorption/ionization (MALDI)-FT-ICR-MS. A database search was performed with the NCBI database using the Mascot search engine. MALDI-FT-ICR-MS was performed with a Bruker APEX II mass spectrometer on a Scout-100 Source as described.[10]

Results and discussions

Epitope identification of an Alzheimer plaque-specific ß-amyloid antibody using epitope excision MS

Immunization of transgenic mouse models of Alzheimer's disease (AD) with ß-amyloid peptide (Aß) of 40 to 42 amino acids (Aß1-42; s. Figure 1) has been recently effective to generate antibodies that inhibit and disaggregate Aß-fibrils and to reduce AD-related neuropathology and memory impairments;[15] however, the mechanism underlying these therapeutic effects has been unclear. Using epitope excision in combination with MALDI- and electrospray ionization (ESI)-FT-ICR-MS, the epitope recognized by the therapeutically-active antibodies was identified as the N-terminal Aß(4-10) sequence.[15,16] Essential MS data for identification of the Aß(4-10) epitope are illustrated in Figure 1. A series of proteolytic enzymes was employed (trypsin; Lys–C; α-chymotrypsin; Glu–C-, Asp–N-protease), all of which provided the corresponding N-terminal Aß-epitope fragments. The minimal core epitope, Aß(4-10) (FRHDSGY), was identified by additional aminopeptidase treatment and was ascertained by the essential residues of the epitopes F4, R5, H6 and S8 which were all shielded from proteolytic degradation in the immune complex. Epitope excision MS studies with the therapeutic Aß-antibodies were performed with synthetic Aß(1-42), Aß-aggregates, brain tissue and mouse plaques and all provided the identical Aß-N-terminal epitope; in addition, the specificity and affinity of the plaque-specific epitope were ascertained by ELISA and alanine mutation studies with the synthetic Aß-epitope peptide.

These results provided a basis for the design of improved immunogens containing the Aß(4-10) epitope as new AD vaccine lead structure,[16] that could overcome the high inflammatory toxicity observed in initial clinical trials by active immunization with the highly neurotoxic Aß(1-42) peptide aggregates. Furthermore, the results suggested a model in which the active antibodies recognize and effectively target the N-terminal, unstructured epitope in protofibrillar oligomeric Aß-aggregates which have been suggested as the critical, highly neurotoxic intermediates in the formation of AD plaques. The specific recognition of the N-terminal Aß-epitope has been confirmed by all recent clinical studies employing antibodies directed against AD established plaques (Clinical reports; 8th Int. Alzheimer Congress AD/PD 2007, March 14–18, 2007, Salzburg, Austria).

"Affinity-proteomics": protein identification from biological material using mass spectrometric epitope mapping

In a methodology variation of epitope excision MS, a proteome analysis approach has been developed for the direct affinity-based identification from biological material. The identification of proteins from complex biological mixtures employs, most commonly, the combination of 2D-electrophoresis (or alternative high resolution separation techniques) and mass spectrometry. The mass spectrometric analysis of the eluted epitope–peptide fragments provides a unique set of peptide masses which can be compared with the theoretical masses from a sequence database in which proteins are digested *in silico*,[8,9] but using the specific restriction that all fragments must contain

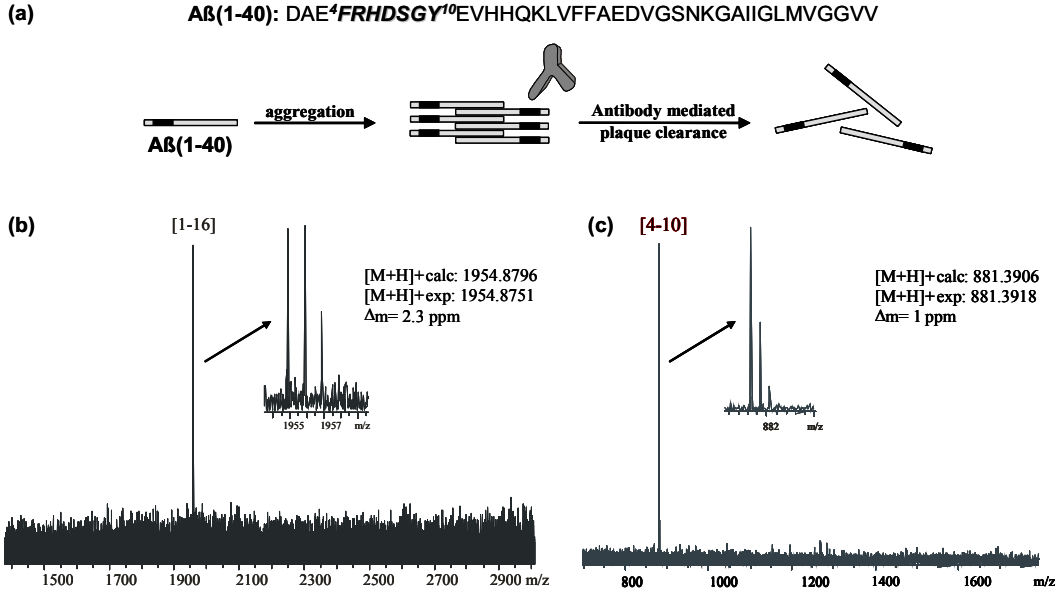

Figure 1. Mass spectrometric identification of the Aß(4-10) epitope recognized by purified plaque-specific antisera from TGR8-transgenic mice.[15] (a), Scheme of Aß-aggregation and suggested mechanism of action of plaque-disaggregating antibody; (b) and (c), MALDI-FT-ICR-MS of epitope elution fraction after epitope excision using trypsin (b) and α-chymotrypsin/ aminopeptidase M (c). Epitope excision was performed with Aß(1-40) or Aß-plaque material bound to the Sepharose-immobilized antibody. The resultant antigen-antibody complex was subjected to proteolysis, followed by removal of supernatant non-bound material and washing as described.[15] Dissociation of the immune complex was performed by addition of 0.1% trifluoroacetic acid (pH 2).

an identical *joint epitope motif*.[11] While not providing information about specific protein interactions or molecular recognition structures due to the denaturing conditions of gel electrophoresis, this approach enable the direct identification of any binding partner for which an affinity reagent is available by combining the affinity MS approach for epitope identification with the peptide mass fingerprinting data of proteome analysis.[11,17,18]

The efficiency of the affinity-proteomics approach was illustrated by the direct identification of the cardiac muscle protein Troponin T (Tn-T), from bovine heart cell lysate, bound to a Sepharose-immobilized anti-troponin T antibody (IgG1-M7; Figure 2). Following removal of supernatant unbound material, the affinity-bound protein was partially degraded by trypsin, thereby generating a set of affinity-bound, overlapping peptide fragments comprising the epitope, Tn-T(118-126).[8,11] Following dissociation from the antibody, the FT-ICR-MS data were used for a proteomics search and provided the unambiguous identification of Troponin T. Only four proteolytic peptide masses, with an average mass accuracy of 6 ppm, were required at a mass resolution of *ca* 60,000; three peptide masses were from different (5+, 4+ and 3+) charge states of the epitope-containing peptide (111–136), while a fourth mass was the 3+ charge-state ion of the epitope sequence (113–131). No proteolytic degradation was observed at any of the shielded tryptic cleavage sites (Lys–121, Arg–123, Lys–126, Arg–127, Arg–128) thus illustrating the specificity of antibody binding. These data demonstrate, thus, the potential of the affinity-MS approach for direct protein identification from highly complex biological material.

Identification of antibody-paratope peptides by proteolytic excision and high resolution FT-ICR-MS (PAREXPROT)

Antibodies have been successfully developed as diagnostic tools and therapeutics for several diseases, such as rheumatoid arthritis[19] and different types of cancer.[20] However, for most applications, human antibodies are required which may be difficult or unfeasible to produce. Due to the highly difficult and time consuming process of classical human antibody extraction and with the consideration that predominantly the complementarity determining regions (CDRs) of an antibody are responsible for antigen binding specificity, a procedure providing information on specific CDR sequences would be of great advantage. Such a procedure has recently been developed in our laboratory, based on a "*retro*"-version of epitope excision MS, which has been modified so as to enable the identification of paratope sequences from intact antibodies. A comparison of the epitope excision MS and the proteolytic paratope excision approach is shown in Figure 3.

Crucial differences between both methods are the proteolytic accessibility of antigens and antibodies: in contrast to the usually proteolytically unstable antigens, native antibodies are stable to proteolysis and must be converted into proteolytically accessible, yet still immunoreactive fragments prior to proteolytic excision. This approach has been accomplished (i) by mild chemical reduction of antibody with dithiothreitol (DTT) and (ii) enzymatically by selective papain or pepsin digestion before paratope excision.[13] Since the complete primary structures of most antibodies are unknown, the identification of paratope-containing peptides (or affinity-analogous sequences therefrom) is

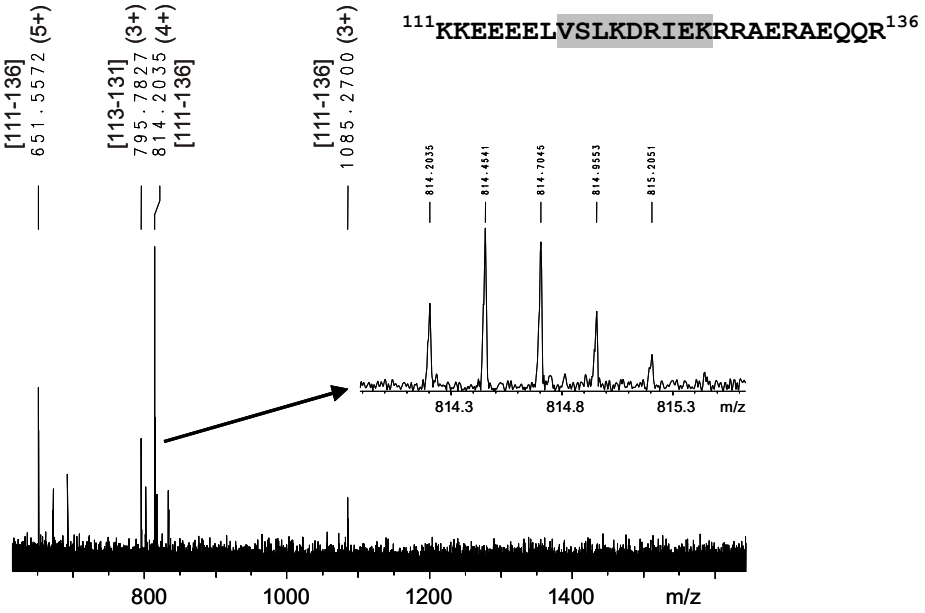

Figure 2. Identification of Troponin T from bovine heart lysate by nano-ESI-FT-ICR-MS after partial epitope excision using an immobilized IgG-M7 monoclonal anti-Troponin T antibody. The insert shows the $[M + 4H]^{4+}$ ion of the epitope containing tryptic peptide [111–136]. The grey box indicates the epitope sequence of the IgG-M7 antibody. Detailed conditions for epitope excision and proteome analysis were as previously described.[11]

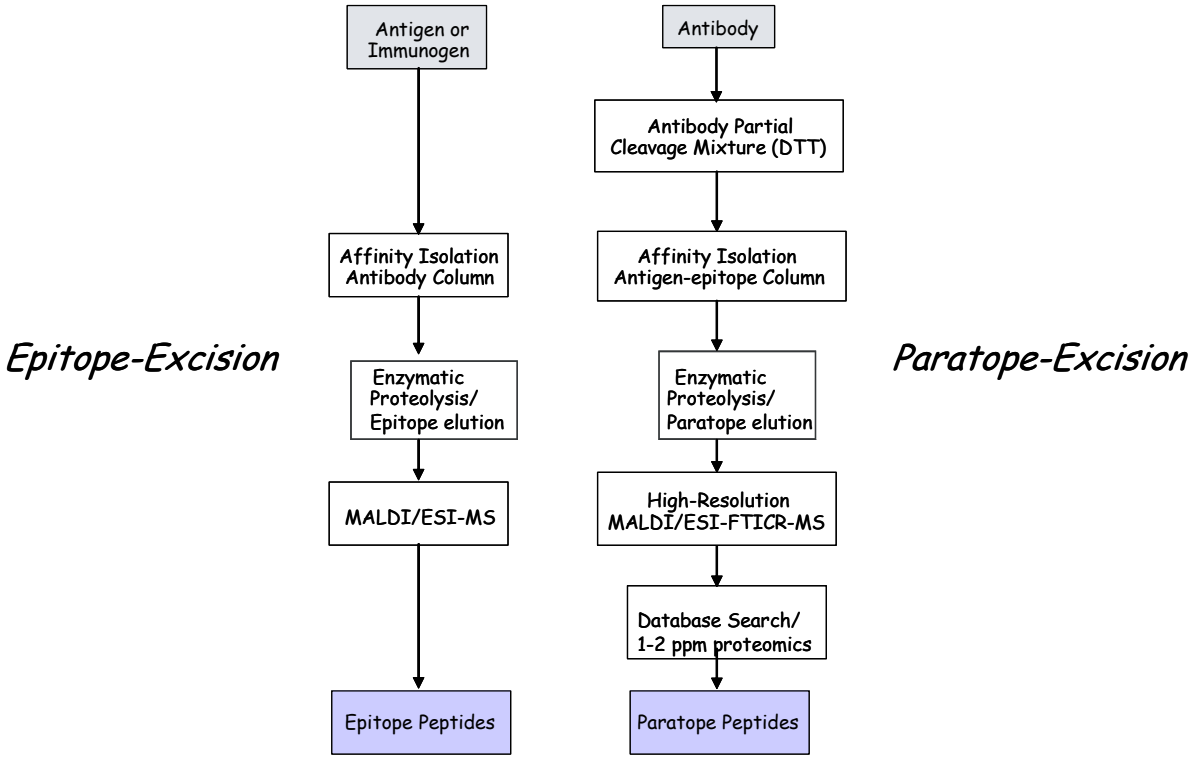

Figure 3. Comparison of the epitope excision MS method (left) and the antibody- paratope excision procedure (PAREXPROT) in conjunction with high resolution MS and high accuracy proteomics and database search.

crucially dependent on high-resolution FT-ICR-MS data, in order to use database search procedures for assigning sequences to the peptide masses determined at high accuracies (typically 1–2 ppm).[10] An example for the determination of heavy- and light-chain paratope sequences from an anti-lysozyme antibody is shown in Figure 4. The peptide fragments, upon tryptic paratope excision of the DTT-reduced, denatured antibodies, were purified from unbound supernatant peptides, then eluted from the column and analyzed by MALDI-FT-ICR-MS. High-accuracy masses from database searches using MASCOT mass fingerprinting at 1–2 ppm threshold identified two specific variable region

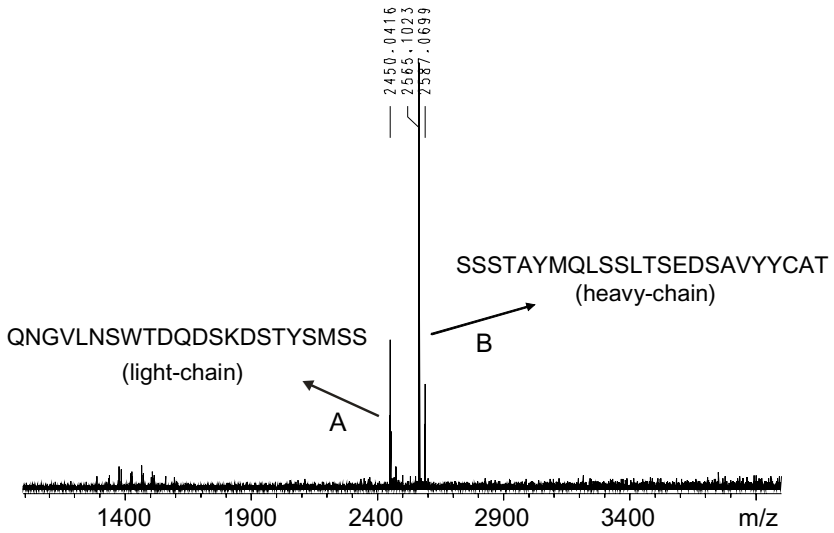

Figure 4. Identification of two paratope peptides derived from a polyclonal anti-lysozyme antibody using the PAREXPROT method in conjunction with MALDI-FT-ICR-MS proteome analysis. Determinations of heavy- and light-chain paratope peptide masses and database search procedures were carried with mass accuracy thresholds < 2 ppm.[13]

peptides with high binding affinities to lysozyme. First applications of the paratope excision method have shown successful determinations of paratope recognition structures using FT-ICR-MS at high mass accuracy for both mono- and poly-clonal antibodies.[13,14]

Conclusions

Recent developments and approaches summarized in this article clearly show that selective proteolytic excision and mass spectrometric mapping present high potential and molecular application for the determination of "*molecular antibody-recognition signatures*", both for antigen-epitope mapping and the identification of relevant antibody paratope sequences. The major value of epitope excision mass spectrometry is in the chemical determination of epitopes that enable a usefull B-cell presentation for vaccine development and provide a molecular "*quality-control*" of antibodies. In contrast, the antibody paratope excision requires high resolution MS, providing lead structures for the production of new antibodies. Therefore, these methods offer considerable application perspectives for (i) the development of new molecular diagnostics, (ii) the characterization of receptor binding structures and (iii) the evaluation of new vaccine lead structures.

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References

1. K.M. Downard, "Contributions of mass spectrometry to structural immunology", *J. Mass Spectrom.* **35,** 493 (2000). doi: 10.1002/(SICI)1096-9888(200004)35:4<493::AID-JMS987>3.0.CO;2-I
2. B.P. Imbimbo, "Toxicity of beta-amyloid vaccination in patients with Alzheimer's disease", *Ann. Neurol.* **51,** 794 (2002). doi: 10.1002/ana.10218
3. E.M. Sigurdsson, T. Wisniewski and B. Frangione, "A safer vaccine for Alzheimer's disease?", *Neurobiol. Aging* **23,** 1001 (2002). doi: 10.1016/S0197-4580(02)00124-0
4. M.H. van Regenmortel, "Which value of antigenic valency should be used in antibody avidity calculations with multivalent antigens?", *Mol. Immunol.* **25,** 565 (1988). doi: 10.1016/0161-5890(88)90078-8
5. A.G. Amit, R.A. Mariuzza, S.E. Phillips and R.J. Poljak, "Three-dimensional structure of an antigen-antibody complex at 2.8 A resolution", *Science* **233**, 747 (1986). doi: 10.1126/science.2426778
6. Y. Paterson, S.W. Englander and H. Roder, "An antibody binding site on cytochrome *c* defined by hydrogen exchange and two-dimensional NMR", *Science* **249**, 755 (1990). doi: 10.1126/science.1697101
7. D. Suckau, J. Kohl, G. Karwath, K. Schneider, M. Casaretto, D. Bitter-Suermann and M. Przybylski, "Molecular epitope identification by limited proteolysis of an immobilized antigen-antibody complex and mass spectrometric peptide mapping", *Proc. Natl. Acad. Sci. USA* **87,** 9848 (1990). doi: 10.1073/pnas.87.24.9848
8. M. Macht, W. Fiedler, K. Kurzinger and M. Przybylski, "Mass spectrometric mapping of protein epitope structures of myocardial infarct markers myoglobin and troponin T", *Biochemistry* **35,** 15633 (1996). doi: 10.1021/bi961727w
9. C. Hager-Braun and K.B. Tomer, "Determination of protein-derived epitopes by mass spectrometry", *Expert Rev. Proteomics* **2,** 745 (2005). doi: 10.1586/14789450.2.5.745
10. E. Damoc, N. Youhnovski, D. Crettaz, J.D. Tissot and M. Przybylski, "High resolution proteome analysis of cryoglobulins using Fourier transform-ion cyclotron resonance mass spectrometry", *Proteomics* **3,** 1425 (2003). doi: 10.1002/pmic.200300482
11. M. Macht, A. Marquardt, S.O. Deininger, E. Damoc, M. Kohlmann and M. Przybylski, "Affinity-proteomics: direct protein identification from biological material using mass spectrometric epitope mapping", *Anal. Bioanal. Chem.* **378,** 1102 (2004). doi: 10.1007/s00216-003-2159-8
12. A. Bauer and B. Kuster, "Affinity purification-mass spectrometry. Powerful tools for the characterization of protein complexes", *Eur. J. Biochem.* **270,** 570 (2003). doi: 10.1046/j.1432-1033.2003.03428.x
13. M. Przybylski, E. Amstalden, A. Marquardt, R. Iacob, R. Stefanescu, I. Perdivara and M. Manea, "Identification of functional antibody-paratopes by proteolytic excision from immobilised immune complexes and high resolution mass spectrometry", *Nature Meth.*, submitted (2007).
14. M. Przybylski and K.B. Tomer, "Application of mass spectrometry to molecular immunology", *Mass Spectrom. Rev.* submitted (2007).
15. J. McLaurin, R. Cecal, M.E. Kierstead, X. Tian, A.L. Phinney, M. Manea, J.E. French, M.H. Lambermon, A.A. Darabie, M.E. Brown, C. Janus, M.A. Chishti, P. Horne, D. Westaway, P.E. Fraser, H.T. Mount, M. Przybylski and P. St George-Hyslop, "Therapeutically effective antibodies against amyloid-beta peptide target amyloid-beta residues 4-10 and inhibit cytotoxicity and fibrillogenesis", *Nat. Med.* **8,** 1263 (2002). doi: 10.1038/nm790
16. M. Manea, F. Hudecz, M. Przybylski and G. Mezo, "Synthesis, solution conformation, and antibody recognition of oligotuftsin-based conjugates containing a beta-amyloid(4-10)

plaque-specific epitope", *Bioconjug. Chem.* **16,** 921 (2005). doi: 10.1021/bc0500037

17. W.J. Henzel, T.M. Billeci, J.T. Stults, S.C. Wong, C. Grimley and C. Watanabe, "Identifying proteins from two-dimensional gels by molecular mass searching of peptide fragments in protein sequence databases", *Proc. Natl. Acad. Sci. USA* **90,** 5011 (1993). doi: 10.1073/pnas.90.11.5011
18. R. Aebersold and M. Mann, "Mass spectrometry-based proteomics", *Nature* **422,** 198 (2003). doi: 10.1038/nature01511
19. P.V. Voulgari, Y. Alamanos, S.N. Nikas, D.V. Bougias, T.I. Temekonidis and A.A. Drosos, "Infliximab therapy in established rheumatoid arthritis: an observational study", *Am. J. Med.* **118,** 515 (2005). doi: 10.1016/j.amjmed.2005.01.029
20. S. Rueckert, I. Ruehl, S. Kahlert, G. Konecny and M. Untch, "A monoclonal antibody as an effective therapeutic agent in breast cancer: trastuzumab", *Expert Opin. Biol. Ther* **5,** 853 (2005). doi: 10.1517/14712598.5.6.853

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